PJN
ISSN 1680-5194

PAKISTAN JOURNAL OF NUTRITION

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Pakistan Journal of Nutrition 1 (1): 1-7, 2002

High-Genistin Isoflavone Supplementation Modulated Erythrocyte Antioxidant Enzymes and Increased Running Endurance in Rats Undergoing One Session of Exhausting Exercise – A pilot study

Chung Yen Chen[1], Golde I. Holtzman[2] and Raga M. Bakhit[3]
[1]Antioxidants Research Lab.Jean Mayer USDA Human Nutrition Research Center on Aging Tufts University Boston, MA 02111
[2]Department of Statistics 210B Hutcheson Hall Virginia Polytechnic Institute and State University Blacksburg, VA 24061-0439
[3]Department of Human Nutrition, Foods and Exercise, Virginia Polytechnic Institute and State University, Balcksburg, VA 24060 0430
email:bakhit@vt.edu

Abstract:Genistein putatively acts as an antioxidant *in vitro*. To investigate the *in vivo* antioxidative activity of genistein, forty-eight male rats were divided into four groups and fed diets with or without 598 mg isoflavone extract per kg of diet for four weeks. On the final day of the study, twenty-four rats were exercised to exhaustion (22 meters/minute at 10% inclination on the treadmill) and then all the rats were sacrificed. The high-genistin isoflavone extract (HGI) diet significantly increased the running time (GE vs. CE: 54 vs. 48 min) and genistein concentrations in the plasma, liver, and gastrocnemius muscle (GE vs. GS: 730.3 vs. 348.5 ng/ml, 529.3 vs. 216.9, and 59.0 vs. 24.9 ng/g, respectively). Exercise doubled genistein concentrations in all tissues and significantly enhanced liver malondialdehyde (MDA). HGI supplementation did not prevent the increase of MDA; instead, it substantially increased MDA levels in muscle tissue (HGI vs. control: 0.46 vs. 0.29 mg/kg). HGI supplementation also maintained the activities of catalase and glutathione peroxidase (GPx) decreased due to exercise (GE vs. GS: 0.113 vs. 0.101 unit/g RBC protein and 0.412 vs. 0.403 μmol/s/mg protein, respectively). It can be concluded that even though HGI modulates erythrocyte antioxidant enzymes against oxidative stress and increases endurance capacity, the supplemented level of HGI does not seem to be optimal for defending the liver and skeletal muscles against oxidative stress.

Key words: antioxidant enzymes, exercise, genistein, malondialdehyde, oxidative stress

Introduction

The role of exercise and physical activity in the prevention of chronic disease and promotion of optimal health has drawn the attention of the public (Singh, 1992). However, research on dietary intervention that protects body tissues from damage during vigorous exercise is in its infancy. This damage is mostly attributed to the sharply increased reactive oxygen species (ROS) in the body during exercise (Davies *et al.*, 1982 and Packer, 1997). Olinescu *et al.* (1995) reported that the increase of urinary excretion of peroxides demonstrated the presence of ROS during exercise. These highly reactive free radicals are known to cause damage to mitochondrial membranes and cytoplasmic structures through peroxidation of phospholipids, proteins, and nucleotides (Jenkins, 1993 and Packer, 1997). Fortunately, endogenous and exogenous antioxidant defense systems in the body can cope with ROS, including vitamin E, vitamin C, beta-carotene, and antioxidant enzymes (SOD, catalase, and GPx). However, an imbalance occurs when ROS, generated during exercise, overcome antioxidant defense systems, a state known as oxidative stress.

Generally, regular physical activity or participation in a sport will increase the total antioxidant capability of the body (Powers *et al.*, 1999). According to Ji (1995), the activities of antioxidant enzymes provide the first line of defense against ROS increase in the heart, liver, lung, blood platelets, and skeletal muscle, in order to cope with oxidative stress induced by acute or exhausting exercise. However, it is possible that acute or irregular participation in exercise will result in oxidative stress due to the elevated use of antioxidants during the defense against ROS. Therefore, more investigations have focused on whether dietary antioxidant supplementation will boost the antioxidant defense systems and overcome oxidative stress. Goldfarb *et al.* (1994) found that rats fed a 250 IU vitamin E/ kg diet for five weeks had lower thiobarbituric acid reactive substance (TBARS) and lipid peroxide levels in plasma and leg muscles after one hour of treadmill exercise, than rats fed a control diet. This finding suggested that antioxidant supplementation in humans and animals may be needed to protect tissues against ROS attack induced by exercise.

Since isoflavones from soy clearly exhibit antioxidant activity *in vitro* and *in vivo* (Kurzer and Xu, 1997) and Brandi (1997) surmised that isoflavones in soy might be responsible for the beneficial effect of lowering the incidence of diseases in Asians. There are many varieties of isoflavones found in soy protein. Genistein and daidzein, and their corresponding glucose conjugated forms, genistin and daidzin, account for the majority of isoflavones in soy.

Isoflavones have a chemical structure similar to estrogen, which has been reported to have a weak antioxidant activity because of the hydroxyl group on its "A" ring in the same location as in vitamin E (Tiidus, 1995). In addition, isoflavones are effective antioxidants, because their phenolic rings have multiple hydroxyl groups that reduce peroxyradicals by donating hydrogen atoms (Tikkanen *et al.*, 1998). With the highest antioxidant characteristic among isoflavones, genistein has been shown to prevent LDL oxidation initiated by oxidizing agents *in vitro* (Bakhit and Potter, 1995; Kapiotis *et al.*, 1997) and to protect microsomal lipid peroxidation induced by a Fe_2^+-ADP complex (Jha *et al.*, 1985). Additionally, Cai and Wei (1996) have observed that dietary administration of genistein (50 and 250 PPM) for 30 days significantly increased

the activities of antioxidant enzymes in small intestine and skin of SENCAR mice.

Most prior studies have used *in vitro* measures to evaluate the antioxidative ability of isoflavones. However, it is not completely understood if isoflavones can defend against ROS or boost up total antioxidant defenses *in vivo*. In order to initiate the maximum impact of oxidative stress on rats, one single exhaustive exercise, without exercise training, was given to rats in an exercised group. We hypothesized that exercise would initiate oxidative stress in the rats, further resulting in changes of erythrocyte antioxidant enzymes and increased products of lipid peroxidation. We also hypothesized that four-week HGI supplementation would modulate activities of erythrocyte antioxidant enzymes and decrease products of lipid peroxidation in tissues after the rats were exercised on the treadmill. The objective of this study was to investigate the *in vivo* antioxidant ability of the isoflavones, especially genistein, in rats undergoing acute exhausting exercise. One high dose challenge was used in this pilot study to assist in determining the range of dose supplementation in a subsequent dose response study.

Materials and Methods

This study was approved by the Virginia Polytechnic Institute and State University Institutional Review Board.

Animals:Forty-eight one-year-old Sprague-Dawley male rats were purchased from Harlan Industries (Indianapolis, IN). The rats were housed at 25°C with a 12-hr light/dark cycle with free access to feed and water throughout the study. Upon delivery to the Virginia Tech animal research facility, the rats were acclimatized on a chow diet for one week.

Experimental design: The rats were randomly divided into four treatments of equal number. The four treatments were control diet and exercise (CE), control diet and sedentary (CS), high-genistin isoflavone extract (HGI) diet and exercise (GE), and HGI diet and sedentary (GS). All semipurified ingredients were purchased from ICN (ICN Pharmaceuticals Inc., Costa Mesa, CA). The control diet was prepared according to the formula of the American Institute of Nutrition (AIN 93 M). The HGI diet was identical to control diet except that 598 mg isoflavone extract per kg diet was added to replace an equal amount of cornstarch. The composition of isoflavones added in this diet was 84.4% genistein, 14.8% daidzein, and 0.8% glycitein. Each rat's feed intake was measured daily throughout the study. All the rats were given the experimental diets for 28 days.

On the final day of the study, all the rats in the CE and GE groups were exercised vigorously in one session until they were exhausted. The exercise protocol was 22 meters/minute at 10% inclination on a treadmill (Exer-4/8 treadmill, Columbus, OH). In order to familiarize the rats with the treadmill, they all were trained to walk on the treadmill before the final acute exercise session. The protocol for the walking training was ten meters/minute for five minutes, twice a week, for the first three weeks. The treadmill was equipped with an electric shocking grid on the rear barrier to motivate the rats to exercise. A rat was considered exhausted when it would not be prompted to run any more. The rats would stop running and when overturned would continue to lie on their backs disregarding gentle prods and electric shock. The running time of each rat in the CE and GE groups was recorded.

Sample collections: Immediately after acute exercise, all the rats were anesthetized using a halogen and nitrogen gas mixture and the maximum possible volume of blood was drawn via the heart puncture. Blood samples were immediately kept in ice. Plasma and red blood cells (RBC) were harvested following centrifugation at 1000 x g for 20 minutes at 4°C. After blood collection, the rats were sacrificed by cervical dislocation and the liver and legs were both removed. In order to diminish tissue exposure to heat and air, whole liver and legs were immediately wrapped in aluminum foil and instantly frozen in liquid nitrogen. All samples, including the liver, plasma, RBC, and muscle were stored at –80°C until further analyses.

Biochemical measurements: Genistein concentrations in the plasma, gastrocnemius muscle, and liver were determined by a HPLC method, modified from methods of Wang and Murphy (1994) and Xu *et al.* (1994). *In vivo* thiobarbituric acid reactive substances (TBARS) method was used to measure MDA concentrations in the liver and gastrocnemius muscle tissues (Pikul *et al.*, 1989). Before the analysis, whole liver and muscle tissues were gently thawed on the ice. Subsequently, gastrocnemius muscle was collected from whole leg via the dissection. One gram of liver or muscle tissue, 5 ml of 5% trichloroacetic acid, and 200 μl of 0.15 % butylated hydroxyltoluene were homogenized for one minute. Following homogenization, the mixture was spun at 17,000 x g for 15 minutes at 4°C. One ml of supernatant was filtered by using 9 cm GF/C filter paper and small funnels, and then was mixed with one ml of 0.67% thiobarbituric acid (TBA) solution. The absorbance was read on a spectrophotometer (532 nm) within 1 hour after incubation in a 95°C water bath for 40 minutes. The concentration of the MDA product was calculated by comparison with a standard curve established from different concentrations of tetraethoxypropane. RBCs were used to measure the activities of antioxidant enzymes, SOD, catalase, and GPx. The activities of antioxidant enzymes were standardized by erythrocyte protein. Superoxide dismutase (EC 1.15.11.) was determined by the method of Xin *et al.* (1991). The activities of GPx (EC 1.11.1.9) and catalase (1.11.1.6) were determined according to the methods developed by Agergaard and Jenson (1982) and Aebi (1983), respectively.

Statistical analysis: A two-way ANOVA was used to compare the effects of exercise and diet, and their interactions on the means of MDA concentrations in tissues and activities of antioxidant enzymes in the rats (Sokal and RohIf, 1995). The significant level was set at $p<0.05$. When the interactions between exercise and dietary supplementation were statistically significant, Tukey's HSD procedure at the 0.05 level experimentwise was used to compare group means. A Student's t-test was used to contrast mean genistein concentrations between the GE and GS groups and the running time between the NE and GE groups (Sokal and RohIf, 1995). The computer software program JMP (SAS Institute Inc., Cary, NC) was used for all computations.

Results

Body weight and running ability: At the time of sacrifice, mean body weights among the CE, CS, GE, and GS groups were not significantly different, 545.3, 549.9, 583.8, and 544.8 gm, respectively (n =12, SE = 12.7, 20.2, 15.6, and 16.6 gm). High-genistin isoflavone supplementation did not affect body weight gain or feed intake. The average running time of the rats in the GE group, 54 minutes (n = 12, SE = 2 minutes), was significantly longer than that of the rats in the CE group,

Table 1: Genistein concentrations in plasma, liver, and gastrocnemius muscle in rats

			Plasma (ng/ml)		Liver (ng/g)		Muscle (ng/g)	
Diet	Exercise level	n	Mean	SE	Mean	SE	Mean	SE
HGI	exercised	12	730.3[a]	70.6	529.3[a]	96.7	59.0[a]	6.1
HGI	sedentary	12	348.5[b]	41.0	216.9[b]	33.9	24.9[b]	4.5
control	exercised	12	0[c]	0	0[c]	0	0[c]	0
control	sedentary	12	0[c]	0	0[c]	0	0[c]	0

[abc]Means with different letters were significantly different, using a student's t-test, $P < 0.05$.

Table 2: Mean liver concentration of malondialdehyde by dietary supplement and exercise

			MDA (mg/kg)	
Diet	Exercise level	n	Mean	SE
HGI	exercised	11*	0.640[a]	0.111
HGI	sedentary	12	0.311[b]	0.044
control	exercised	12	0.449[ab]	0.071
control	sedentary	12	0.366[ab]	0.062

[ab]Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey's HSD, Sokal and Rohlf, 1995). Exercise effect was significant, $P = 0.008$.
*One sample was deleted from statistical analysis because it was an outlier.

which was 48 minutes (n = 12, SE = 1.4 minutes) ($P < 0.05$).

Genistein concentrations: High-genistin isoflavone supplementation led to significant ($P < 0.001$, 0.001, and 0.002, respectively) increases in genistein concentrations in plasma, liver, and gastrocnemius muscle in sedentary rats (Table 1). After the rats underwent one session of acute exhausting exercise on the treadmill, all genistein concentrations in plasma, liver, and gastrocnemius muscle were significantly higher than those were in the sedentary rats. Exercise more than doubled the concentrations of genistein in plasma, liver, and gastrocnemius muscle (109, 144, and 137% increase, respectively) as compared to sedentary HGI fed rats. There was no significant correlation between genistein concentration and other biochemical parameters.

Products of lipid peroxidation: Malondialdehyde is an index of the extent of lipid peroxidation. MDA concentration in the liver and gastrocnemius muscle were approximated by the TBARS method.
According to the results of a two-way ANOVA (Table 2), the interactions between exercise and diet were not significant ($P = 0.10$) and the main effects of exercise led to significant increase of MDA concentrations in the liver ($P = 0.008$), while the main effects of diet did not ($P = 0.37$). Multiple comparisons of the means suggested synergy of exercise and HGI supplementation on the increase of liver MDA, but the relationship was weak, with HGI and exercise explaining only $R^2 = 20\%$ of the variation in the liver MDA concentration.
The two-way ANOVA revealed no significant interaction effects between diets and exercise levels ($P = 0.47$) on MDA concentrations in the gastrocnemius muscle, and one session of acute exhausting exercise did not result in a significant augmentation of MDA concentrations in rats fed control diet and HGI diet ($P = 0.75$). Yet, gensitein supplementation caused a slightly significant increase of MDA ($P = 0.044$) in both sedentary and exercised rats (Table 3). Mean MDA concentration was 0.454 mg/kg (n = 24, SE = 0.049 mg/kg) with HGI supplementation, and was 0.322 mg/kg (n = 24, SE = 0.040 mg/kg) with no supplementation.

Activities of antioxidant enzymes : Two-way ANOVA analysis showed that one session of exercise significantly increased SOD activity in rats fed control and HGI diets ($P = 0.037$); however, HGI supplementation led to an inhibition of SOD activity in rats ($P = 0.031$) (Table 4). There was no significant interaction between exercise and dietary supplementation on SOD activity ($P = 0.19$).
The main effects of HGI supplementation and exercise on catalase activity (Table 5) were not significant ($P = 0.42$ and 0.18, respectively), while the interactions between diet and exercise were significant ($P = 0.003$). Comparing means by Tukey's HSD procedure, one session of acute exhaustive exercise significantly decreased catalase activities in the rats fed the control diet compared to those in sedentary rats, but exercise did not result in a reduction in erythrocyte catalase activities in rats fed the HGI diet.
The interactions between diet and exercise were significant ($P = 0.018$) in GPx activities of RBC, and the main effects of exercise were significant ($P = 0.038$), but the main effects of diet were not statistically significant ($P = 0.12$) (Table 6). The mean activity of GPx in rats that underwent one session of acute exhaustive exercise was 0.353 μmol/s/mg RBC protein (n = 24, SE = 0.018 μmol/s/mg RBC protein), and was 0.415 μmol/s/mg RBC protein (n = 24, SE = 0.026 μmol/s/mg RBC protein) in sedentary rats. One session of acute exhausting exercise significantly decreased GPx activities in RBC in rats fed the control diet, based on multiple comparisons of means by Tukey's HSD procedure. However, GPx activities in the GE rats did not change with the acute exhausting exercise.

Discussion

This study assessed the effects of dietary high-genistin isoflavone supplementation on immediate post-exercise indices of tissue oxidative damage and modulations antioxidant enzymes (SOD, GPx, and catalase) in erythrocytes. It is the first study to examine the antioxidant potential of isoflavones using an *in vivo* animal exercise model. It was shown that significant accumulation of genistein in the plasma, skeletal muscle, and liver occurred after dietary HGI supplementation for four weeks but this did not increase the antioxidative capability of the liver and skeletal muscle in these rats. Nor did an increased genistein concentration in the liver provide significant protection against the oxidative stress due to acute exhaustive exercise. However, the HGI supplementation significantly extended the rats' running time to reach exhaustion on the treadmill. Furthermore, in regard to the antioxidant enzymes in erythrocytes, HGI, while it significantly prevented the decrease of GPx and catalase activities by exercise, did not significantly influence the activities of GPx and catalase in the sedentary rats. In addition, the HGI supplementation significantly decreased erythrocyte SOD activity in both exercised and sedentary rats.
The major isoflavones in soy are the conjugated forms genistin and daidzin, which are glycosylated genistein and daidzein,

Table 3: Malondialdehyde (MDA) concentrations in the gastrocnemius muscle

			MDA (mg/kg)	
Diet	Exercise level	n	Mean[a]	SE
HGI	exercised	12	0.441	0.052
HGI	sedentary	12	0.468	0.086
control	exercised	12	0.356	0.066
control	sedentary	12	0.228	0.046

aThere was a statistical difference due to diets, P = 0.044 (Tukey's HSD, Sokal and Rohlf, 1995).

Table 4: The activities of superoxide dismutase (SOD) in RBC in rats

			SOD (unit/mg RBC protein)	
Diet	Exercise level	n	Mean	SE
HGI	exercised	12	0.091[ab]	0.011
HGI	sedentary	12	0.080[a]	0.013
control	exercised	12	0.138[b]	0.016
control	sedentary	12	0.092[ab]	0.012

[ab]Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey's HSD, Sokal and Rohlf, 1995).

respectively. It has been suggested that conjugated isoflavones derived from foods could not be absorbed from the small intestine (Hollman, 1997). Hydrolysis of the mostly beta-glucosidic bonds that attach isoflavones to sugars by the gut microorganisms is necessary for absorption of genistin (Hollman 1997; King and Bursill, 1998; and Zhang *et al.*, 1999). Significantly increased genistein concentrations in the plasma, skeletal muscle, and liver in rats fed at 500 mg genistin containing compounds per kg diet indicated that isoflavones from food supplementation could lead to a significant accumulation of genistein in tissues. However, genistein concentrations varied among each of sedentary rats fed the HGI diet. It has been suggested that high inter-individual variation of isoflavone bioavailability in humans may be attributed to existence of gut bacteria, which are necessary for the degradation of conjugated isoflavones into aglycone forms for absorption (Xu *et al.*, 1994 and Wiseman, 1999). The current results support such a suggestion in rats. It may be that degradation of conjugated isoflavones in the colon possibly plays a role in their bioavailability in rats.

Genistein concentrations in the plasma, skeletal muscle, and liver of rats fed the HGI diet doubled after acute exhausting exercise. Exercise can significantly enhance antioxidants in tissues such as vitamin E in plasma and erythrocytes (Pincemail *et al.*, 1988 and Vasankari *et al.*, 1997) and vitamin C in plasma (Maxwell *et al.*, 1993). Pincemail *et al.* (1988) hypothesized that exercise moved vitamin E from other tissues into the plasma and that skeletal muscle utilized circulating vitamin E for protection against oxidative damage. Like vitamin E, genistein might be mobilized from some tissues other than the liver and skeletal muscle because both the above tissues and plasma had significantly higher genistein concentrations in exercised rats than in sedentary rats in this study. Chang *et al.* (2000) reported the presence of genistein in the prostate gland, testes, thyroid gland, and brain of rats fed a genistein diet. Therefore, it can be hypothesized that genistein may be transported from these tissues to the plasma, liver, and muscle tissue. In addition to mobilization from storage sites, acute exhausting exercise might also decrease the clearance of genistein from the liver. Like endogenous estrogen, glucuronidated genistein is excreted through urine and bile and undergoes enterohepatic circulation (Kurzer and Xu, 1997). Several reports have noted an increase in plasma estrogen concentrations following acute exercise in women (Bonen *et al.*, 1981). It has been suggested that this increase is primarily due to the decreased metabolic clearance of estrogen as a direct consequence of exercise induced reduction in hepatic blood flow during exercise (Sutton *et al.*, 1990). Therefore, the increased genistein concentrations in the plasma, liver, and gastrocnemius muscle may be attributed to a movement of genistein from other tissues and low clearance from the liver.

Genistein also has a structure similar to estrogen and has been suggested to have estrogenic and anti-estrogenic responses in mammalian tissues (Kurzer and Xu, 1997). Estrogens were suggested to act as antioxidants in a manner similar to vitamin E to terminate peroxidation chain reactions (Burton and Ingold, 1989), however, they were reported to act as prooxidants and elevate plasma TBARS concentrations in rats when administrated in the form of pharmacological contraceptives (Kose *et al.*, 1993 and Pizzichini *et al.*, 1993). In the present study, 500 mg genistin-containing compounds per kg diet from isoflavone extract given to rats may be too high to act as an antioxidant. Thus, a significantly increased MDA concentration in the gastrocnemius muscle in rats fed HGI diet means that genistein at high levels may act as a prooxidant rather than an antioxidant. Unpublished data from our lab showed that 399-ppm HGI supplementation led to a slightly lower plasma MDA concentration measured by the TBARS method than 598-ppm HGI supplementation in rats.

Oxidative stress induced by acute exercise can significantly elevate markers of tissue peroxidative damage such as MDA, and possibly elevate tissue antioxidant enzyme activities (Davies *et al.*, 1982; Ji, 1995; Sen, 1995; and Somani and Arroyo, 1995) because physical exercise promotes the production of ROS due to a substantial increase in oxygen consumption (Davies *et al.*, 1982; Packer, 1986; and Ayres *et al.*, 1998). The present study showed that exercise at 22 meters/min at 10% inclination for 1 hour significantly elevated MDA levels in the livers of rats fed control and HGI diets. However, one session of acute exhausting exercise did not result in a significant accumulation of MDA in the gastrocnemius muscle. Ji and Fu (1992) also observed that exhausting exercise (20 meters/min and 0% inclination) did not significantly elevate the MDA level in muscle, but significantly increased it in the liver. Therefore, acute exercise might not lead to lipid peroxidation in all tissues equally. It is plausible to speculate that products of lipid peroxidation may be transported from the muscle into the circulation, and possibly to the liver.

One session of acute exhaustive exercise significantly increased MDA levels in the liver; however, HGI supplementation did not diminish the increase of MDA. Although genistein has been strongly suggested as an antioxidant *in vitro* and *in vivo* (Kurzer and Xu, 1997), in this study, genistein did not perform as an antioxidant in the liver. Further research is necessary to explore whether there is a dose-dependent response of isoflavones on increased liver MDA initiated by exhaustive exercise.

Every antioxidant, including vitamin antioxidants, is a redox agent, protecting against ROS in some circumstances, but also promoting ROS generation in others (Herbert, 1996). Herbert (1994 and 1995) reported that antioxidant vitamin supplements at pharmacological levels might promote heart disease, cancer, and liver and kidney disease. The present study's use of genistin-containing compounds from isoflavone

Table 5: The activity of catalase in RBC

			Catalase (unit/g RBC protein)	
Diet	Exercise level	n	Mean	SE
HGI	exercised	12	0.113[a]	0.008
HGI	sedentary	12	0.101[ab]	0.005
control	exercised	12	0.086[b]	0.004
control	sedentary	12	0.116[a]	0.009

[ab]Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey's HSD, Sokal and Rohlf, 1995).

Table 6: The activity of glutathione peroxidase (GPx) in RBC

			GPx (imol/s/mg RBC protein)	
Diet	Exercise level	n	Mean	SE
HGI	exercised	12	0.412[a]	0.024
HGI	sedentary	12	0.403[ab]	0.029
control	exercised	12	0.294[b]	0.010
control	sedentary	12	0.428[a]	0.043

[ab]Means followed by the same superscript were not significantly different at the 0.05 level of significance experimentwise (Tukey's HSD, Sokal and Rohlf, 1995).

extract in the diet may have been too high, thereby promoting ROS generations in tissues. The results of this study showed genistein not only enhanced MDA in the gastrocnemius muscle of exercised and sedentary rats, but also provided no significant protection against increased MDA in the liver due to exercise.

Although dietary HGI supplementation did not prevent MDA increase in the liver of rats run to exhaustion on the treadmill, the running time was significantly longer than rats fed the control diet. Because oxidative damage may occur with exercise, antioxidant administration has drawn much attention both in terms of preventing damage and in terms of affecting performance. Packer (1997) suggested that oxidative stress might play a role on the fatigue process, and antioxidant administration might reduce the fatigue, leading to an increase in performance. Yet, the reports from most studies showed that antioxidant supplementation exhibited no effect on performance in humans (Clarkson, 1995). Although antioxidant supplementation may not bolster the performance in humans, exogenous glutathione supplementation was found to increase swimming time in mice (Novelli *et al.*, 1991). Furthermore, Balakrishnan and Anuradha (1998) reported that exogenous glutathione influenced the endurance capacity of athletes. Studies conducted in our laboratory, demonstrated that isoflavones increased GSH concentration in the blood. In addition, Appelt and Reick (1999) observed that feeding rats 810-PPM soy isoflavones led to increased GSH concentration and decreased GSSG concentration in plasma. Therefore, isoflavones may increase endurance capacity in rats by their influence on glutathione concentrations. The present study suggests that HGI supplementation increases endurance capacity in one-year old rats. The further study is needed to investigate the effect of isoflavones on running endurance. It was reported that genistein may modulate antioxidant enzyme activities. Genistein, administrated to female rats at 0.1 g/kg BW levels a day, inhibited the activity of glutathione reductase (GR), catalase, SOD, and GPx in RBC (Breinholt *et al.*, 1999). These researchers observed that the activities of antioxidant enzymes in RBC decreased concurrently with an increase in the antioxidant potential due to administered flavonoids. Others observed that genistein could suppress 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated H_2O_2 production *in vitro* and *in vivo* and inhibit superoxide anion formation by the xanthine-xanthine oxidase system (Wei *et al.*, 1993 and Wei *et al.*, 1995). Hence, Breinholt *et al.* (1999) hypothesized that antioxidant enzymes in RBC were down-regulated by genistein in response to an improved antioxidant status of the RBC due to the increase of high antioxidant potential from genistein supplementation. This study confirms the hypothesis of Breinholt *et al.* (1999), because it showed that HGI supplementation decreased SOD activities in RBC. Physical exercise can cause oxidative stress in erythrocytes because of increased generation of ROS, which may trigger antioxidant enzymes to enhance their activities and reduce ROS to safe compounds (Ji, 1995). However, the increase of activities of antioxidant enzymes by physical exercise is still controversial. A session of acute exhaustive exercise was shown to increase SOD activity, indicating increased superoxide production during exercise (Ji,1993) in a number of biological tissues including heart (Ji, 1993), liver, (Alessio and Goldfarb, 1988; Ji *et al.*, 1988; and Ji *et al.*, 1990), lung (Reddy *et al.*, 1992), blood platelets (Buczynski *et al.*, 1991), skeletal muscle (Ji *et al.*, 1990 and Lawler *et al.*, 1993), and erythrocytes (Somani *et al.*, 1995). Ohno *et al.* (1986) and Kaczmarski *et al.* (1999), however, reported that physical exercise did not increase erythrocyte SOD activity in humans, and Ji *et al.* (1990) and Alessio and Goldfarb (1988) observed that one session of acute exercise did not increase SOD activity in muscle from rats. In contrast, most of the literature revealed no significant alternation in catalase activity with acute exercise (Ji *et al.*, 1990 and Meydani *et al.*, 1993), while studies conducted by Somani *et al.* (1995) and Kaczmarski *et al.* (1999) reported that erythrocyte catalase activity decreased after acute exercise. The effect of an acute session of exercise on GPx activity in various tissues has not been reported consistently in the literature (Ji, 1995). Erythrocyte GPx activity was slightly reduced after a brief (30 min) physical exercise in sedentary students (Ohno *et al.*, 1986), yet, Ji *et al.* (1990) observed that acute exercise did not affect GPx activity in the livers of rats. There is yet no clear explanation for these discrepancies (Ji, 1995). This study agrees with Somani *et al.* (1995) that erythrocyte catalase activities were significantly decreased by one session of acute exhausting exercise in the rats fed the control diet, while erythrocyte SOD activity significanly increased. In addition, RBC GPx activity was significantly decreased by exercise. In general, an increased generation of ROS during an acute session of strenuous exercise, causing the activation of antioxidant enzymes, remained the most viable explanation (Ji *et al.*, 1988; Ji *et al.*, 1990; and Ji, 1993). In the present study, only erythrocyte SOD in rats reflected the increased ROS production, because its activity was significantly enhanced by acute exercise. However, this explanation could not effectively account for the decrease of erythrocyte GPx and catalase activities in the study.

Erythrocyte SOD activity was significantly increased by acute exercise in the rats, while the activity of the GE rats was maintained by HGI administration at the same level as that of the NS rats. As Wei *et al.* (1993 and 1995) observed that genistein could suppress H_2O_2 production and inhibit superoxide formation, genistein may diminish superoxide anion generated by one session of acute exhaustive exercise. In regard to GPx and catalase activities in erythrocytes of exercised rats, HGI prevented the decrease of GPx and catalase activities due to acute exercise. Although the physiological justification in the decrease of enzymes' activities is not clear, the homeostasis of antioxidant defense is disturbed by oxidative stress due to one session of acute

exhaustive exercise. The HGI supplementation could prevent this disturbance of oxidative stress due to exercise, further leading to maintenance of a more reductive environment in erythrocytes. Therefore, we can speculate that HGI administration in the diet of the rats maintained redox status, because genistein acts as an antioxidant or exerts its estrogenic effect to modulate antioxidant enzyme activities. This study demonstrated that HGI supplementation modulated erythrocyte antioxidant enzyme activities in response to acute exhaustive exercise probably through either the antioxidative or estrogenic effect of genistein. Isoflavones also enhanced the endurance capacity in rats possibly by their influence on glutathione homeostasis. The increased genistein in the liver, skeletal muscle, and plasma may have been mobilized from other tissues such as the prostate, testes, thyroid gland, and brain, during exercise, or was due to reduced clearance from the liver. However, elevated genistein concentrations in the liver did not provide an extra protection against oxidative stress due to acute exhaustive exercise. The dose of HGI administered in this study might be too high, and consequently genistein may have acted as both an antioxidant and a prooxidant. It can be concluded that genistein may elevate antioxidant defense in erythrocytes against oxidative stress, and increase endurance capacity by its antioxidative effect. This may be either directly, or indirectly through its estrogenic effect. However, the amount of HGI used in this pilot study, may not be optimal to defend the liver and skeletal muscle against oxidative stress. Further study is necessary to explore the optimal dose for dietary genistein supplementation to produce a beneficial effect on antioxidant defense systems and to avoid the disadvantageous effect of increased MDA in skeletal muscle.

References

Aebi, H.E, 1983. Catalase. In: Bergmeyer, H.U. (Eds.), Methods of enzymatic analysis. 3rd ed. Weinheim. Deerfield Beach, FL, pp: 273-285.

Agergaard, N. and P.T. Jensen, 1982. Procedure for blood glutathione peroxidase determination in cattle and swine. Acta. Vet. Scand., 23: 515-527.

Alessio, H.M. and A.H. Goldfarb, 1988. Lipid peroxidation and scavenger enzymes during exercise: adaptive response to training. J. Appl. Physiol., 64: 1333-1336.

Appelt, L.C. and M.M. Reicks, 1999. Soy induces phase II enzymes but does not inhibit dimethylbenz[a]anthracene-induced carcinogenesis in female rates. J. Nutr., 129: 1820-1826.

Ayres, S., W. Abplanalp, J.H. Liu and M.T. Ravi Subbiah, 1998. Mechanisms involved in the protective effect of estradiol-17betta on lipid peroxidation and DNA damage. Am. J. Physiol., 274: E1002-E1008.

Bakhit, R.M. and S.M. Potter, 1995. Crude isoflavone extract inhibits LDL oxidation *in vitro*. FASEB J., 9: 4: A767.

Balakrishnan, S.D. and C.V. Anuradha, 1998. Exercise, depletion of antioxidants and antioxidant manipulation. Cell Biochem. Funct., 16: 269-275.

Bonen, A., A. Belcastro, W. Ling and A. Simpson, 1981. Profiles of selected hormones during menstrual cycles of teenage athletes. J. Appl. Physiol., 50: 545-551.

Brandi, M.L., 1997. Natural and synthetic isoflavones in the prevention and treatment of chronic disease. Calcif. Tissue Int., 61 Suppl., 1: S5-S8.

Breinholt, V., S.T. Lauridsen and L.O. Dragsted, 1999. Differential effects of dietary flavonoids on drug metabolizing and antioxidant enzymes in female rats. Xenobiotica, 12: 1227-1240.

Buczynski, A.J., J. Kedziora, W. Tkaczewski and B. Wachowicz, 1991. Effect of submaximal physical exercise on antioxidative protection of human blood platelets. Int. J. Sports Med., 12: 52-54.

Burton, G. and K. Ingold, 1989. Vitamin E as an *in vitro* and *in vivo* antioxidant. Annal. NY Acad. Sci., 570: 7-22.

Cai, Q. and H. Wei, 1996. Effect of dietary genistein on antioxidant enzyme activities in SENCAR mice. Nutr. Cancer, 25: 1-7.

Chang, H.C., M.I. Churchwell, K.B. Delclos, R.R. Newbold and D.R. Doerge, 2000. Mass spectrometric determination of genistein tissue distribution in diet-exposed Sprague Dawley rats. J. Nutr., 130: 1963-1970.

Clarkson, P., 1995. Antioxidants and physical performance. Criti. Rev. Food Sci. Nutr., 35: 131-141.

Davies, K.J.A., A.T. Quintanilha, G.A. Brooks and L. Packer, 1982. Free radicals and tissue damage produced by exercise. Biochem. Biophys. Res. Commun., 107: 1198-1205.

Goldfarb, A.H., M.K. McIntosh, B.T. Boyer and J. Fatouros, 1994. Vitamin E effects on indexes of lipid peroxidation in muscle from DHEA-treated and exercised rats. J. Appl. Physiol., 76: 1630-1635.

Herbert. V., 1994. The antioxidant supplement myth. Am. J. Clin. Nutr., 60: 157-158.

Herbert. V., 1995. Editorial: Vitamin C supplements and disease-counterpoint. J. Am. Coll. Nutr., 14: 112-113.

Herbert. V., 1996. Symposium: prooxidant effects of antioxidant vitamins. Introduction. J. Nutr., 126: 1197S-1200S.

Hollman, P.C.H., 1997. Bioavailability of flavonoids. Eur. J. Clin. Nutr., 51: S66-S69.

Jenkins, R.R., 1993. Exercise, oxidative stress, and antioxidants: a review. Int. J. Sport Med., 3: 356-375.

Jha, H.C., V. Von Recklinghausen, and F. Zilliken, 1985. Inhibition of *in vitro* microsomal lipid peroxidation by isoflavnoids. Biochem. Pharmacol., 34: 1367-1369.

Ji. L.L., 1993. Antioxidant enzyme response to exercise and aging. Med. Sci. Sports Exerc., 25: 225-231.

Ji, L.L., 1995. Exercise and oxidative stress: role of cellular antioxidant systems. Exerc. Sport Sci. Rev., 23:135-166.

Ji. L.L., F.W. Stratman and H.A. Lardy, 1988. I. Enzymatic down regulation with exercise in rat skeletal muscle. II. Antioxidant enzyme systems in rat liver and skeletal muscle. Arch. Biochem. Biophys., 263: 137-160.

Ji. L.L., D. Dillon and E. Wu, 1990. Alternation of antioxidant enzymes with aging in rat skeletal muscle and liver. Am. J. Physiol., 258: R918-R923.

Ji, L.L., and R. Fu, 1992. Responses of glutathione system and antioxidant enzymes to exhaustive exercise and hydroperoxide. J. Appl. Physiol., 72: 549-554.

Kaczmarski, M., J. Wójcicki, T. Samochowiec, T. Dutkiewicz and Z. Sych, 1999. The influence of exogenous antioxidants and physical exercise on some parameters associated with production and removal of free radicals. Pharmazie., 54: 303-306.

Kapiotis, S., M. Hermann, I. Held, C. Seelos, H. Ehringer and B.M. Gmeiner, 1997. Genistein, the dietary-derived angiogenesis inhibitor, prevents LDL oxidation and protects endothelial cells from damage by atherogenic LDL. Arterioscler. Thromb. Vasc. Biol., 17: 2868-2874.

King, R.A. and D.B. Bursill, 1998. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. Am. J. Clin. Nutr., 67: 867-872.

Kose, K., P. Dogan and C. Ozesmi, 1993. Contraceptive steroids increase erythrocyte lipid peroxidation in female rats. Contraception, 47: 421-425.

Kurzer, M.S. and X. Xu, 1997. Dietary phytoestrogen. Annu. Rev. Nutr., 17: 353-381.

Lawler, J.M., S.K. Powers, T. Visser, H. Van Dijk, M.J. Korthuis and L.L. Ji, 1993. Acute exercise and skeletal muscle antioxidant and metabolic enzymes: effect of fiber type and age. Am. J. Physiol., 265: R1344-R1355.

Maxwell, S.R.J., P. Jakeman and H. Thomason, 1993. Changes in plasma antioxidant status during eccentric exercise and the effect of vitamin supplementation. Free Radic. Res. Commun., 19: 191-201.

Meydani, M. and W.J. Evans, 1993. Free radicals, exercise, and aging. In: Yu, B.P. (Eds.), Free Radical in Aging. CRC Press, Boca Raton, FL, pp: 183-204.

Novelli, G.P., S. Falsini and G. Bracciotti, 1991. Exogenous glutathione increases endurance to muscle effort in mice. Pharmacol. Res., 23: 149-155.

Ohno, H., Y. Sato, K. Yamashita, R. Doi, K. Arai, T. Kondo and N. Taniguchi, 1986. The effect of brief physical exercise on free radical scavenging enzyme systems in human red blood cells. Can. J. Physiol. Pharmacol., 64: 1263-1265.

Olinescu, R., D. Talaban, S. Nita and G. Mihaescu, 1995. Comparative study of the presence of oxidative stress in sportsmen in competition and aged people, as well as the preventive effect of selenium administration. Rev. Roum. Med. Int., 33: 47-54.

Packer, L., 1986. Oxygen radicals and antioxidants in endurance exercise. In: Benzi, S., L. Packer and N. Siliprandi (Eds.), Biochemical Aspects of Physical Exercise. Elsevier, New York, pp: 73-92.

Packer, L., 1997. Oxidants, antioxidant nutrients and the athlete. J. Sports Sci. 15: 353-363.

Pikul, J., D.E. Leszcynski and F.A. Kummerow, 1989. Evaluation of three modified TBA methods for measuring lipid peroxidation in chicken meat. J. Agric. Food Chem., 37: 1309-1313.

Pincemail, J., C. Deby, G. Camus, F. Pirnay, R. Bouchez, L. Massaux and R. Goutier, 1988. Tocopherol mobilization during intensive exercise. Eur. J. Appl. Physiol. Occup. Physiol., 57: 189-191.

Pizzichini, M., G. Cinci, M.L. Pandolfi, L. Arezzini and R. Pagani, 1993. Estradiol and phospholipid metabolism in the rat. Biochem. Soc. Trans., 21: 190S.

Powers, S.K., L.L. Ji and C. Leeuwenburgh, 1999. Exercise training-induced alterations in skeletal muscle antioxidant capacity: a brief review. Med. Sci. Sports Exerc., 31: 987-997.

Reddy, V.K., C.T. Kumar, M. Prasad and P. Reddanna, 1992. Exercise-induced oxidant stress in the lung tissue: role of dietary supplementation of vitamin E and selenium. Biochem. Int., 26: 863-871.

Sen, C.K., 1995. Oxidants and antioxidants in exercise. J. Appl. Physiol., 79: 675-686.

Singh, V.N., 1992. A current perspective on nutrition and exercise, J. Nutr., 122: 760-765.

Sokal, R.R. and F.J. Rohlf, 1995. Biometry, Three edition. W. H. Freeman Co. New York.

Somani, S.M. and C. Arroyo, 1995. Exercise training generates ascorbate free radicals in the rat heart. Indian J. Physiol. Pharmacol., 39: 323-329.

Somani, S.M., S. Frank and L.P. Rybak, 1995. Responses of antioxidant system to acute and trained exercise in rat heart subcellular fractions. Pharmacol. Biochem. Behav., 51: 627-634.

Sutton, J., P. Farrel and V. Harber, 1990. Hormonal adaptation to physical activity. In: Bouchard, C., R.J. Sephard, T. Stephens, J.R. Sutton and B.D. McPherson (Eds.), Exercise, Fitness and Health, a Consensus of Current Knowledge. Human Kinetics, Champaign, IL, pp: 217-258.

Tikkanen, M.J., K. Wähälä, S. Ojala, V. Vihma and H. Adlercreutz, 1998. Effect of soybean phytoestrogen intake on low density lipoprotein oxidation resistance. Proc. Natl. Acad. Sci. U S A., 95: 3106-3110.

Tiidus, P.M., 1995. Can estrogens diminish exercise induced muscle damage? Can. J. Appl. Physiol., 20: 26-38.

Tiidus, P.M., E. Bombardier, N. Hidiroglou and R. Madere, 1998. Estrogen administration, postexercise tissue oxidative stress and vitamin C status in male rats. Can. J. Physiol. Pharmacol., 76: 952-960.

Vasankari, T.J., U.M. Kujala, T.M. Vasankari, T. Vuorimaa and M. Ahotupa, 1997. Effects of acute prolonged exercise on-serum and LDL oxidation and antioxidant defenses. Free Radic. Biol. Med., 22: 509-513.

Wang, H. and P.A. Murphy, 1994. Isoflavone content in commercial soybean foods. J. Agric. Food Chem., 42: 1666-1673.

Wei, H., L. Wei, K. Frenkel, R. Bowen and S. Barnes, 1993. Inhibition of tumor promoter-induced hydrogen peroxide formation *in vitro* and *in vivo* by genistein. Nutr. Cancer, 20: 1-12.

Wei, H., R. Bowen, Q. Cai, S. Barnes and Y. Wang, 1995. Antioxidant and antipromotional effects of the soybean isoflavone genistein. Proc. Soc. Exp. Biol. Med., 208: 124-130.

Wiseman, H., 1999. The bioavailability of non-nutrient plant factors: dietary flavonoids and phyto-oestrogens. Proc. Nutr. Soc., 58: 139-146.

Xin, Z., D.F. Waterman, R.M. Hemken and R.J. Harmon, 1991. Effects of copper status on neutrophil function, superoxide dismutase, and copper distribution in steers. J. Dairy Sci., 74: 3078-3085.

Xu, X., H. Wang, P.A. Murphy, L. Cook and S. Hendrick, 1994. Daidzein is a more bioavailable soy milk isoflavone than is genistein in adult women. J. Nutr., 124: 825-832.

Zhang, Y., G-J. Wang, T.T. Song, P.A. Murphy and S. Hendrich, 1999. Urinary disposition of the soybean isoflavones daidzein, genistein, and glycitein differs among humans with moderate fecal isoflavone degradation activity. J. Nutr., 129: 957-962.

Abbreviations Key: CE: Control diet and Exercise, MDA: Malondialdehyde, ROS: Reactive Oxygen Species, SOD: Superoxide dismutase,

Pakistan Journal of Nutrition 1(1) : 8-19, 2002

Recent Advances in Minerals and Vitamins on Nutrition of Lactating Cows

Lee R. McDowell
Department of Animal Sciences, University of Florida,
P.O. Box 110910, Gainesville, FL 32611-0910, USA
E-mail: mcdowell@animal.ufl.edu

Abstract: Highly productive lactating cows have much greater needs for minerals and vitamins than low-producing animals. The antioxidant vitamins, vitamin E and β-carotene are beneficial in reducing mastitis. Vitamins (D, E, C and β-carotene) and minerals (Cu, Zn, and Se) are needed for an optimum immune response. Vitamin E is effective in counteracting gossypol toxicity. New research suggests the need for supplemental biotin for dairy cattle. Milk fever is best prevented with anionic diets. There is environmental concern from use of excess P. Grazing lactating cows should have access to high quality free-choice mineral mixtures.

Key words: Lactating cows, vitamins, minerals, nutritient values

Introduction

A great deal of information has recently become available for better nutrition strategies for feeding minerals and vitamins to livestock, including lactating cows. Textbooks and new publications are available for minerals (McDowell, 1992; 1999; McDowell and Valle, 2000). In 1999, a publication titled "Minerais para Ruminantes sob Pastejo em Regiões Tropicais, Enfatizando o Brasil" (McDowell, 1999) was available. This publication and a recent journal article (Tokarnia *et al*., 2000) review both older literature and the newest findings of mineral research in Brazil and specifies regions of Brazil with reported deficiencies. Due to the enormity of this subject, this report will emphasize newer information most applicable to lactating dairy cows. The report will attempt to discuss problems for high density dairy operations feeding considerable concentrates to smaller operations that are dependent on grazing cows that receive minimal or no concentrations.

Mineral and vitamin requirements for cattle: Mineral requirements and toxic levels and vitamin requirements suggested by the National Research Council (NRC) for lactating dairy cows are presented in Table 1. Mineral and vitamin requirements are highly dependent on the level of productivity. Increased growth rates and milk production will greatly increase mineral requirements. Improved management practices that lead to improved milk production and growth rates for cattle will necessitate more attention to mineral and vitamin nutrition. Marginal mineral and vitamin deficiencies, under low levels of production, become more severe with increased levels of production, and previously unsuspected nutritional deficiency signs usually occur as production levels increase.

Immunity and antioxidant roles of trace minerals and vitamins: Both trace minerals and vitamins play important roles in the health of cattle. For lactating dairy cows, nutrient supplementation for trace minerals and vitamins go beyond correcting for deficiencies but are aimed rather at minimizing stress and optimizing production efficiency.

Free radicals can be extremely damaging to biological systems (Padh, 1991). Also, phagocytic granulocytes undergo respiratory burst to produce oxygen radicals to destroy intracellular pathogens. However, these oxidative products can, in turn, damage healthy cells if they are not eliminated. Antioxidants serve to stabilize these highly reactive free radicals, thereby maintaining the structural and functional integrity of cells (Chew, 1995). Therefore, antioxidants are very important to immune defense and health of humans and animals.

Tissue defense mechanisms against free-radical damage generally include vitamin C, vitamin E, and β-carotene as the major vitamin antioxidant sources. In addition, several metalloenzymes which include glutathione peroxidase (Se), catalase (Fe), and superoxide dismutase (Cu, Zn, and Mn) are also critical in protecting the internal cellular constituents from oxidative damage. The dietary and tissue balance of all these nutrients are important in protecting tissues against free-radical damage. Both *in vitro* and *in vivo* studies show that these nutrients generally enhance different aspects of cellular and non-cellular immunity. The antioxidant function could, at least in part, enhance immunity by maintaining the functional and structural integrity of important immune cells. A compromised immune system will result in reduced animal production efficiency through increased susceptibility to diseases, thereby leading to increased animal morbidity and mortality.

Trace minerals: There are numerous potential sites for trace elements to affect immune function. Virtually every aspect of immunity involves Zn. A nutritional deficiency of Zn is consistently associated with increased morbidity and mortality (Kincaid, 1999). The immune response to many pathogens cause a rapid decline in blood Zn, perhaps a 50% drop within a few hours. Zinc deficiency is associated with reduced phagocytosis and killing by macrophages. Zinc deficiency results in a decrease in blood lymphocyte population (Fraker and King, 1998) and atrophy of the spleen and thymus. The responsiveness of T-lymphocytes to mitogens (Droke *et al*., 1993) and the cytokines (Tanaka *et al*., 1990) is inhibited in zinc-deficient animals. Zinc also is important in B-cell activation. Calves fed zinc methionine had greater antibody response against bovine herpesvirus (Spears *et al*., 1991). Zinc as Zn methionine has reduced somatic cell counts (SCC) about 22% in some trials (Kincaid *et al*., 1999).

Copper deficiency reduces the number of circulating T cells, B cells, and neutrophils. Impairment of bactericidal activity can occur early in the development of Cu deficiency in cattle and sheep. Copper-deficient sheep had increased mortality from bacterial infection (Chew, 2000). Antibody titers to *Brucella abortus* and proliferation responses to concanavalin A and soluble antigen-stimulated mononuclear cells were lower in

Table 1: Suggested mineral and vitamin requirements and trace element toxicities for lactating dairy cows (dry basis)

Required elements	Lactating dairy cows[a] Suggested value
Macroelements (%)	0.43 - 0.77
Calcium	0.25 - 0.49
Phosphorus (P)	0.20 - 0.25
Magnesium (Mg)	0.90 - 1.00
Potassium (K)	0.18
Sodium (Na)	0.20 - 0.25
Sulfur (S)	
Microelements (mg/kg)	
Cobalt (Co)	0.1
Copper (Cu)	10.0
Iodine (I)	0.6
Iron (Fe)	50.0
Manganese (Mn)	40.0
Molybdenum (Mo)	---
Selenium (Se)	0.3
Zinc (Zn)	40.0
Vitamins (IU/kg)	
Vitamin A	3200
Vitamin D	1000
Vitamin E	15
Toxic Elements[b] (mg/kg)	
Copper (Cu)	80
Fluorine (F)	30
Molybdenum (Mo)	6
Selenium (Se)[c]	5
Zinc (Zn)	500

[a]National Research Council (1989)
[b]National Research Council (1980)
[c]McDowell (1992)

Cu-deficient heifers (Cerone *et al.*, 1995). Ceruloplasmin, an acute-phase protein, did not have the normal post-inoculant (bovine herpesvirus-1) increase in Cu-deficient calves (Arthington *et al.*, 1996).

Selenium deficient animals have impaired bactericidal activity. Selenium, as part of the enzyme glutathione peroxidase, protects the cytosol against peroxides produced during respiratory burst. There may be an increase in reactive oxygen species in Se deficiency and this could affect immune responses (Kincaid, 1999). Accordingly, cows supplemented with Se have neutrophils in milk with increased intracellular kill of bacteria, and reduced extracellular hydrogen peroxide concentrations. Mean plasma selenium concentration was inversely correlated with bulk tank (SCC in dairy herds (Weiss *et al.*, 1990).

Vitamins: In addition to the relationship of Se to Vitamin E, Vitamin E, Vitamin C and β-carotene as antioxidant vitamins together have important tissue defense mechanisms against free-radical damage. Even Vitamin D [1,25 $(OH)_2$ D_3] has been shown to booster humoral responses to vaccines (Reinhardt *et al.*, 1999). Vitamin A, not so much as an antioxidant, has important immune functions.

Vitamin A deficiency affects immune function, particularly the antibody response to T-cell-dependent antigens (Ross, 1992). The RAR-αmRNA expression and antigen-specific proliferative responses to T lymphocytes are influenced by vitamin A status *in vivo* and are directly modulated by retinoic acid (Halevy *et al.*, 1994). Vitamin A deficiency affects a number of cells of the immune system, and that repletion with retinoic acid effectively reestablishes the number of circulating lymphocytes (Zhao and Ross, 1995).

A diminished primary antibody response could also increase the severity and/or duration of an episode of infection, whereas a diminished secondary response could increase the risk of developing a second episode of infection. Vitamin A deficiency causes decreased phagocytic activity in macrophages and neutrophils. The secretory immunoglobulin (Ig) A system is an important first line of defense against infections of mucosal surfaces (McGhee *et al.*, 1992). Several studies in animal models have shown that the intestinal IgA response is impaired by vitamin A deficiency (Wiedermann *et al.*, 1993; Stephensen *et al.*, 1996).

Vitamin A-deficient cattle have depressed activity of natural killer cells, decreased antibody production, decreased responsiveness of lymphocytes to mitogenic stimulation, and increased susceptibility to infection (Ross, 1992; Nonnecke *et al.*, 1993; Michal *et al.*, 1994; Rajaraman *et al.*, 1998).

Carotenoids have been shown to have biological actions independent of vitamin A (Chew, 1995; Burton, 1989; Aréchiga *et al.*, 1998). University of Florida research indicated significantly increased milk production in dairy cows receiving supplemental β-carotene (Aréchiga *et al.*, 1998). In this large, three-part study using intensive management, the supplemented cows (400 mg/day) produced from 6.2 to 11.3 percent more milk than the unsupplemented controls. Also, for cows fed supplemental β-carotene for ≥90d, pregnancy rate at 120 d postpartum was increased in Experiment 1 (35.4% vs. 21.1%).

Recent animal studies indicate that certain carotenoids with antioxidant capacities, but without vitamin A activity, can enhance many aspects of immune functions, can act directly as antimutagens and anticarcinogens, can protect against radiation damage, and can block the damaging effects of photosensitizers. β-carotene can function as a chain-breaking antioxidant, it deactivates reactive chemical species such as single oxygen, triplet photochemical sensitizers and free radicals which would otherwise induce potentially harmful processes (e.g., lipid peroxidation).

Vitamin A and β-carotene have important roles in protecting animals against numerous infections including mastitis. Potential pathogens are regularly present in the teat orifice, and under suitable circumstances can invade and initiate clinical mastitis. Any unhealthy state of the epithelium would increase susceptibility of a mammary gland to invasion by pathogens. There are reports of improved mammary health in dairy cows supplemented by β-carotene and vitamin A during the dry (Dahlquist and Chew, 1985) and lactating (Chew and Johnston, 1985) periods.

Polymorphonuclear neutrophils (PMN) are the major line of defense against bacteria in the mammary gland. β-carotene supplementation seems to exert a stabilizing effect on PMN and lymphocyte function to the period around dry off (Tjoelker *et al.*, 1990). Daniel *et al.* (1991a, b) reported that β-carotene enhanced the bactericidal activity of blood and milk PMN, against *S. aureus* but did not affect phagocytosis. Vitamin A either had no effect or suppressed bactericidal activity and phagocytosis. Control of free radicals is important for bacterial activity but not for phagocytosis. The antioxidant activity of vitamin A is not important; it does not quench or remove free radicals. β-carotene, on the other hand, does have significant antioxidant properties and effectively quenches singlet oxygen free radicals (Mascio *et al.*, 1991; Zamora *et al.*, 1991).

One of the protective effects of vitamin C may partly be mediated through its ability to reduce circulating glucocorticoids (Degkwitz, 1987). The suppressive effect of

corticoids on neutrophil function in cattle was alleviated with vitamin C supplementation (Roth and Kaeberle, 1985). In addition, ascorbate can regenerate the reduced form of α-tocopherol, perhaps accounting for observed sparing effect of these vitamins (Jacob, 1995). In the process of sparing fatty acid oxidation, tocopherol is oxidized to the tocopheryl free radical. Ascorbic acid can donate an electron to the tocopheryl free radical, regenerating the reduced antioxidant form of tocopherol.

Vitamin C is the most important antioxidant in extracellular fluids and can protect biomembranes against lipid peroxidation damage by eliminating peroxyl radicals in the aqueous phase before the latter can initiate peroxidation (Frei *et al.*, 1989). Vitamin C and E supplementation resulted in a 78% decrease in the susceptibility of lipoproteins to mononuclear cell-mediated oxidation (Rifici and Khachadurian, 1993). Ascorbic acid is very high in phagocytic cells with these cells using free radicals and other highly reactive oxygen containing molecules to help kill pathogens that invade the body. In the process, however, cells and tissues may be damaged by these reactive species. Ascorbic acid helps to protect these cells from oxidative damage.

Considerable attention is presently being directed to the role vitamin E and Se play in protecting leukocytes and macrophages during phagocytosis, the mechanism whereby animals immunologically kill invading bacteria. Both vitamin E and Se may help these cells to survive the toxic products that are produced in order to effectively kill ingested bacteria (Badwey and Karnovsky, 1980). Macrophages and neutrophils from vitamin E-deficient animals have decreased phagocytic activity.

Since vitamin E acts as a tissue antioxidant and aids in quenching free-radicals produced in the body, any infection or other stress factors may exacerbate depletion of the limited vitamin E stores from various tissues. The protective effects of vitamin E on animal health may be involved with its role in reduction of glucocorticoids, which are known to be immunosuppressive. Vitamin E also most likely has an immune enhancing effect by virtue of altering arachidonic acid metabolism and subsequent synthesis of prostaglandin, thromboxanes and leukotrienes. Under stress conditions, increased levels of these compounds by endogenous synthesis or exogenous entry may adversely affect immune cell function (Hadden, 1987).

The effects of vitamin E and Se supplementation on protection against infection by several types of pathogenic organisms, as well as antibody titers and phagocytosis of the pathogens have been reported for calves (Reddy *et al.*, 1987a). As an example, calves receiving 125 IU of vitamin E daily were able to maximize their immune responses compared to calves receiving low dietary vitamin E (Ready *et al.*, 1987b). Antioxidants, including vitamin E, play a role in resistance to viral infection. Vitamin E deficiency allows a normally benign virus to cause disease (Beck *et al.*, 1994). A Se or vitamin E deficiency leads to a change in viral phenotype, such that an avirulent strain of a virus becomes virulent and a virulent strain becomes more virulent (Beck, 1997).

Supplemental levels of vitamin E higher than recommended by the dairy cattle NRC (1989) have been beneficial in the control of mastitis. Smith and Conrad (1987) reported that intramammary infection was reduced 42.2% in vitamin E-selenium supplemented versus unsupplemented controls. The duration of all intramammary infections in lactation was reduced 40 to 50% in supplemented heifers. Weiss *et al.* (1990) reported that clinical mastitis was negatively related to plasma Se concentration and concentration of vitamin E in the diet.

Many new intramammary infections (IMI) occur in the 2 weeks before and after calving. Deficiencies of either vitamin E or Se have been associated with increased incidence and severity of IMI, increased clinical mastitis cases, and higher somatic cell counts (SCC) in individual cows and bulk tank milk. Somatic cell counts are a primary indicator of mastitis and milk quality in dairy herds. The polymorphonuclear neutrophil (PMN) is a major defensive mechanism against infection in the bovine mammary gland. A known consequence of vitamin E and Se deficiency is impaired PMN activity and postpartum vitamin E deficiencies are frequently observed in dairy cows. Dietary supplementation of cows with Se and vitamin E results in a more rapid PMN influx into milk following intramammary bacterial challenge and increased intracellular kill of ingested bacteria by PMN. Subcutaneous injections of vitamin E approximately 10 and 5 d before calving successfully elevated PMN α-tocopherol concentrations during the periparturient period and negated the suppressed intracellular kill of bacteria by PMN that commonly is observed around calving (Smith *et al.*, 1997).

Diets of multiparous dairy cows were supplemented with either 0 or 1,000 IU vitamin E (as *dl*- α-tocopheryl acetate) during the dry period (Smith *et al.*, 1984). Cows were additionally administered Se at the rate of 0 or 0.1 mg per kg body weight via i.m. injection 21 days prepartum. No vitamin E or Se were supplemented during lactation. Incidence of new clinical cases of mastitis was reduced by 37% in both groups receiving vitamin E compared to controls. The reduction in clinical mastitis was only 12% when cows were injected with Se but not supplemented with dietary vitamin E. These authors also reported that clinical cases in the vitamin E supplemented-selenium injected cows were consistently of shorter duration than those occurring in all other groups. Erskine *et al.* (1989) investigated specific effects of Se status of dairy cattle on the induction of mastitis by *E. coli*. Bacterial concentrations were significantly higher in Se-deficient than in Se-adequate cows and Se supplementation reduced both severity and duration of clinical mastitis.

Plasma concentrations of α-tocopherol decreased at calving for cows fed dietary treatments with low or intermediate concentrations of vitamin E, but not for cows fed the high vitamin E treatment (Weiss *et al.*, 1997). High dietary vitamin E increased concentration of α-tocopherol in blood neutrophils at parturition. The high vitamin E treatment was 1,000 IU/d of vitamin E during the first 46 d of the dry period, 4,000 IU/d during the last 14 d of the dry period, and 2,000 IU/d during lactation.

The percentage of quarters with new infections at calving was not different (32.0%) between cows receiving treatments that contained low and intermediate concentrations of vitamin E but was reduced (11.8%) in cows receiving the high vitamin E treatment. Clinical mastitis affected 25.0, 16.7 and 2.6% of quarters during the first 7 d of lactation for cows receiving the low, intermediate, and high vitamin E treatments, respectively. Cows with plasma concentrations of α-tocopherol > 3.0 μg/ml at calving were 9.4 times more likely to have clinical mastitis during the first 7 d of lactation than were cows with plasma concentrations of α-tocopherol < 3.0 μg/ml (Weiss *et al.*, 1997).

Additional benefits of vitamin E supplementation: There have been more recent reports on benefits of vitamin E supplementation for livestock than any other vitamin (McDowell *et al.*, 1996). Vitamin E was originally supplemented to poultry and livestock for prevention of exudative diathesis, encephalomalacia, white muscle disease,

Table 2: Relationship of gossypol and vitamin E on semen characteristics of dairy bulls[a]

	Treatment		
Item	TRT[b]	TRT2[c]	TRT3[c]
Normal, %	64.7 ± 6.4[h]	31.4 ± 7.4[i]	54.6 ± 6.4[h]
Abnormal[e], %	4.4 ± 1.3[h]	13.4 ± 1.5[i]	4.8 ± 1.2[h]
DSPG[f] (x10^6/g)	14.6 ± 1.0[h]	10.2 ± 1.0[i]	17.6 ± 1.0[h]
DSP[g] (x10^9)	3.2 ± 3.0[h]	2.2 ± 3.0[i]	4.1 ± 3.0[h]

[a] Least square means ± SEM. [b]Diet based on SBM, corn and 30 IU vitamin E/kg of supplement. [c]Diet containing 14 mg free gossypol/kg BW/d and 30 IU vitamin E/kg of supplement. [d]Diet containing 14 mg free gossypol/kg BW/d and 4,000 IU vitamin E/bull/d. [e]Midpiece abnormalities evaluated in isotonic formal saline. [f]Daily sperm production per gram of parenchyma. [g]Daily sperm production total. [h,i]Means in a row with different superscript differ $p < 0.05$.

liver degeneration and other degenerative diseases. Recent research has revealed the benefits of improving disease resistance (see previous section) as well as improving product quality. Supplementing vitamin E in well balanced diets has been shown to increase humoral immunity for ruminants (Hoffmann-La Roche, 1994). These results suggest that the criteria for establishing requirements based on overt deficiencies or growth do not consider optimal health.

Recent research has shown a beneficial response for vitamin E supplementation on male reproduction for Holstein bulls fed high concentrations of gossypol. Velasquez-Pereira *et al*. (1998) reported that bulls which received 14 mg free gossypol/kg body weight had a lower ($p < 0.05$) percentage of normal sperm than those which also received supplemental vitamin E, 31 vs 55%, respectively (Table 2). Likewise, sperm production per gram of parenchyma and total daily sperm production were higher ($p < 0.05$) when gossypol treated animals also received vitamin E. Bulls receiving gossypol exhibited more sexual inactivity ($p > 0.05$) than bulls in other treatments. Vitamin E supplementation to bulls receiving gossypol improved number of mounts in the first test and time of first service in the second test. The final conclusion of the Florida data is that vitamin E is effective in reducing or eliminating important gossypol toxicity effects for male cattle.

Many attempts have been made to control lipid oxidation in meats through the use of antioxidants. Dietary supplementation of vitamin E, and intravenous infusion of vitamin C immediately before harvest, are efficacious techniques for increasing the concentration of these vitamins in beef skeletal muscle (Schaefer *et al*., 1995). Meat with elevated levels of either and probably both of these antioxidant vitamins possesses greater stability of oxymyoglobin and lipid, which results in less discoloration and rancidity. Vitamin E would seem to be the most practical since it is administered dietetically.

Dramatic effects of vitamin E supplementation (500 IU per head daily) to finishing steers on the stability of beef color have been observed (Faustman *et al*., 1989). Loin steaks of control steers discolored two to three days sooner than those supplemented with vitamin E. Supplemental dietary vitamin E extended the color shelf life of loin steaks from 3.7 to 6.3 days. This was most likely due to the increased α-tocopherol content of the loin tissue of the supplemented animals, which was approximately 4-fold greater than controls (Faustman *et al*., 1989). Color is an extremely critical component of fresh red meat appearance and greatly influences the consumer perception of meat quality.

Feeding supplemental vitamin E at levels of 1,000 to 2,000 mg of naturally-occurring mixed tocopherols per cow per day increased the vitamin E content of milk and its stability against oxidized flavor (Neilsen *et al*., 1953). The vitamin E content of milk from cows fed stored feeds was lower than that of milk from cows on pasture and their milk was more susceptible to development of oxidized flavor. Feeding supplemental vitamin E as *dl*- α-tocopheryl acetate, providing an equivalent of 500 mg of *dl*- α-tocopheryl per cow per day, increased the vitamin E content and oxidative stability of milk (Dunkley *et al*., 1967). Nicholson *et al*. (1991) suggest that adequate selenium improves the transfer of dietary tocopherol to milk.

The ability of vitamin E to affect growth, health and reproduction of animals is documented. A vitamin E supplementation program utilizing both parenteral and oral administration is often suggested, particularly when fresh green pasture is lacking.

The need for supplementation of vitamin E is dependent on conditions of production, and in relation to available vitamin E in food or feed sources. The primary factors that influence the need for supplementation include (1) vitamin E- and or Se-deficient concentrates and roughages; (2) excessively dry ranges or pastures for grazing livestock; (3) confinement feeding where vitamin E-rich forages are not included or only forages of poor quality are provided; (4) diets that contain predominantly non-α-tocopherol and thereby are less biologically active; (5) diets that include ingredients that increase vitamin E requirements (e.g., unsaturated fats, waters high in nitrates); (6) harvesting, drying, or storage conditions of feeds that result in destruction of vitamin E and/or selenium; (7) accelerated rates of gain, production and feed efficiency that increase metabolic demands for vitamin E; and (8) intensified production that also indirectly increases vitamin E needs of animals by elevating stress, which often increases susceptibility to various diseases (McDowell, 1992; 2000). After stress, livestock may have reductions in α-tocopherol concentrations in certain tissues. Supplemental vitamin E may be required after stress to restore α-tocopherol in tissues (Nockels *et al*., 1996).

Biotin, niacin and thiamin: Due to ruminal and intestinal synthesis of biotin, a need for supplemental sources was at one time not expected for ruminants. Nevertheless, promising preliminary results in preventing lameness in dairy cattle with biotin supplementation were reported (Frigg *et al*., 1993). Hoof disease is considered by many experts in dairy cattle health to be the most costly health problem in the dairy industry. Hoof disorders occur in both confinement and pasture-based dairy production systems, and result in considerable economic loss to the dairy industry. In the U.S., it is estimated that hoof disorders cost an average of $345 per case in treatment and lost milk production. Successful biotin treatment of dairy cows with claw problems has been reported (Midla *et al*., 1998; Fitzgerald *et al*., 2000). From Australia (Fitzgerald *et al*., 2000), biotin-supplemented herds exhibited better locomotion scores than the unsupplemented herds. In the wet summer period, the number of lame cows, as observed by the farmer, were significantly fewer during the rainy period for the biotin-supplemented herds and required fewer antibiotic treatments than unsupplemented herds.

In biotin-deficient dairy cows, the hoof horn is of poor quality, soft, and crumbling, with no distinct separation of keratinizing and cornified cells. This results in the omission of the granular layer at the epidermis of the bulb of the heel. Decreased stabilizing filaments in the upper spiny layer of the hoof corium in biotin-deficient cows reveals the depressed hormone-like activity of biotin in the synthesis of protein.

Increased plasma biotin levels have been associated with hardness and conformational changes in the bovine hoof (Higuchi and Nagahata, 2000). Dairy cows supplemented with

20 mg of biotin per cow over an 11-month period expressed a steepened angle of the dorsal border and height of the heel; length of the diagonal and size of the ground surface increased (Distl and Schmid, 1994). The hardness of the hoof was also significantly greater in the biotin-treated group. Feeding dairy and beef cows 20 mg/day of supplemental biotin not only resulted in reduced incidence of hoof lesions but also increased milk production (Zimmerly, 2000). Zimmerly (2000) indicated that biotin supplementation increased milk and milk protein yield.

"Fatty Liver" syndrome is a metabolic disorder that occurs primarily around the days surrounding parturition when energy intake is low. Low energy intake causes the cow to mobilize fat from its adipose tissue and deposit it in the liver. The consequences of a fatty liver can be significant. A fatty liver is less able to perform its necessary functions such as to synthesize glucose, detoxify ammonia to urea, and oxidize fat to obtain energy. Cows with fatty liver are more likely to develop ketosis and suffer milk production losses.

During development of fatty liver, the rate of gluconeogenesis and key rate-limiting enzyme activities may be suboptimal (Rukkwamsuk *et al.*, 1999). If hepatic lipidosis occurs, a number of multi-factorial associated diseases such as ketosis, retained placenta, metritis, milk fever, mastitis and even laminitis may appear at parturition (Bruss, 1993; Breukink and Wensing, 1998). On the basis of a positive effect of biotin on reducing fatty liver conditions in other species, current University of Florida research is studying the effect of supplemental biotin on reducing fatty liver syndrome in lactating Holsteins. We hypothesized that short-term supplemental biotin to dry and periparturient cows might alter the occurrence of metabolic disorders such as fatty liver and ketosis through regulatory effects of the products from hepatic biotin-dependent carboxylases.

Niacin is the most common water-soluble vitamin added to dairy cow diets. One report indicated that about 50% of dairy cows in high-production herds go through borderline ketosis during early lactation. The concentration of β-hydroxybutyrate was reduced from 1.24 to 0.74 mmol/L after 5 days, when 10 g of niacin was given to cows with ketosis (Flachowsky *et al.*, 1988). Fronk and Schultz (1979) indicated that treating ketotic dairy cows with 12 g of nicotinic acid daily had a beneficial effect on the reversal of both subclinical and clinical ketosis. Other studies indicate that 6 g of niacin may be sufficient (Hoffmann-La Roche, 1994). Klippel *et al.* (1993) reported that supplemental niacin decreased milk short- and medium-chain fatty acids and increased monounsaturated fatty acids. Feeding cows niacin has been shown to correct fat-induced milk protein depression (Driver *et al.*, 1990; Cervantes *et al.*, 1996).

Thiamin deficiency is common in feedlot cattle fed high concentrate diets, with the condition (nervous disorder) referred to as polioencephalomalacia (PEM). The condition affects mainly calves and young cattle between 4 months and 2 years old. The incidence of PEM is reported to be between 1 and 20%, and mortality may reach 100%. Clinical signs in mild cases include dullness, blindness, muscle tremors (especially of the head) and opisthotonos. High-sulfur diets and antithiamin compounds (e.g., thiaminases) are associated with thiamin deficiency and PEM (Gould, 1998; McDowell, 2000). Lactating dairy cows seem less affected by PEM.

Milk fever (parturient paresis) relationship to CA, P and vitamin D: Milk fever (parturient paresis) in dairy cows is caused by a temporary imbalance between Ca availability and high Ca demand following the onset of lactation (Oetzel, 1996). Calcium leaves the extracellular fluid to enter the mammary gland faster than it can be replaced by intestinal Ca absorption or bone Ca resorption (Goff and Horst, 1993). Despite much research, milk fever incidence has remained steady in the United States at 8 to 9%. Milk fever is an economically important disease and can reduce the productive life of a dairy cow by 3.4 years. Each case of milk fever leads to a loss of $334 to the producer by way of treatment charges and milk loss (Horst *et al.*, 1997). If left untreated, about 60 to 70% of cows die.

Aged cows are at the greatest risk of developing milk fever. Heifers almost never develop milk fever. Older animals have a decreased response to dietary Ca stress due to both decreased production of 1,25-$(OH)_2D_2$ and decreased response to the 1,25-$(OH)_2D$ Target tissues of cows with milk fever may have defective hormone receptors, and the number of receptors declines with age. In older animals, fewer osteoclasts exist to respond to hormone stimulation, which delays the ability of bone to contribute Ca to the plasma Ca pool. The aging process is also associated with reduced renal 1α-hydroxylase response to Ca stress, therefore reducing the amount of 1,25-$(OH)_2D$ produced from 25-OHD (Goff *et al.*, 1991).

Special Ca and P supplementation is required for high-producing dairy cows to prevent parturient paresis. Parturient paresis can be prevented effectively by feeding a prepartum diet low in Ca and adequate in P. Prepartal low-Ca diets are associated with increased plasma parathyroid hormone (PTH) and 1,25-$(OH)_2D_2$ and 1,25-$(OH)_2D_3$ concentrations during the prepartal period. These increased PTH and 1,25-$(OH)_2D$ concentrations resulted in "prepared" and effective intestinal and bone Ca homeostatic mechanisms at parturition that prevented parturient paresis.

Supplemental vitamin D has been used to prevent parturient paresis in dairy cows for a number of years. Treatment with high levels of vitamin D has been successful, but toxicity problems have sometimes resulted, and for some animals, the disease has been induced by treatment. Hodnett *et al.* (1992) used a combination of 25-OHD_3 plus 1α-hydroxycholecalciferol to reduce parturient paresis in dairy cows fed high dietary Ca. The incidence of the disease was reduced from 33 to 8%.

Anion-cation balance of prepartum diets (sometimes referred to as acidity or alkalinity of a diet) can also influence the incidence of milk fever (Gaynor *et al.*, 1989; Horst *et al.*, 1997; Pehrson *et al.*, 1999; Vagnon and Oetzel, 1998). Diets high in cations, especially Na and K, tend to induce milk fever, but those high in anions, primarily C1 and S, can prevent milk fever. The incidence of milk fever depended on the abundance of the cations Na^+ and K^+ relative to the anions $C1^-$ and SO_4^{2-}. This concept is now generally referred to as the cation-anion difference (CAD). Because most legumes and grasses are high in K, many of the commonly used prepartum diets are alkaline. There are large variations in the mineral content of roughages fed on different farms, and that the mineral content of grass, and consequently, the CAD of a diet can be significantly altered by different types of fertilization (Pehrson *et al.*, 1999). Addition of anions to a prepartal diet is thought to induce in the cow a metabolic acidosis, which facilitates bone Ca resorption and intestinal Ca absorption (Horst *et al.*, 1997). Diets higher in anions increase osteoclastic bone resorption and synthesis of 1,25-$(OH)_2D_3$ in cows (Goff *et al.*, 1991). Both of these physiologic processes are controlled by PTH. Workers at the Rowett Research Institute (Abu Damir *et al.*, 1994) have also recently reported that 1,25-$(OH)_2D_3$ production is enhanced in cows fed acidifying diets.

Collectively, these data suggest that a major underlying cause of milk fever is metabolic alkalosis, which causes an inability

of cow tissues to respond adequately to PTH (Horst *et al*., 1997). This lack of response in turn reduces the ability of the cow to draw on bone Ca stores, and production of the second Ca-regulating hormone, 1,25-$(OH)_2D$, which is needed for active transport of Ca within the intestine. The presumption is that metabolic alkalosis somehow disrupts the integrity of PTH receptors on target tissues. Low CAD diets prevent metabolic alkalosis, increasing target tissue responsiveness to PTH, which controls renal 1α-hydroxylase and resorption of bone calcium.

Several options exist regarding methods for the control of milk fever (Horst *et al*., 1997). The current understanding of the CAD concept suggests that milk fever could be managed more effectively if dietary K as reduced (Goff and Horst, 1997). Calcium chloride has been used to reduce blood pH (Dhiman and Sasidharan, 1999; Schonewille *et al*., 1999b). This reduction is beneficial but excessive oral calcium chloride can induce metabolic acidosis (Goff and Horst, 1994), which can cause inappetence at a time when feed intake is already compromised. Dietary acidity can be monitored via the pH of urine, which should be below 7.5. Calcium propionate treatment has been beneficial in reducing subclinical hypocalcemia in all trials and reduced the incidence of milk fever in a herd having a problem with milk fever (Goff *et al*., 1996; Pehrson *et al*., 1998). Commercial preparations of HC1 mixed into common feed ingredients as a premix could offer an inexpensive and palatable alternative to anionic salts as a means of controlling the incidence of milk fever in dairy cows (Goff and Horst, 1998).

Treatment of milk fever returns serum Ca concentration to the normal range and must be carried out at the earliest possible opportunity to avoid muscular and nervous damage of downer cows. This is facilitated by maintaining close surveillance over cows that have calved in the preceding 72 hr. Calcium borogluconate is most commonly used, with Mg added to the injectable Ca preparation when hypomagnesemia is in evidence. The produce is preferably administered intravenously for rapid response, but subcutaneous administration permits slow absorption of the Ca ion and may lessen the danger of cardiac arrest.

Dietary mineral buffers: Mineral buffers are included in cow diets to improve lacational performance, milk composition and a favorable acid-base balance. Buffers are used in the diets of dairy cows to combat milk fat depression. Downer and Cummings (1987) estimated that more than 50% of all dairy farms in the US may be using dietary buffers, such as $NaHCO_3^-$, for this purpose. Researchers have concluded that responses to dietary buffers occur via reduced ruminal acidity and subsequent improvements in systemic acid-base status, particularly during sudden ration changes.

Schneider *et al*. (1986) hypothesized that responses of lactating cows to dietary buffers are the result of both the HCO_3 (buffering effect) and Na moiety (solute effect). Russell and Chow (1993) suggested that bicarbonates function not by increasing ruminal buffering capacity, but by increasing water intake, ruminal fluid dilution, and flow of undegraded starch and by reducing ruminal propionate production (Staples and Lough, 1989).

Metabolic acidosis is a complicating factor in a number of diseases that affect cattle, including ketoacidosis, lactic acidosis (grain overload), enterotoxigenic diarrhea of calves, and some enteric diseases of adult cattle. Treatment with $NaHCO_3$, i.v. or orally, is an effective method to restore blood pH to normal (Kasari, 1990; Roussel, 1990).

Orally administered $NaHCO_3$ and Na propionate were equally effective in correcting the acid-base balance of blood (Bigner *et al*., 1997). Sodium propionate may be considered a more effective treatment of metabolic acidosis in diseases such as ketosis because the added propionate can serve as a source of glucose for the cow.

Phosphorus and environmental concerns: Increasing environmental concerns and proposed regulations have also stimulated renewed interest in the role of P in dairy cattle and feedlot rations (Spears, 1996; Erickson *et al*., 1999; Satter and Wu, 1999; Valk and Sebek, 1999). Environmental regulation, which limits the quantity of P applied to land, are either in place or are being considered in a number of countries.

Society wishes to maintain a reasonable level of productivity in lakes and rivers, but this requires the presence of very low levels of mineral nutrients. Several bodies of water have progressed from oligotrophic conditions (low mineral and high dissolved oxygen) to mesotrophic conditions and finally to eutrophic conditions (high mineral and low dissolved oxygen). Eutrification is the over enrichment of surface water with mineral nutrients. This results in the excessive production of algae and cyanobacteria. High levels of algae and cyanobacteria, with their high respiration rates, lead to low levels of dissolved oxygen, which in turn leads to a loss of aquatic animals (Correll, 1999). Phosphorus is the limiting element for freshwater algae and aquatic plants; therefore, P is the limiting nutrient for the eutrification process. Most discussions of pollution focus on reduction techniques in excreta. Phosphorus-reducing techniques center on better knowledge of the requirements and using more available sources of P. Excessive field trials in the UK and The Netherlands have shown that P surplus can be reduced by up to 90% by dietary manipulations without apparent detrimental effect on milk production (Valk *et al*., 2000). Recently published data showed dietary P level of 80% of current feeding practices had no effect on milk production or reproductive performance (Wu and Satter, 2000).

An additional environmental concern relates to fertilization with biosolids (municipal sewage sludge). The use of biosolids as pasture fertilizer is of interest to animal scientists because some contain high Mo, as well as other metals, which could be absorbed by plants and ingested by grazing species and thereby induce toxicity. Use of biosolids as fertilizer could prove beneficial, if they increase the often-deficient mineral status of tropical forages without creating an environment in which plants could accumulate excessive levels of undesirable metals.

Tiffany *et al*. (2000) reported mineral status of cattle that grazed forage fertilized with two high Mo (12 or 33 ppm) containing biosolids for 176 days. Forage Mo uptake was low due to good drainage and acid soils. However, resulting forages contained high S (>0.4%) which significantly reduced animal Cu status (liver and plasma). High dietary S reduces Cu absorption, possibly due to unabsorbable Cu sulfide formation, independent from its part in thiomolybdate complexes (Underwood and Suttle, 1999). If cattle are to graze pastures treated with high levels of biosolids where forages with low Cu status are grown, Cu supplementation is essential.

Providing free-choice minerals to grazing lactating cows: The most efficient methods of providing supplemental minerals in through use of mineral supplements combined with concentrates. This assures an adequate intake of mineral elements by each animal as it consumes other nutrients. This procedure represents an ideal system for providing

supplemental minerals to lactating cows under more intensive production system but it cannot be used with grazing cattle which receive little concentrates and depend on forages.

Cattle not consuming concentrates are less likely to receive an adequate mineral supply; free-choice minerals are much less palatable than concentrates and are often consumed irregularly. Intakes of free-choice mineral mixtures by grazing cattle are highly variable and not related to mineral requirements (McDowell, 1985, 1999). Factors that affect the consumption of mineral mixtures have been listed by Cunha *et al*. (1964) and McDowell (1992, 1999) as follows: (1) soil fertility and forage type consumed, (2) season of year, (3) available energy-protein supplements, (4) individual requirements, (5) salt content of drinking water, (6) palatability of mineral mixture, (7) availability of fresh mineral supplies, and (8) physical form of minerals.

Biological availability of mineral sources: There is considerable difference in the availability of a mineral element provided from different sources. The bioavailability and percentage of mineral elements in inorganic sources commonly used in mineral supplements have been reported (McDowell, 1999). These variations in bioavailability of sources must be taken into consideration when evaluating or formulating a mineral supplement.

Excellent reviews on the significance of chelates and complexes of minerals for the feed industry have been prepared (Nelson, 1988; Kincaid, 1989; Patton, 1990; Spears *et al*., 1991). In a review, Spears (1991) concluded that the use of certain organic trace mineral complexes or chelates in ruminant diets has increased performance (growth and milk production), carcass quality and immune responses and decreased somatic cell counts in milk compared with animals fed inorganic forms of the mineral. Trace minerals sequestered as amino acid or polysaccharide complexes have the highest biological availability and also have a higher stability and solubility. These mineral forms also have a lack of interaction with vitamins and other ions and are effective at low levels. In cases where there is high dietary Mo, Cu in chelated form would have an advantage over an inorganic form as it may escape the complexing that occurs in the digestive system among Mo, Cu and S (Nelson, 1988).

Some studies have shown no benefit from chelated and complex minerals, but most have shown positive responses when compared to inorganic sources. Zinc and Cu complexed with proteins or amino acids, such a methionine or lysine, tended to have an advantage over inorganic forms of trace elements when given to stressed cattle.

Weaning weights were higher for zinc methionine and manganese methionine supplemented calves compared to control or oxide supplemented calves (Spears and Kegley, 1991). Herrick (1989) reviewed zinc-methionine feeding in four dairy trials and concluded that the Zn complex treated animals had lower somatic cell counts and higher milk yields than control cows.

Kincaid *et al*. (1986) compared copper proteinate and copper sulfate in terms of their ability to increase copper status in calves fed a diet naturally high in Mo (3.1 ppm) and low in Cu (2.8 ppm). Calves fed Cu proteinate had higher plasma (0.87 vs. 0.75 mg/1) and liver (325 vs. 220 ppm) Cu concentrations than calves supplemented with a similar level of Cu from the sulfate form after 84 days. Zinc in the form of zinc lysine resulted in the highest levels of metallothionein in liver, pancreas and kidney compared to other Zn sources, thus indicating a more bioavailable source of Zn (Rojas, 1994). Copper lysine at 16 ppm Cu was more beneficial for cattle that were borderline to deficient in Cu status versus copper sulfate (Rabiansky *et al*., 1999).

Much more needs to be learned about the selectivity of chelating agents toward minerals, the kind and quantity most effective, their mode of action, and their behavior with different species of animals and with varying diets. Dietary requirements for minerals may be greatly reduced by the addition of chelating agents to animal diets, but cost-to-benefit relationships need to be established.

The supplemental form of Se most widely used is the inorganic form of sodium selenite. An alternative organic Se source derived from yeast has been developed whereby Se is incorporated into the protein structure of growing yeast cells (Mahan, 1996). Dairy cattle have exhibited a higher glutathione peroxidase activity when the organic form of selenium is fed compared to when selenite was provided (Pehrson *et al*., 1998, cited by McDowell, 1999). In the future, organic Se will likely be an extremely important source of supplementation of this element.

Typical free-choice mixtures: Even though grazing cattle have been found not to balance their mineral needs perfectly when consuming a free-choice mixture, there is usually no other practical way of supplying mineral needs under grazing conditions. As a low-cost insurance to provide adequate mineral nutrition, modified complete mineral supplements should be available free-choice to grazing cattle (Cunha *et al*., 1964). A modified complete mineral mixture usually includes salts, a low fluoride-phosphorus source, Ca, Co, Cu, Mn, I, Fe and Zn. Except where selenosis is a problem, most free-choice supplements should contain Se. Magnesium, K, S, or additional elements can also be incorporated into a mineral supplement or can be included at a later date as new information suggests a need.

Calcium, Cu, or Se, when in excess, can be more detrimental to ruminant production than any benefit derived by providing a mineral supplement. In regions where high forage Mo predominates, three to five times the Cu content in mineral mixtures is needed to counteract Mo toxicity (Cunha *et al*., 1964). As little as 3 ppm Mo has been shown to decrease Cu availability by 50%. Sulfur at 0.4% can have the same effect. Thus, the exact level of Cu to use in counteracting Mo or S antagonism is a complex problem and should be worked out for each area. Table 3 lists the characteristics of a modified complete mineral supplement (McDowell, 1992).

Special free-choice mixtures: A oral Mg supplement is of value only during seasonal occurrences of grass tetany (Allcroft, 1961). Unfortunately, many commercial Mg-containing, free-choice mineral supplements are often of little value because (1) they contain inadequate amounts of Mg to protect against tetany during susceptible periods, and (2) provision of such supplements to normal animals during non-susceptible periods is useless as a prophylactic measure, since additional Mg will not provide a depot of readily available Mg for emergency use. Some producers feed Mg supplements about a month before the Mg tetany season, to decrease the amount of Mg needed daily during the susceptible period.

The provision of special high-Mg mineral blocks or mineral salt mixtures on pasture was more effective in raising blood Mg levels quickly after the initial drop than was the Mg fertilization (Reid *et al*., 1976). Various combinations of magnesium oxide with salt, protein supplements, molasses, other concentrate ingredients and other feeds have been used to obtain optimal Mg intakes. From West Virginia, average consumption of Mg by beef cows given a free-choice mixture of 40% salt, 40%

Table 3: Characteristics of a recommended complete free-choice cattle mineral supplement

An acceptable complete cattle mineral supplement should be as follows.
1. Contains a minimum of 6-8% total P. In areas where forages are consistently lower than 0.20% P, mineral supplements in the 8-10% P are preferred.
2. Has a Ca:P ratio not substantially over 2:1.
3. Provides a significant proportion (e.g. about 50%) of the trace mineral requirements for Co, Cu, I, Mn, and Zn.[a] In known trace mineral deficient regions, 100% of specific trace minerals should be provided.
4. Includes high-quality mineral salts that provide the best biologically available forms of each mineral element, and avoidance of minimal inclusion of mineral salts containing toxic elements. As an example, phosphates containing high F should be either avoided or formulated so that breeding cattle would receive no more than 30-50 mg/kg F in the total diet. Fertilizer or untreated phosphates could be used to a limited extent for feedlot cattle.
5. Is sufficiently palatable to allow adequate consumption in relation to requirements.
6. Is backed by a reputable manufacturer with quality-control guarantees as to accuracy of mineral-supplement label.
7. Has an acceptable particle size that will allow adequate mixing without smaller size particles settling out.
8. Is formulated for the area involved, the level of animal productivity, the environment (temperature, humidity, etc.) in which it will be fed., and is as economical as possible in providing the mineral elements used.

[a]For most regions it would be appropriate to include Se, unless toxicity problems have been observed. Iron should be included in temperate region mixtures but often both Fe and Mn can be eliminated for acid soil regions. In certain areas where parasitism is a problem, Fe supplementation may be beneficial.

dicalcium phosphate and 20% magnesium oxide ranged from 1.3 to 4.2 g per head per day (Reid *et al.*, 1976). This compared to an intake level of 5-10 g Mg from a similar mixture containing 20% dried molasses, or 4.1-8.8 g Mg from commercial molasses-magnesium oxide blocks (15% mg).

Several relatively successful free-choice consumption formulas of both liquid and dry supplements are as follows: (1) magnesium oxide plus molasses at a ratio of 1:1; (2) 97% molasses plus 3% magnesium chloride (often with urea and a source of P); (3) equal parts of magnesium oxide, salt, bonemeal and grain; (4) a 1:1 ratio of salt and magnesium oxide. In the southeastern US, a complete mineral mixture with 25% magnesium oxide (14% Mg) has been effective in preventing a grass tetany in beef cattle (Cunha, 1973). Licking wheels or licking belts are sometimes used to slowly dispense Mg oxide or magnesium sulfate in molasses.

Other methods of tetany control, including administration of Mg through fertilizer, foliar application, enemas, water and injections, have been reviewed (McDowell, 1992; McDowell, 1999). Berger (1992) calculated that, in a 100-cow herd, preventing the loss of a single cow every 3 years from grass tetany would more than pay the cost of Mg supplementation.

Often young forages contain high concentrations of K, with K level an important risk factor in the development of tetany. Potassium decreases Mg absorption (Schonewille *et al.*, 1999). However, there are some conditions where cattle need supplemental K. Generally, forages contain considerably more K than required by cattle. However, mature pastures that have weathered or hay that has been exposed to rain and sun or was overly mature when harvested can have K levels less than adequate for good nutrition (Karn and Clanton, 1977; McDowell, 1985). Potassium is a very soluble element, and dead material that is allowed to leach will have a reduced K content.

Even though mature forages are low in K, deficiency does not occur if ruminants are provided in the winter or dry season with a molasses-urea supplement. Molasses counteracts low forage K, as it has a high K level (about 4.0%). When molasses is too expensive for ruminant livestock supplementation, the chances of K deficiency are greatly increased.

Most often S is not included in free-choice supplements. Sulfur supplementation will most likely be needed to meet the requirements of ruminants when poor quality roughages grown on S-deficient soils or feed combined with urea are fed. There is no S in urea, therefore the element may need to be added when high levels of urea are fed. Pasture fertilization programs have changed in recent years from using a source of S in single-superphosphate (approx. 12% S) to triple-superphosphate and other high-analysis fertilizers that contain little or no S. In a review (Miles and McDowell, 1983) which summarized four cattle supplementation trials, control diets contained between 0.04 and 0.10% S. Intake by S-supplemented cattle increased from 7 to 260% and production of milk and meat increased by 6 to more than 400%.

Some reports from tropical regions indicate that S fertilization may increase forage intake by improving palatability of less palatable species. Low levels of S have been found in young regrowths of *Digitaria decumbens* and feeding a S supplement has increased voluntary feed intake by 28% (Rees *et al.*, 1974). Holstein cows fed S-fertilized Bermudagrass hay had improved apparent digestibility of nitrogen and tended to consume more hay dry matter (Mathews *et al.*, 1994).

Free-choice mineral supplement evaluation: Problems concerned with mineral supplementation programs in diverse world regions have been summarized (McDowell, 1999) and include: (1) insufficient chemical analyses and biological data to determine which minerals are required and in what quantities; (2) lack of mineral consumption data needed for formulating supplements; (3) inaccurate and/or unreliable information on mineral ingredient labels; (4) supplements that contain inadequate amounts or imbalances; (5) standardized mineral mixtures that are inflexible for diverse ecological regions (e.g. supplements containing Se distributed in a Se-toxic region); (6) farmers not supplying mixtures as recommended by the manufacturer (e.g. mineral mixtures diluted 10:1 and 100:1 with additional salt); (7) farmers not keeping minerals in front of animals continually; (8) difficulties involved with transportation, storage and cost of mineral supplements. Many of these problems are more related to tropical versus temperate regions, as in temperate regions (more developed countries) there is better quality control of products produced. However, some of the problems with temperate mineral mixes are related to inadequate quantities of Cu and Zn in mixtures, with some products low in P while others still not providing Se.

Responsible firms that manufacture and sell high-quality mineral supplements provide a great service to individual farmers. However, there are companies that are responsible for exaggerated claims of advertising, and some that produce

Table 4: An inferior mineral mixture available in Latin America[a,b,c]

	Mineral dietary allowance	Amount in mixture (%)	Allowance provided from mineral mix	% Allowance from mineral mixture (%)
Sodium Chloride	0.50%	20.00	0.10%	20.0
Calcium	0.30%	29.44	0.147%	49.1
Phosphorus	0.25%	1.80	0.009%	3.6
Magnesium	2000.0 mg/kg	3.2	0.016%	8.0
Iron	100.0 mg/kg	0.88	44.0 mg/kg	44.0
Zinc	50.0 mg/kg	0.02	1.0 mg/kg	2.0
Cobalt	0.1 mg/kg	0.002	0.1 mg/kg	100.0
Iodine	0.80 mg/kg	0.001	0.05 mg/kg	6.25
Copper	10.0 mg/kg	0.015	0.75 mg/kg	7.5
Manganese	25.0 mg/kg	0.075	3.75 mg/kg	15.0
Selenium	0.10 mg/kg	0.0005	0.025 mg/kg	25.0

[a]From McDowell *et al*., 1999). [b]Mineral mixture is recommended by the manufacturers for cattle, sheep, pigs and chickens. It is assumed that mineral consumption will average approximately 0.5% of the total dietary intake. This is based on an estimated intake of 50 g of mineral mixture for cattle and 10 kg of total dry feed per head daily. [c]Criticisms of mineral mixture are as follows: (1) the mixture is extremely low in P and exceptionally high in Ca. The Ca:P ratio is 16.4:1; (2) the supplement does not provide a significant proportion (i.e. 50%) of the trace mineral requirements of Cu, I, Mn and Zn; (3) the majority of the Fe is from ferric oxide, an unavailable form of this element; (4) since this contains 29.4% Ca and only 20% salt (NaC1), it is likely to be of low palatability.

inferior products that are of little value, or worse, those likely to be of detriment to animal production. Table 4 provides an example of an inferior mineral mixture available in Latin America. This particular mineral supplement is recommended for cattle, sheep, pigs and chickens. It is impossible to adequately meet requirements of both ruminants and monogastric animals with the same mixture. This unbalanced mineral mixture, which is extremely high in Ca (29.4%) and low in P (1.8%), would likely be more detrimental to grazing cattle than having no access to supplemental minerals, and may actually contribute to a P deficiency.

References

Abu Damir, H, M. Phillippo, B.H. Thorp, J.S. Milne, L. Dick and I.M. Nevison, 1994. Effects of dietary acidity on calcium balance and mobilization, bone morphology and 1,25 dihydroxy-vitamin D in prepartal cows. Res. Vet. Sci., 56:310.

Allcroft, R., 1961. The use and misuse of mineral supplements. Vet. Rec., 73:1255.

Aréchiga, C.F., C.R. Staples, L.R. McDowell and P.J. Hansen, 1998. Effects of timed insemination and supplemental β-carotene on reproduction and milk yield of dairy cows under heat stress. J. Dairy Sci., 81:390.

Arthington, J.D., L.R. Corah and F. Blecha, 1996. The effect of molybdenum-induced copper deficiency on acute-phase protein concentrations, superoxide dismutase activity, leukocyte numbers and lymphocyte proliferation in beef heifers inoculated with bovine herpevirus-1. J. Anim. Sci., 74:211.

Badwey, J.A. and M.L. Karnovsky, 1980. Active oxygen species and the functions of phagocytic leukocytes. Ann. Rev. Biochem., 49:695.

Beck, M.A., 1997. Increased virulence of coxsackievirus B3 in mice due to vitamin E or selenium deficiency. J. Nutr., 127:966S.

Beck, M.A., P.C. Kolbeck, L.H. Kohr, Q. Shi, V.C. Morris and O.A. Lavander, 1994. Vitamin E deficiency intensifies the myocardial injury of coxsackievirus B3 infection in mice. J. Nutr., 124:345.

Berger, L.L.. 1992. Grass tetany; causes and prevention. Salt Trace Miner., 24:1.

Bigner, D.R., J.P. Goff, M.A. Faust, H.D. Tyler and R.L. Horst, 1997. Comparison of oral sodium compounds for the correction of acidosis. J. Dairy Sci., 80:2162.

Breukink, H.J. and T. Wensing, 1998. Pathophysiology of the liver in high yielding dairy cows and its consequences for health and production. The Bovine Pract., 32:73.

Bruss, M.L., 1993. Metabolic fatty liver of ruminants. Adv. Vet. Sci. Comp. Med., 37:417.

Burton, G.W., 1989. Antioxidant action of carotenoids. J. Nutr., 119:109.

Cerone, S., A. Sansinanea and N. Auza, 1995. Copper deficiency alters the immune response of bovine. Nutr. Res., 15:1333.

Cervantes, A., T.R. Smith and J.W. Young, 1996. Effects of nicotinamide on milk composition and production in dairy cows fed supplemental fat. J. Dairy Sci., 79:105.

Chew, B.P., 1995. Antioxidant vitamins affect food animal immunity and health. J. Nutr., 125:1804S.

Chew, B.P., 2000. Micronutrients play role in stress, production in dairy cattle. Feedstuffs., 72:11.

Chew, B.P. and L.A. Johnson, 1985. Effects of supplemental vitamin A and β-carotene on mastitis in dairy cows during the early dry period. J. Dairy Sci., 68(Suppl. 1):191.

Correll, D.L., 1999. Phosphorous: A rate limiting nutrient in surface waters. Poult. Sci., 78:674.

Cunha, T.J., 1973. Recent developments in mineral nutrition. A look at the highlights of research involving mineral requirements for swine, beef, cattle and horses. Feedstuffs, 45 :27.

Cunha, T.J., R. L. Shirley, H.L. Chapman, Jr., C.B. Ammerman, G. K. Davis, W. G. Kirk and J. F. Hentges, 1964. Minerals for beef cattle in Florida. Fla. Agric. Exp. Stn. Bull., 683.

Dahlquist, S.P. and B.P. Chew, 1985. Effects of vitamin A and β-carotene on mastitis in dairy cows during the early dry period. J. Dairy Sci., 68(Suppl. 1):191.

Daniel, L.R., B.P. Chew, T.S. Tanaka and L.W. Tjoelker, 1991a. β-carotene and vitamin A effects on bovine phagocyte function *in vitro* during the peripartum period. J. Dairy Sci., 74:124.

Daniel, L.R., B.P. Chew, T.S. Tanaka and L.W. Tjoelker, 1991b. *In vitro* effects of β-carotene and vitamin A on peripartum bovine peripheral blood mononuclear cell proliferation. J. Dairy Sci., 74:911.

Degkwitz, E., 1987. Some effects of vitamin C may be indirect, since it affects the blood levels of cortisol and thyroid hormones. Ann N.Y. Acad. Sci., 498-470.

Dhiman, T.R. and V. Sasidharan, 1999. Effectiveness of calcium chloride in increasing blood calcium concentrations of periparturient dairy cows. J. Anim. Sci., 77:1597

Distl, O. and D. Schmid, 1994. Influence of biotin supplementation on the formation, hardness and health of claws in dairy cows. Tierarztliche Umschau, 49:581.

Downer, J.F. and K.R. Cummings, 1987. Sodium bicarbonate: a ten year review of lactation studies. J. Dairy Sci., 70(Suppl. 1):152..

Driver, L.S., R.R. Grummer and L.H. Schultz, 1990. Effects of feeding heat-treated soybeans and niacin to high producing cows in early lactation. J. Dairy Sci., 73:463.

Droke, E.A., J.W. Spears, T.T. Brown and M.A. Qureshi, 1993. Influence of dietary zinc and dexamethasone on immune responses and resistance to *Pastuerella hemolytica* challenge in growing lambs. Nutr. Res., 13:1213.

Dunkley, W.L., M. Ronning, A.A. Franke and J. Robb, 1967. Supplementing rations with tocopherol and ethoxyquin to increase oxidative stability of milk. J. Dairy Sci., 50:492.

Erickson, G.E., T.J. Klopfenstein, C.T. Milton, D. Hanson and C. Calkins, 1999. Effect of dietary phosphorus on finishing steer performance, bone status and carcass maturity. J. Anim. Sci., 77:2832.

Erskine, R.J., R.J. Eberhart, P.J. Grasso and R.W. Schulz, 1989. Induction of *Escherichia coli* mastitis in cows fed selenium-deficient or selenium-supplemented diets. Am. J. Vet Res., 50:2093.

Faustman, C., R.G. Cassens, D.M. Schaefer, D.R. Buege and K.K. Scheller, 1989. Vitamin E supplementation of Holstein steer diets improves sirloin steak color. J. Food Sci., 54 :485.

Fitzgerald, T., B.W. Norton, R. Elliot, H. Podlich and O.L. Svendsen, 2000. The influence of long-term supplementation with biotin on prevention of lameness in pasture fed cows. J. Dairy Sci., 83:338.

Flachowsky, G., U. Lober, H. Ast, D. Wolfram and M. Matlhey, 1988. Wiss. Z. Karl-Marx-Univ. Leipzig Math..-Naturwiss R., 37:55.

Fraker, P. and L. King, 1998. Changes in regulation of lymphoporesis and myeloporesis in the zinc-deficient mouse. Nutr. Rev., 56:S65.

Frei, B., L. England and B. N. Ames, 1989. Ascorbate is an outstanding antioxidant in human blood plasma. Proc. Natl. Acad. Sci., 86:6377.

Frigg, M., O.C. Straub, D. Harmann, 1993. The bioavailability of supplemental biotin in cattle. Int. J. Vit. Nutr. Res., 63:122.

Fronk, T.J. and L. H. Schultz, 1979. Oral nicotinic acid as a treatment for ketosis. J. Dairy Sci., 62:1804.

Gaynor, P.J., F. J. Mueller, J. K. Miller, N. Ramsey, J. P. Goff and R. L. Horst, 1989. Parturient hypocalcemia in Jersey cows fed alfalfa haylage diets with different cation and anion rations. J. Dairy Sci., 71:2525.

Goff, J.P. and R.L. Horst, 1993. Oral administration of calcium salts for treatment of hypocalcemia in cattle. J. Dairy Sci., 76:101.

Goff, J.P. and R.L. Horst, 1994. Calcium salts for treating hypocalcemia: Carrier effects acid-base balance and oral versus rectal administration. J. Dairy Sci., 77:1456.

Goff, J.P. and R.L. Horst, 1997. Effects of the addition of potassium or sodium, but not calcium to prepartum rations on milk fever in dairy cows. J. Dairy Sci., 80:176.

Goff, J.P. and R.L. Horst, 1998. Use of hydrochloric acid as a source of anions for prevention of milk fever. J. Dairy Sci., 81:2874.

Goff, J.P., R.L. Horst, P.W. Jardon, C. Borelli and J. Wedam, 1996. Field trials of an oral calcium propionate paste as an aid to prevent milk fever in periparturient dairy cows. J. Dairy Sci., 79:378.

Goff, J.P., T.A. Reinhardt and R.L. Horst, 1991. Enzymes and factors controlling vitamin D metabolism and action in normal and milk fever cows. J. Dairy Sci., 74:4022.

Gould. D.H. 1998. Polioencephalomalacia. J. Anim. Sci., 76:309.

Hadden, J.W., 1987. Neuroendocrine modulation of the thymus-dependent immune system. Ann NY. Acad. Sci., 496:39.

Halevy, O., Y. Arazi, D. Melamed, A. Friedman and D. Sklan, 1994. Retinoic acid receptor - α gene expression is modulated by dietary vitamin A and by retinoic acid in chicken T lymphocytes. J. Nutr., 124:2139.

Herrick, J., 1989. Zinc methionine: feedlot and dairy indications. Large Anim. Vet., 44:35.

Higuchi, H. and N. Nagahata, 2000. Relationship between serum biotin concentration and moisture content of sole horn in cows with clinical laminitis and in cows with sound hooves. Report Animal Health Laboratory, Faculty of Veterinary Medicine, Rakomo Gakuen University, Hokkaido, Japan. pp: 7

Hodnett, D.W., N.A. Jorgensen and H.F. DeLuca, 1992. 1α-hydroxyvitamin D_3 plus 25-hydroxyvitamin D_3 reduces parturient paresis in dairy cows fed high dietary calcium. J. Dairy Sci., 75:485.

Hoffmann-La Roche, 1994. Vitamin Nutrition for Ruminants. RCD 87751/894. Hoffmann-La Roche, Inc., Nutley, NJ.

Horst, R.L., J.P. Goff, T.A. Reinhardt and D.R. Buxton, 1997. Strategies for preventing milk fever in dairy cattle. J. Dairy Sci., 80:1269.

Jacob, R.A., 1995. The integrated antioxidant system. Nutr. Res., 15:755.

Karn, J.F. and D. C. Clanton, 1997. Potassium in range supplements. J. Anim. Sci., 45:1426.

Kasari, T.R., 1990. Metabolic acidosis in diarrheic calves: the importance of alkalinizing agents in therapy. Vet. Clin. North Am. Food Anim. Pract., 6:29.

Kincaid, R.L., 1989. Availability, biology of chelated, sequestered minerals explored. Feedstuffs, 61 :22.

Kincaid, R.L., R.M. Blauwiekel and J.D. Cronrath, 1986. Supplementation of copper as copper sulfate or copper proteinate for growing calves fed forages containing molybdenum. J. Dairy Sci., 69:160.

Kincaid, R.L., 1999. Critical role of trace minerals in the animal's immune response. Proc. 1st International Nutrition Conference. pp: 1. Salt Lake City, UT.

Klippel, M., P. Mockel and G. Flachowski, 1993. Symposium Vitamine und weitere Zusatzstoffe bei Mensch und Tier: 4, G. Flachowsky and R. Schubert (eds.), pp: 194. Jena Germany.

Mahan, D., 1996. Organic selenium for the livestock industry: Conclusions and potential from research studies. North American University Tour, Alltech Biotechnology Center, Nicholasville, KY.

Mascio, P.E., M.E. Murphy and H. Sies, 1991. Antioxidant defense system: The role of carotenoids, tocopherols and thiols. Am. J. Clin. Nutr., 53:194S.

Mathews, B.W., L. E. Sollenberger and C.R. Staples, 1994. Sulfur fertilization of bermudagrass and effect on digestion of nitrogen, sulfur and fiber by nonlactating cows. J. HAW. Pas. Agri., 5:21.

McDowell, L.R., 1985. Nutrition of Grazing Ruminants in Warm Climates. Academic Press, New York.

McDowell, L.R., 1992. Minerals in Animal and Human Nutrition. Academic Press, San Diego, CA. pp: 524

McDowell, L.R., 1999. Minerais para Ruminantes sob Pastejo em Regiões Tropicais Enfatizando o Brasil, University of Florida, Gainesville, FL.

McDowell, L.R., S.N. Williams, N. Hidiroglou, C.A. Njeru, G.M. Hill, L. Ochoa and N.S. Wilkinson, 1996. Vitamin E. supplementation for the ruminant. Anim. Feed Sci. Tech., 60:273.

McDowell, L.R. and G. Valle, 2000.Givens, D.I., Owen,E., Oxford, R.F.E. and Omed, H.M., eds. In: Forage Evaluation in Ruminant Nutrition pp: 373, CAB International, Wallingford, UK.

McGhee, J.R., J. Mestecky, M.T. Dertzbaugh, J.H. Eldridge, M. Hirasawa and H. Kiyono, 1992. The mucosal immune system: From fundamental concepts to vaccine development. Vaccine, 10:75.

Michal, J.J., L.R. Heirman, T.S. Wong and B.P. Chew, 1994. Modulatory effects of dietary β-carotene on blood and mammary leukocyte function in preparturient dairy cows. J. Dairy Sci., 77:1408.

Midla, L.T., K.H. Hoblet, W.P. Weiss and M. Moeschberger, 1998. Supplemental dietary biotin for prevention of lesions associated with aseptic subclinical laminitis (pododermatitis aseptica diffusa) in primiparous cows. AJVR, 59:733.

Miles, W. H. and L. R. McDowell, 1983. Mineral deficiencies in the llanos rangelands of Colombia. World Anim. Rev., 46:2.

Neilsen, J.A., N. Fisher and A.H. Pederson, 1953. The influence of feeding tocopherol to dairy cows on the yield of milk and milk fat and on the tocopherol content and keeping quality of butter. J. Dairy Res., 20:333.

Nelson, J., 1988. Review of trace mineral chelates and complexes available to the feed industry. Western Nutrition Conference, Winnipeg, Manitoba, September, p:1.

Nicholson, J.W.G., A.M. St. Laurent, R.E. McQueen and E. Charmley, 1991. The effect of feeding organically bound selenium and α-tocopherol to dairy cows on susceptibility of milk to oxidation. Can. J. Anim. Sci., 71:143.

Nockels, C.F., K.G. Odde and A.M. Craig, 1996. Vitamin E supplementation and stress affect tissue α-tocopherol content of beef heifers. J. Anim. Sci., 74:672.

Nonnecke, B.J., T.A. Reinhardt and S.T. Franklin, 1993. Retinoid-induced modulation of immunoglobulin M secretion by bovine mononuclear leukocytes in vitro. J. Dairy Sci., 76:2175.

NRC., 1980. Mineral tolerance of domestic animals. National Academy of Science - National Research Council, Washington, DC.

NRC., 1989. Nutrient Requirements of dairy cattle. (6th Rev. Ed.) Natl. Academy Press, Washington, DC.

Oetzel, G.R., 1996. Effect of calcium chloride gel treatment in dairy cows on incidence of periparturient diseases. J. Am. Vet. Med. Assoc., 209:958.

Padh, H., 1991. Vitamin C: Newer insights into its biochemical functions. Nutr. Rev., 49:65.

Patton, R.S., 1990. Chelated minerals: what are they, do they work? Feedstuffs, 62 :14.

Pehrson, G., C. Svensson, I., Gruvaeus and M. Virkki, 1999. The influence of acidic diets on acid-base balance of dry cows and the effect of fertilization on the mineral content of grass. J. Dairy Sci., 82:1310.

Pehrson, B., C. Svensson and M. Jonsson, 1998. A comparative study of the effectiveness of calcium propionate and calcium chloride for the prevention of parturient paresis in dairy cows. J. Dairy Sci., 81:2011.

Rabiansky, P.A., L. R. McDowell, J. Velasquez-Pereira, N.S. Wilkinson, S.S. Percival, F.G. Martin, D.B. Bates, A.B. Johnson, T.R. Batra and E. Salgado-Madriz, 1999. Evaluating copper lysine and copper sulfate for heifers. J. Dairy Sci., 82:2642.

Rajaraman, V., B.J. Nonnecke, S.T. Franklin, D.C. Hammell and R.L. Horst, 1998. Effect of vitamins A and E on nitric oxide production by blood mononuclear leukocytes from neonatal calves fed milk replacer. J. Dairy Sci., 81:3278.

Reddy, P.G., J.C. Morrill and R.A. Frey, 1987a. Vitamin E requirements of dairy calves. J. Dairy Sci., 70:123.

Reddy, P.G., J.L. Morrill, H.C. Minocha and J.S. Stevenson, 1987b. Vitamin E is immunostimulatory in calves. J. Dairy Sci., 70:993

Rees, M.C., D. J. Minson and F.W. Smith, 1974. The effect of supplementary and fertilizer sulphur on voluntary intake, digestibility, retention time in the rumen and site of digestion on pangola grass in sheep. J. Agr. Sci., 82:419.

Reid, R. L., G. A. Jung and C.F. Gross, 1976. Grass tetany as a metabolic problem in the eastern United States. Proc. Hill Lands Int. Symp., West Virginia University, Morgantown, WV, pp: 640.

Reinhardt, T.A., J.R. Stabel and J.P. Goff, 1999. 1, 25-dihydroxyvitamin D_3 enhances milk antibody titers to Escherichia coli J5 Vaccine. J. Dairy Sci., 82:1904.

Rifici, V.A. and A.K. Khachadurian, 1993. Dietary supplementation with vitamins C and E inhibits in vitro oxidation of lipoproteins. Am. J. Coll. Nutr., 12:631.

Rojas, L.X., 1994. Relative bioavailability of different organic and inorganic zinc and copper sources in ruminants and rats. Ph.D. Dissertation, University of Florida, Gainesville.

Roth, J.A. and M.L. Kaeberle, 1985. *In vivo* effect of ascorbic acid on neutrophil function in healthy and desamethasone-treated cattle. Am. J. Vet. Res., 46:2434.

Ross, A.C., 1992. Vitamin A status: Relationship to immunity and the antibody response. Proc. Soc. Exp. Biol. Med., 200:303.

Roussel, A.J., 1990. Fluid therapy in mature cattle. Vet. Clin. North Am. Food Anim. Pract., 6:111.

Rukkwamsuk, T., T. Wensing and M.J.H. Geelen, 1999. Effect of fatty liver on hepatic gluconeogenesis in periparturient dairy cows. J. Dairy Sci., 82:500.

Russel, J. B. and J. M. Chow, 1993. Another theory for the action of ruminal buffer salts: decreased starch fermentation and propionate production. J. Dairy Sci., 76:826.

Satter, L.D. and Z. Wu, 1999. In: Cornell Nutrition Conference for Feed Manufacturers. p: 72. Cornell University, Ithaca, NY.

Schaefer, D.M., Q. Liu, C. Faustman and M. Yin, 1995. Supranutritional administration of vitamins E and C improves oxidative stability of beef. J. Nutr., 125:1792S.

Schneider, P.L., D. K. Beede and C.J. Wilcox, 1986. Responses of lactating cows to dietary sodium source and quantity and potassium quantity during heat stress. J. Dairy Sci., 69:99.

Schonewille, J.T., A.T. Van't Klooster, H. Wouterse and A.C. Beynen, 1999a. Effects of intrinsic potassium in artificially dried grass and supplemental potassium bicarbonate on apparent magnesium absorption in dry cows. J. Dairy Sci., 82:1824.

Schonewille, J.T., A.T. Van't Klooster, H. Wouterse and A.C. Beynen, 1999b. Hypocalcemia induced by intravenous administration of disodium ethylenediaminotetraacetate and its effects on excretion of calcium in urine of cows fed a high chloride diet. J. Dairy Sci., 82:1317.

Smith, K.L., J.H. Harrison, D.D. Hancock, D.A. Todhunter and H.R. Conrad, 1984. Effect of vitamin E and selenium supplementation on incidence of clinical mastitis and duration of clinical symptoms. J. Dairy Sci., 67:1293.

Smith, K.L. and H.R. Conrad, 1987. Vitamin E and selenium supplementation for dairy cows. RCD 7442, Proc. Roche Technical Symposium, The role of Vitamins on Animal Performance and Immune Response. Daytona Beach, FL. p: 47.

Smith, K., J.S.Hogan and W.P. Weiss, 1997. Dietary vitamin E and selenium affect mastitis and milk quality. J. Anim. Sci., 75:1659.

Spears, J.W., 1991. Chelated trace minerals in ruminant nutrition. Proc. 2nd annual Florida Ruminant Nutrition Symp., 23-24 January 1991, Gainesville, FL p: 1.

Spears, J.W., 1996. In: E.T. Kornegay (Ed.) Nutrient Management of Food Animals to Enhance and Protect the Environment. p: 259. CRC Press, Boca Raton, FL.

Spears, J.S. and E. B. Kegley, 1991. Effect of zinc and manganese methionine on performance of beef cows and calves. J. Anim. Sci., 69(Suppl. 1):59 .

Spears, J.W., R.W. Harvey, T.T. Brown, Jr., 1991. Effects of zinc methionine and zinc oxide on performance, blood characteristics and antibody titer response to viral vaccination in stressed feeder calves. Am. Vet. Med. Assn., 199:1731.

Spears, J.W., E.B. Kegley and J.D. Ward, 1991. Bioavailability of organic, inorganic trace minerals explored. Feedstuffs, 27 :12.

Staples, C.R. and D.S. Lough, 1989. Efficacy of supplemental dietary neutralizing agents for lactating dairy cows. A Review. Anim. Feed Sci. Technol., 23:277.

Stephensen, C.B., Z. Moldoveanu and N.N. Gangopadhyay, 1996. Vitamin A deficiency diminishes the salivary immunoglobulin A response and enhances the serum immunoglobulin G response to influenza A virus infection in BALB/c mice. J. Nutr., 126:94.

Tanaka, U., S. Shiozawa, I. Morimonto and T. Fujita, 1990. Role of zinc in interleukin 2 (IL-2) - mediated T-cell activation. Scan. J. Immunol., 31:547.

Tiffany, M.E., L.R. McDowell, G.A. O'Connor, F.G. Martin, N.S. Wilkinson, E.C. Cardoso, S.S. Percival and P.A. Rabiansky, 2000. Effects of pasture applied biosolids on performance and mineral status of grazing beef heifers. J. Anim. Sci, 78:1331.

Tjoelker, L.W., B.P. Chew, T.S. Tanaka and L.R. Daniel, 1990. Effect of dietary vitamin A and β-carotene on polymorphonuclear leukocyte and lymphocyte function in dairy cows during the early dry period. J. Dairy Sci., 73:1017.

Tokarnia, C.H., J. Döbereiner and P.V. Piexoto, 2000. Topico: Deficiências minerais em animais de fazenda, principalmente bovinos em regime de campo. Pesquisa Veterinária Brasileria., 20:127.

Underwood, E.J. and N.F. Suttle, 1999. The mineral nutrition of livestock (3rd ed.). Midlothian, UK.

Vagnon, D.G. and G.R. Oetzel, 1998. Effects of dietary cation-anion difference on acid-base balance of dry cows. J. Dairy Sci., 81:1643.

Valk, H., J.A. Metcalf and P.J.A. Withers, 2000. Prospects for minimizing phosphorus excretion in ruminants by dietary manipulations. J. Environ. Qual., 29:28.

Valk, H. and L.B.J. Sebek, 1999. Influence of long-term feeding of limited amounts of phosphorus on dry matter intake, milk production and body weight of dairy cows. J. Dairy Sci., 82:2157.

Velasquez-Pereira, J., C.A. Risco, P.J. Chenoweth, L.R. McDowell, D. Prichard, F.G. Martin, N.S. Wilkinson, S.N. Williams and C.R. Staples, 1998. Reproductive effects of feeding gossypol and vitamin E to bulls. J. Anim. Sci., 76:2894.

Weiss, W.P., J.S. Hogan, K.L. Smith and K.H. Hoblet, 1990. Relationships among selenium, vitamin E and mammary gland health in commercial dairy herds. J. Dairy Sci., 73:381.

Weiss, W.P., J.S. Hogan, D.A. Todhunter and K.L. Smith, 1997. Effect of vitamin E supplementation in diets with low concentration of selenium on mammary gland health of dairy cows. J. Dairy Sci., 80:1728.

Widermann, U., L.A. Hanson, J. Holmgren, H. Kahu and U.I. Dahlgren, 1993. Impaired mucosal antibody response to cholera toxin in vitamin A-deficient rats immunized with oral cholera vaccine. Infect. Immun., 61:3952.

Wu, Z. and L.D. Satter, 2000. Milk production and reproductive performance of dairy cows fed two concentrations of phosphorus for two years. J. Dairy Sci., 83:1052.

Zamora, R., F.J. Hidalgo and A.L. Tappel, 1991. Comparative antioxidant effectiveness of dietary β-carotene, vitamin E, selenium and coenzyme Q in rat erythrocytes and plasma. J. Nutr., 121:30.

Zhao, Z. and C. Ross, 1995. Retinoic acid repletion restores the number of leukocytes in vitamin A-deficient rats. J. Nutr., 125:2064.

Zimmerly, C.A., 2000. Effect of supplemental dietary biotin on performance of Holstein cows in early lactation. M.S. Thesis, Ohio State University, Wooster, OH.

Pakistan Journal of Nutrition 1(1): 20-24, 2002

Role of Lactic Acid Bacteria (LAB) in Food Preservation and Human Health – A Review

Soomro, A.H., T. Masud and Kiran Anwaar*
Department of Food Technology *Department of Bio-Chemistry,
University of Arid Agriculture, Rawalpindi, Pakistan

Abstract: Fermentation of various food stuffs by lactic acid bacteria (LAB) is one of the oldest forms of biopreservation practiced by mankind . Bacterial antagonism has been recognized for over a century but in recent years this phenomenon has received more scientific attention , particularly in the use of various strains of lactic acid bacteria One important attribute of many LAB is their ability to produce antimicrobial compounds called bacteriocins. In recent years interest in these compounds has grown substantially due to their potential usefulness as natural substitute for chemical food preservatives in the production of foods with enhanced shelf life and / or safety. There is growing consumer awareness of the link between diet and health. Recent scientific evidence supports the role of probiotic LAB in mediating many positive health effects.Traditional probiotic dairy strains of lactic acid bacteria have a long history of safe use and most strains are considered commensal microorganisms with no pathogenic potential.

Key words: Human health, food preservatin, lactic acid bacteria

Introduction:

The single most important development permitting the formation of civilization was the ability to produce and store large quantities of food . Hunter-gatherer societies lived from day to day either starving or gorging themselves based upon the amount of food they could find in a day. When it became possible for one person to produce more food then they needed, time from gathering food could be apportioned to culture and science. Following this trend, it became beneficial to be able to store as much food as possible in order to minimize the amount of time spent gathering that food. Food storage has always been at odds with food spoilage. Some of the earliest evidence of food preservation comes from the post-glacial era, from 15,000 to 10,000 BC. The first use of biological methods was from 6000 to 1000 BC when fermentation was used to produce beer, bread, wine, vinegar, yoghurt, cheese and butter. In 1864, Louis Pasteur proved that microorganisms in foods were the cause of food spoilage, that heat treatment of food killed these microbes and that sealed containers helped to preserve food by preventing recontamination from atmospheric air. A major development in the distribution and storage of foods came in 1940 with the availability of low cost home refrigerators and freezers. Other developments included the artificial drying, vaccum packaging, ionizing radiations and chemical preservation.

Now-a-days consumers are concerned about the synthetic chemicals used as preservatives in food , and there is resulting trend towards less processed food. These untreated foods can harbour dangerous pathogens which can multiply under refrigeration and without oxygen . A solution to this dilemma is the use of antimicrobial metabolites of fermentative microorganisms . Many antimicrobial chemicals have been in use for a long time without any known adverse effects. Many of the organic compounds which have stirred interest are antimicrobial metabolites of bacteria used to produce , or associated with fermented foods.

In fermentation, the raw materials are converted by microorganisms (bacteria , yeast and molds) to products that have acceptable qualities of food. In common fermented products such as yogurt, lactic acid is produced by the starter culture bacteria to prevent the growth of undesirable microorganisms (Ray and Daeschel 1992). Food fermentations have a great economic value and it has been accepted that these products contribute in improving human health. LAB have contributed in the increased volume of fermented foods world wide especially in foods containing probiotics or health promoting bacteria.

Micro-organisms of genera *Lactococcus, Lactobacillus, Leuconostoc, Streptococcus* and *Pediococcus* are involved in these fermentations. In addition, *Lactobacillus spp.* and species of *Bifidobacterium* which is not LAB in nature are part of normal human intestinal microflora and they exert a positive effect on human health. (Daly and Davis, 1998).

This review will focus on some of the properties of LAB that contribute to their roles in biopreservation and in modulating the health of their hosts.

Development in the biotechnology of LAB: One area where genetic engineering would be of particular benefit to the dairy industry is in the genetic modification of lactic acid bacteria (LAB) which are commonly used as starter cultures in the production of fermented dairy foods . During past 20 years much of the research on LAB focused on dairy *lactococci* , investigations now include different LAB involved in wide variety of fermentation processes and, various *lactobacilli* and *bifidobacteria* belonging to the human microbiota. However, significant development in bacteriophage biology and resistance mechanisms, pyruvate metabolism and production of bacteriocins have also been made (Fitzgerald and Hill, 1996; von Wright and Sibakov, 1998).Where as research on chromosomal genetics of LAB is also progressing rapidly.Physical & genetic map of many of these strains have been constructed (Davidson *et al.*, 1996). New techniques such as ability to sequence large tracts of 16s and 23s rRNA genes using polymerase chain reaction (RAPD-PCR) and the use of pulsed field gel electrophoresis (PFGE) to finger print genomic restriction patterns have contributed enormously in strain identification and classification (Axelsson, 1998). This relates to field of probiotics where the ability to monitor strains through clinical trials and to evaluate their effects on the gastrointestinal tract microflora as well as the protection of their proprietary value depends on exact and reproducible strain identification.

LAB bacteriocins as biopreservatives: Despite improved

manufacturing facilities and implementation of effective process control procedures such as Hazard Analysis and Critical Control Points (HACCP) in the food industries, the number of food borne illnesses has increased. Nowadays consumers favour food with few chemical preservatives (Daeschel, 1993). As a result there is increased interest in the preservation through LAB because of their safe association with human fermented foods. Several metabolic products produced by these bacteria have antimicrobial effects, including organic acids, fatty acids, hydrogen peroxide and diacetyl (Holzapfel *et al.*, 1995; Ouwehand, 1998). However attention has focused on the ability of LAB to produce specific proteinaceous substances, bacteriocins that inhibit the growth of pathogens such as *Listeria, Clostridium, Staphylococcus, Bacillus spp.* and *Enterococcus spp.*, therefore they enhance the shelf life of foods.

Bacteriocins produced by LAB are the subject of intense research because of their antibacterial activity against foodborne bacteria. Bacteriocin producing strain of LAB may be very important in competing with other organisms in the intestine. They consists of a biologically active protein moiety, have a bactericidal mode of action and attach to specific cell receptors. Bacteriocins are heterogeneous group of bacterial antagonists that vary considerably in molecular weight, biochemical properties, range of sensitive hosts and mode of action. Klaenhammer (1988) define them as, protein or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium. Both Gram negative and Gram positive bacteria produce them. The Gram-negative bacteriocins are colicin, which are produced by strain of *E.coli* (Braun *et al.*, 1994). These are large, complex proteins, 29-90 kDa, with characteristic structural domains involved in cell attachment, translocation and bactericidal activity. They bind to specific receptors on the outer membrane of the target cell. The bacteriocins produced by Gram-positive bacteria are small peptides 3-6 kDa, in size (Nes *et al.*, 1996), although there are exceptions (Joerger and Klaenhammer, 1990). They fall with in two broad classes,viz the lantibiotics (Jack *et al.*, 1995) and the non- lantibiotic bacteriocins (Nes *et al.*, 1996).Most of the Gram positive bacteriocins are membrane active compounds that increase the permeability of the cytoplasmic membrane (Jack *et al.*, 1995). They often show a much broarder spectrum of bactericidal activity than the colicins. There is currently much interest in the application of bacteriocins in both food preservation and the inhibition of pathogenic bacteria (Liao *et al.*, 1994; Young and Ray, 1994; Delves-Broughton, 1996). Most of the bacteriocins have been isolated from organisms involved in food fermentation. Bacteriocin production and resistance is considered as an important property in strains used as commercial inoculants to eliminate or reduce growth of undesirable or pathogenic organisms.

At present four classes of LAB bacteriocins have been identified (Table 1). Nisin which is produced by *Lactobacillus lactis subsp lactis* strains, belongs to the class 1 lantibiotics (Dodd and Gasson, 1994; Jack *et al.*,1995) and is active against many gram positive bacteria including *Listeria spp.* It prevents the growth of germinating *bacillus* and *clostridial* spores and through the addition of calcium chelator, it is active against some gram negative bacteria (Stevens *et al.*, 1991). The mature nisin molecule is just 34 amino acids long and undergoes certain post translational modifications in which serine and threonine residues are dehydrated and several thio-ether bridges are formed, this results in the formation of five ring structures. The primary target of Nisin's antimicrobial action is the cell membrane. It is thought that nisin interferes with the energy supply, of the cell by creating pores in the membrane and dissipating its potential (Sahl *et al.*, 1995). Another compound, lacticin 3147 was identified from *lactococcal* isolate of Irish Kefir grains used in the manufacture of butter milk (Ryan *et al.*, 1996) This bacteriocin inhibits gram positive food borne pathogens such as *staphylococcus, clostridium* and *listeria spp.* as well as several mastitis producing *staphylococci* and *streptococci* (Meaney *et al.*, 1997) Lacticin 3147 requires two peptides for activity.

Class II bacteriocins are relatively small cationic peptides (30-100 amino acids) exhibiting a high degree of heat stability. Class III bacteriocins are produced by members of the *Lactobacillus* genera. Helveticin J is the best known compound of this class.

Many bacteriocins of LAB are safe and effective natural inhibitors of pathogenic and food spoilage bacteria in various foods. Nisin is the classic example, it prevents *clostridial* spoilage of processed and natural cheeses, inhibits the growth of some psychrotrophic bacteria in cottage cheese, extends the shelf life of milk in warm countries, prevents the growth of spoilage *lactobacilli* in beer and wine fermentations and provides additional protection against *bacillus* and *clostridial* spores in canned foods. Nisin is a permitted food additive in more than 50 countries including the US and Europe under the trade name Nisaplin (Vandenberg, 1993; Delves- Broughton *et al.*, 1996).

Listeria monocytogenes is common contaminant of raw foods such as milk, meat and vegetables and results in the serious foodborne illness in consumers (Ryser and Marth, 1991). Therefore bacteriocins of sub class IIa pediocin PA-1/AcH produced by *Pediococcus acidilactici* have ability to control *Listeria* in cheese, vegetables and meat. *P. acidilactici* is used as a starter culture in the production of many fermented meat (Vandenberg, 1993; Stiles, 1996). Ryan *et al.* (1996) developed lacticin 3147 producing starter strains for cheese making, these strains effectively controlled the growth of a non starter LAB in cheddar cheese and completely eliminated *L. monocytogenes* from cottage cheese. Lacticin 3147 is effective at neutral pH and starter cultures producing this bacteriocins have good acid producing and bacteriophage resistance properties. Unfortunately the LAB bacteriocins are not effective against gram negative bacteria and yeasts and moulds.

Recently, studies aimed at broadening the bactericidal activity of LAB bacteriocins are focused on the synergistic effects of bacteriocins most notably nisin, with other antibacterial factors such as the lactoperoxides system present in milk, hydrolytic enzymes, various chelating agents (including siderophores)and other bacteriocins (Helander *et al.*, 1997). Now-a-days nisin remains the only LAB bacteriocin legally used as a food additive. Two products ALTA 2431 and Microgard have been developed as shelf life extenders based on crude LAB fermentation products.

ALTA 2341 is produced from *Pediococcus acidilactici* fermentation and have to rely on the inhibitory effects of pediocin PA-1/AcH. It is added to Mexican soft cheese which is susceptible to *listerial* contamination (Glass *et al.*, 1995) Microgard is the result of a *propionibacterium* fermentation. It is active against gram negative bacteria such as *Pseudomonas, Salmonella, Yersinia* as well as yeast and moulds. Microgard's protective action is probably due to the presence of propionic acid. It has been approved by FDA for use in cottage cheese and fruit flavoured yoghurts. Another product Bioprofit is a combination of specific *Lactobacillus* and *Propionibacterium* strains is used in normal starter cultures to inhibit the growth of yeasts, moulds, *Bacillus spp. Clostridium*

Table 1: Properties of some well characterized bacteriocins

Bacteriocin	Producer organism	Properties
Nisin	*Lactococcus lactis subsp. lactis* ATCC 11454	Lantibiotic, broad spectrum,chromosome/plasmid mediated, bactericidal, produced late in the growth cycle
Pediocin A	*Pediococcus pentosaceus* FBB61 and L-7230	Broad spectrum, plasmid mediated
Pediocin AcH	*Pediococcus acidilactici* H	Broad spectrum, plasmid mediated
Leucocin	*Leuconostoc gelidum* UAL 187	Broad spectrum, plasmid mediated, bacteriostatic, produced early in the growth cycle
Helveticin J	*L.helveticus* 481	Narrow spectrum, chromosomally mediated, bactericidal
Carnobacteriocin	*Carnobacterium piscicola* LV17	Narrow spectrum, plasmid mediated, produced early in the growth cycle

spp. and heterofermentative *lactobacili* during dairy fermentations. (Mayra-Makinen and Suomalainen, 1995).

It is proposed that bacteriocins should be used in combination with other physical chemical and microbial preservatives to check the growth of pathogenic or food spoilage bacteria.

LAB and health :Probiotics: As we enter in the new millennium, people are aware that for spending a healthy life style diet play a major role in preventing diseases and promoting health. Therefore there is an increasing trend for foods containing probiotic cultures.

The concept of probiotics was in use in the early 1900s, however, the term was coined in 1965 by Lilly and Stillwell . Probiotic is a preparation of live microorganisms which when applied to man or animal, beneficially affects the host by improving the properties of the indigenous microbiota (Havenaar and Huis in't Veld, 1992).

Probiotics are viable organisms and supportive substances that improve intestinal microbial balance, such as *Lactobacillus acidophilus* and bioactive proteins (Fuller, 1991). The empirical evidence that for many years, linked the use of fermented dairy products such as yogurt and milk with the promotion of intestinal health is today well supported by modern science. The ability of the probiotic *L. acidophilus* to help prevent pathogenic bacteria from proliferating and healthy bacteria from becoming toxic is well documented. (Speck, 1975 ; Wynder, 1977).

Antimicrobial compounds produced by LAB have provided these organisms with a competitive advantage over other microorganisms. Exploitation of antibiosis of LAB is the best choice for not only improving the microbial safety of the food products but as a probiotic preparation because of their natural adaptation to the gut environment. Lactics need to be acid tolerant bacteria and exhibit resistance to lysozyme present in the saliva and other enzymes, gastric juice and duodenal fluids. Many lactics are resistant to the bile salt present in the gut and survive the intestinal motility and adhere well to gastric mucosa. Probiotics act through suppression of viable count by production of antibacterial compounds, competition for nutrients and adhesion sites, alteration of microbial metabolites and stimulation of immunity. (Mishra and Lambert, 1996).

Traditional probiotic dairy strains of LAB have a long history of safe use. There is considerable interest in extending the range of foods incorporating probiotic organisms from dairy foods to infant formulae, baby foods, fruit juice based products, cereal based products and pharmaceuticals (Lee and Salminen, 1995). *Lactobacillus spp.* and *Bifidobacterium spp.* are prominent members of the commensal intestinal flora and are the commonly studied probiotic bacteria. They cause reduced lactose intolerance, alleviation of some diarrhoeas, lowered blood cholesterol, increased immune responses and prevention of cancer (Marteau and Rambaud, 1993, 1996; Gilliland 1996, Salminen *et al.*, 1998a). Salminen *et al.* (1998a) presented a list of successful probiotic strains, including, *L. acidophilus* NCFB 1478, *L. johnsonii* LAI, *L. casei shirota* strain and *L. rhamnosus GG*. The selection criteria for probiotic LAB include: human origin, safety, viability/activity in delivery vehicles, resistance to acid and bile, adherence to gut epithelial tissue, ability to colonise the GIT, production of antimicrobial substances, ability to stimulate a host immune response and the ability to influence metabolic activities such as vitamin production, cholesterol assimilation and lactose activity (Huis in't Veld and Shortt, 1996, Salminen *et al.*, 1996). Probiotic preparations such as *Lactobacillus GG, L. johnsonii* LA1 and NCFB 1748, *L. casei shirota* strain and *L. reuterii* are beneficial in the prevention and treatment of certain GI infections including infantile viral diarrhoea and antibiotic associated diarrhoea (Lee and Salminen, 1995, Salminen *et al.*, 1998a). It is likely that *lactobacilli* suppress the growth of pathogens at the mucosal surface probably by out competing them for nutrients or by producing antibacterial compounds (Salminen *et al.*, 1998a; Isolauri *et al.*, 1998).

Several studies in both animals and humans have showed the ability of LAB to reduce the toxicity of intestinal contents by suppressing the levels of bacterial enzymes such as B-glucoronidase, nitroreductase, azo-reductase and urease, all of which activate procarcinogens (Salminen *et al.*, 1996, 1998a; Isolauri *et al.*, 1998).

In addition many LAB produce metabolic end products (butyrate/ butyric acid) that have antitumorigenic activities in vitro (young, 1996). *Lactobacillus casei shirota* strain when orally administered reduced the recurrence of superficial bladder carcinoma in humans (Aso and Akazan, 1992; Aso *et al.*, 1995). LAB can modulate host immune response. Reports showed increased production of immunoglobulins, interleukins 6 and 10, gamma interferon, tumour necrosis factor-a and increased phagocytic activity. *L. GG* stimulate local and systemic IgA to rota virus during infection of children with this agent (Kaila *et al.*, 1992). *L. salivarius* UCC 118 also exhibits a strong mucosal IgA immune response in humans during clinical trials (Mattila Sandholm, 1997).

Another compound called prebiotics are based on non- or slowly absorbable complex CHO that can be assimilated by beneficial bacteria such as *Bifidobacterium* & *Lactobacillus spp*. Examples of prebiotic substrates are inulin, lactulose, various galacto, fructo, xylo-oligosaccharides and sugar alcohols such as lactitol and xylitol (Salminen *et al.*, 1998b).Many of the functional foods contain a combination of a probiotic culture with a prebiotic substrate that favours its

growth. One fermented drink Fyos (Nutricia) is the synbiotic product which is combination of probiotic culture *L. casei* and prebiotic oligofructose, inulin .Over the years a number of microbes have been utilized as probiotics.

Microorganisms used as probiotics:
Lactobacillus acidophilus
L.plantarum
L. casei
L. casei subsp. rhamnosus
L. delbreuckii subsp. bulgaricus
L. fermentum
L.reuteri
Lactococcus lactis subsp. lactis
L. lactis subsp. cremoris
Bifidobacterium bifidum
B. infantis
B.adolescentis
B. longum
B. breve
Streptococcus salivarius subsp. thermophilus
Enterococcus faecalis
E.faecium
Saccharomyces boulardii
Source: Conway (1996).

The recent advances in biotechnology have significantly increased the production of high quality, nutritious and tasteful foods that remain fresh for long time and are completely safe and less reliant on artificial additives. The potential application of bacteriocins as consumer friendly biopreservatives either in the form of protective cultures are as additives is significant. Besides being less potentially toxic or carcinogenic than current antimicrobial agents , lactic acid bacteria and their by products have been shown to be more effective and flexible in several applications. Evidence is accumulating that confirms that probiotics can benefit the host by improving intestinal well being .In order to have functional probiotic strains with predictable and measurable beneficial effects , strict attention to strain selection is required.

References

Aso, Y. and H.Akazan, 1992. Prophylactic effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer. Urology international, 49:125-129.

Aso,Y., H. Akazan, T. Kotake, T. Tsukamoto, K. Imai and S. Naito, 1995. Preventative effect of a *Lactobacillus casei* preparation on the recurrence of superficial bladder cancer in a double blind trial. European Urology, 27: 104-109

Axelsson, L., 1998. Lactic acid bacteria: classification and physiology. In: Salminen, S. & von Wright, A. (eds). Lactic Acid Bacteria: Microbiology and Functional Aspects 2nd Edition. New York: Marcel Dekker Inc., 1-72.

Braun,V., H. Pilsl and P. Grob,1994. Colicins: Structures, modes of action, transfer through membranes ,and evolution. Arch Microbiol.,161:199-206.

Conway, P.L., 1996. Selection criteria for probiotic microorganisms. Asia Pacific J. Clin. Nutr., 10-14

Daeschel, M.A., 1993. Applications and interactions of bacteriocins from lactic acid bacteria in foods and beverages. In: Hoover, D.B & Steenson. L.R. (eds.). Bacteriocins of Lactic Acid Bacteria. New York: Academic Press Inc., 63-91.

Daly, C. and R. Davis, 1998. The biotechnology of lactic acid bacteria with emphasis on applications in food safety and human health. Agri. and food Sci. in Finland, 7: 2, 251-264.

Davidson. B.E., N. Kordias , M. Dobos and A. J. Hillier, 1996. Genomic organisation of lactic acid bacteria. Antonie van Leeuwenhoek, 70:65-87.

Delves-Broughton, J., P. Blackburn, R.J. Evans and J. Hugenholtz, 1996. Applications of the bacteriocin, nisin. Antonie van Leeuwenhoek, 70: 193-202.

Dodd, H.M. and M.J. Gasson, 1994. Bacteriocins of lactic acid bacteria. In: Gasson, M.J. & de Vos. W.M. (eds.). Genetics and Biotechnology of Lactic Acid Bacteria. Glasgow: Blackie Academic and Professional, 211-251.

Fitzgerald, G.F. and C. Hill, 1996. Genetics of Starter cultures. In: Cogan, T.M. & Accolas, J.P. (eds.). Dairy Starter Cultures. New York: VCH Publishers, 25-46.

Fuller, R., 1991.Probiotics in human medicine. Gut, 32: 439-442

Gilliland, S.E., 1996. Special additional cultures. In: Cogan, T.M. & Accolas, J.P. (eds). Dairy Starter Cultures. New York: VCH Publishers, 25-46.

Glass, K.A., B. Bhanu Prasad, J.H. Schlyter, H.E. Uljas, N.Y. Farkye and J.B.Luchansky, 1995. Effects of acid type and ALTATM 2431 on *Listeria monocytogenes* in a Queso Blanco type of cheese. Journal of Food Protection, 58: 737-741.

Havenaar, R. and J. Huis in't Veld, 1992. Probiotics: A general view. In:wood ed . The lactic acid bacteria in health and disease. London Elsevier Applied Science, 209-224.

Helander, I.M., A. von Wright and T.M. Mattila-Sandholm, 1997. Potential of lactic acid bacteria and novel anti-microbials against Gram-negative bacteria. Trends in Food Science and Technology, 8:146-150.

Holzapfel, W. H., R. Geisen and U. Schillinger, 1995. Biological preservation of foods with reference to protective cultures, bacteriocins and food-grade enzymes. International J. Food Microbiol., 24: 343-362.

Huis in't Veld, J. and C. Shortt, 1996. Selection criteria for probiotic microorganisms. In Leeds, A.R. & Rowland, I.R. (eds.). Gut Flora and Health-Past, Present and Future London: The Royal Society of Medicine Press Ltd., 19-26.

Isolauri, E., E. Salminen and S. Salminen, 1998. Lactic acid bacteria and immune modulation. In: Salminen, S. & von Wright, A. (eds.) Lactic Acid Bacteria: Microbiology and Functional Aspects 2nd Edition. New York: Marcel Dekker Inc., 255-268.

Jack, R.W., J.R. Tagg and B. Ray, 1995. Bacteriocins of gram positive bacteria. Microbiology Reviews, 59: 171-200.

Joerger, M.C and T.R. Klaenhammer, 1990. Cloning , expression and nucleotide sequence of the *Lactobacillus helveticus* 481 gene encoding the bacteriocin helveticin. J. Bacteriol., 172:6339-6347.

Kaila, M., E.Isolauri, E. Soppi , E. Virtanen, S. Laine and H. Arvilommi, 1992. Enhancement of the circulating antibody secreting cell response in human diarrhoea by a human *Lactobacillus* strain. Paediatric Research, 32: 141-144.

Klaenhammer, T.R., 1988. Bacteriocins of lactic acid bacteria. Biochimie., 70 :337-379.

Liao, C.C., A.E. Yuosef, E.R. Chism and E.R. Richter, 1994. Inhibition of *Staphylococcus aureus* in buffer , culture media, and foods by lacidin A, a bacteriocin produced by *Lactobacillus acidophilus*.OSU 133 J. Food Safety, 14:87-101.

Lee, Y. K. and S. Salminen, 1995. The coming of age of probiotics. Trends in Food Science and Technology, 6: 241-245.

Marteau. P. and J.C. Rambaud, 1993. Potential of using lactic acid bacteria for therapy and immunomodulation in man. FEMS Microbiology Reviews, 12: 207220.

Marteau. P. and J.C. Rambaud, 1996. Therapeutic applications of probiotics in humans In: Leeds, A.R. & Rowland. I.R.(eds.) Gut Flora and Health - Past, Present and Future, London: The Royal Society of Medicine Press Ltd. , 47-56.

Mattila-Sandholm, T. ,1997. Demonstration Project FAIR CT96-1028. In:Alander, M. *et al.* (eds.) Novel Methods for Probiotic Research: 2nd Workshop Demonstration of the Nutritional Functionality of Probiotic Foods FAIRCT 96-1028. Technical Research Centre of Finland (VTT) , 11-17.

Mayra-Makinen, A. and T. Suomalainen, 1995. *Lactobacillus casei* spp. rhamnosus, bacterial preparations comprising said strain and use of said strain and preparations for the controlling of yeast and moulds. United States Patent US., 5: 378-458.

Meaney, B., M. Ryan, J. Flynn, C. Hill and P. Ross, 1997 Mastitis control without antibiotics In: O' Rourke, C.(ed) Farm and Food Teagasc., 7 : 23-25.

Mishra,C. and J. Lambert, 1996. Production of antimicrobial substances by probiotics. Asia Pacific J. Clin. Nutr., 5:20-24.

Nes, I.F., D.B. Diep, L.S. Halvarstein, M.B. Brurbeg, V. Eijsink and H. Holo, 1996.Biosynthesis of bacteriocin in lactic acid bacteria .Antonie van Leeuwenhoek Int. J. Gen. Mol. Microbiol., 70:113-128.

Ouwehand, A.C., 1998. Antimicrobial components from lactic acid bacteria. In: Salminen, S. & von Wright, A. (eds.) Lactic Acid Bacteria: Microbiology and Functional Aspects 2nd Edition. New York: Marcel Dekker Inc. , 139-160.

Ray, B. and M. Daeschel, 1992. Food biopreservatives of microbial. origin. CRC Press, In. Boca Raton, Florida:3-11

Ryan, M.P., M.C. Rea, C. Hill and R.P. Ross, 1996. An application in Cheddar cheese manufacture for a strain of *Lactococcus lactis* producing a novel broad-spectrum bacteriocin 3147. Applied and Environmental Microbiology, 62: 612-619.

Ryser, E. and E.H. Marth, 1991. Food borne Listeriosis. In : Ryser, E. and Marth, E.H. (eds.). Listeria, Listeriosis and Food Safety. New York: Marcel Dekker Inc., p: 24o-287.

Sahl, H.G., R.W. Jack and G. Bierbaum, 1995. Biosynthesis and biological activities of lantibiotics with unique post-translational modification. European J. Biochem., 230: 827-853.

Salminen, S., M.A. Deighton, Y. Benno and S.L. Gorbach, 1998a. Lactic acid bacteria in health and disease. In: Salminen, S. & von Wright, A. (eds). Lactic Acids Bacteria: Microbiology and Functional Aspects 2nd Edition. New York: Marcel Dekker Inc., 211-254.

Salminen, S., E. Isolauri and E. Salminen, 1996. Clinical uses of probiotics for stabilising the gut mucosal barrier: successful strains and future challenges. Antonie van Leeuwenhoek, 70: 251-262.

Salminen, S. M. Roberfroid., P. Ramos and R. Fonden, 1998b. Prebiotic substrates and lactic acid bacteria. In: Salminen, S. & von Wright, A. (eds.) Lactic Acid Bacteria: Microbiology and Functional Aspects 2nd Edition New York: Marcel Dekker Inc., 343-350.

Speck, M.L., 1975. Contribution of microorganisms to food and nutrition. Nutr. News, 38:13

Stevens, K. A., B.W. Sheldon, N.A. Klapes and T.R. Klaenhammer, 1991. Nisin treatment for the inactivation of Salmonella species and other Gram negative bacteria. Applied and Environmental Microbiology, 57:3613-3615.

Stiles, M.E., 1996. Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek., 70:235-249.

Vandenberg, P. A., 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiology Reviews., 12:221-238.

Wynder,L., 1977. Colon cancer prevention. Cancer, 40 :2565-71.

von Wright, A. and M. Sibakob, 1998. Genetic modification of lactic acid bacteria. In: Salminen, S. and vonWright, A. (eds.). Lactic Acid Bacteria: Microbiology and Functional Aspects. 2nd Edition. Newyork:Marcel Dekker Inc., p:161-210.

Yang, R. and B. Ray,1994. Prevalence and biological control of bacteriocin producing psychrotrophic *Leuconostocs* associated with spoilage of vacuum packaged processed meats. J.Food Prot., 57:209-217.

Young,G., 1996. Prevention of colon cancer: role of short chain fatty acids produced by intestinal flora. Asia Pacific J. Clin, Nutr., 5:44-47.

Pakistan Journal of Nutrition 1 (1): 25-30, 2002

A Review on the Applications of Organic Trace Minerals in Pig Nutrition

S. P. Acda[1] and B. J. Chae
[1]Institute of Animal Science, University of the Philippines Los Banos,
College, Laguna, Philippines
Division of Animal Resource Sciences, Kangwon National University,
Chunchon 200-701, Korea

Abstract: The purpose of this review is to provide an update on recent informations regarding application of organic trace minerals in pig nutrition. Understanding the efficacy of organic trace minerals has been increasingly important over the past few years as a result of increased customer awareness of their benefits and the increase in the number of commercial products available to the consumer. Organically bound trace minerals of interests in pig nutrition specifically include iron, copper, zinc, chromium and selenium. Organic iron, chromium and selenium have been shown to improve reproductive efficiency as measured by increased farrowing rate, reduced mortality, larger litter size and increased litter weight at birth and at weaning. Additional benefits derived from organic chromium supplementation include improved carcass quality and increased nitrogen retention. Reviews presented reveal no consistent effect of organic copper and zinc on growth performance but could provide the needs of pigs at lower inclusion rate without compromising performance of animals while maintaining serum concentration, and substantially reducing fecal excretions of these elements.

Key words: Organic sources, chelates, reproductive efficiency, growth performance

Introduction

With the increasing number of commercial organic trace mineral products available to the consumer and the increasing use of these products by the pig industry, it is of interest to briefly review the application characteristics of organic trace minerals. The most prudent selection and use of organic trace minerals result from increased customer awareness on the current production performance. So it is very likely that benefits as well as the non-beneficial effects of application be known.

Trace minerals play a vital and important role in nutrition, being part of structural materials, constituents of the soft tissues and cells, and regulate many of the vital biological processes. They occur naturally in most feed ingredients but the amount and bioavailability varies considerably. Although trace minerals are traditionally included in the diet at very small amount in the form of premix of inorganic salts such as sulphates, chlorides, carbonates and oxides, there are several factors that may reduce their availability when ingested by the animals. Some researches in mineral nutrition have shown that the availability of trace minerals can be improved by binding them to organic ligands, usually a mixture of amino acids or small peptides and thus, the so- called organic trace minerals.

A ligand is a molecule containing an atom which has a lone pair of electrons. In the process of chelation, the ligand acts as chelating agent and encircles the metal atom to form a heterocyclic ring structure. That is, the metal atom is bonded to the ligand through donor atoms such as the oxygen of the carboxyl group , nitrogen or sulfur of the amino acid or peptide. As reviewed by Hynes and Kelly (1995), ligands that contain only one donor atom are termed "monodentate" ligands and those that contain two or more donor atoms capable of bonding to a metal ion are termed bi, tri- or tetradentate ligands. When such ligands bond to a metal ion via two or more donor atoms, the complex formed contains one or more heterocyclic rings and such species are called "chelates". Chelates may have four, five- six- and seven-membered rings but it has been shown that chelates having five-membered rings have the greatest stability (Graddon, 1968). There are various categories of organic trace minerals as defined by the Association of American Feed Control Officials (AAFCO,1998) such as:

Metal amino acid chelate - is the product resulting from the reaction of a metal ion from a soluble metal salt with amino acids with a mole of metal to one to three (preferably two) moles of amino acids to form coordinate covalent bonds. The average weight of the hydrolyzed amino acids must be approximately 150 and the resulting molecular weight of the chelate must not exceed 800.

Metal amino acid complex - is the product resulting from complexing of a soluble metal salt with an amino acid(s).

Metal polysaccharide complex - is the product resulting from complexing of a soluble metal salt with a polysaccharide solution.

Metal proteinate - is the product resulting from the chelation of a soluble metal salt with amino acids and/or partially hydrolyzed protein.

Absorption and bioavailability of organic trace minerals: Absorption of trace minerals is often a major limitation of their utilization. Oftentimes absorption is coined with availability because a trace mineral must be certainly absorbed before it can be utilized. However, a trace mineral can also be absorbed but not necessarily be utilized thus making its bioavailability low. During digestion the mineral ions from inorganic sources are released and may re-combine with other digesta components in the intestine forming insoluble complexes and thereby excreted, reducing their absorption across the small intestine. This indicates that the degree to which these dietary minerals are available for absorption depends on the extent to which they form complex molecules in the gut. Whereas the organic minerals utilize peptide and/or amino acid uptake mechanisms in the intestine (Ashmead *et al*., 1985; Ashmead, 1993). The mineral within the complex or chelate is in a chemically inert form due to the coordinate covalent and ionic bonding by the amino ligands hence, more stable and less prone to interactions. The mineral is protected from physiochemical factors or from negative interactions with dietary components such as phytate, which binds cations making them unavailable for absorption (Fairweather-Tait, 1996). In addition, the organic trace minerals remained to be electrically neutral at certain pH conditions. Thus, the mineral chelate/complex is absorbed intact through the intestinal mucosa, traversing the mucosal cell membrane into the plasma (Power and Horgan, 2000). Organic trace minerals have stability constants at such magnitude as to allow the metal ions to be released and transferred to the host's biological system (Hynes and Kelly,1995). Minerals using either amino acid or peptide uptake mechanism would therefore be expected to be absorbed and circulated to target tissues very efficiently (Power and Horgan, 2000) hence, highly bioavailable. However, bioavailability could be influenced by several factors. These include host-related factors such as age and species of the animal, sex, stage of growth, pregnancy, lactation, nutritional status, disease, gastrointestinal secretions and microflora as well as gastrointestinal transit time (Johnson, 1989; Fairweather-Tait,

Table 1: The effect of supplementing the breeder diet with 200 ppb chromium picolinate on reproductive performance

Location	Performance trait	Chromium (ppb)		
		0	200	P-value
Site 1	Sow number	831.0	775.0	
	Farrowing rate, %	82.0	86.3	0.125
	Born alive	12.3	12.4	0.325
	Still births, %	7.2	7.1	0.584
Site 2	Sow number	197.0	222.0	
	Farrowing rate, %	85.0	84.0	0.900
	Born alive	10.9	11.1	0.345
	Still births, %	9.1	8.3	0.885

Adapted from: Campbell (1998)

Table 2: Effect of chromium from chromium chloride and chromium picolinate on growth and serum and carcass traits of growing-finishing pigs

Item	Basal	Cr chlorideb	Cr picolinateb
Gain, kg/d	0.69 0.73	0.72	
Feed intake, kg/d	2.09	2.08	2.25
Gain/feed	0.33	0.35	0.32
Cholesterol, mg/dl	72.30	80.40	68.00
Growth hormone, ng/ml	2.61	2.37	1.55
Insulin, U/ml	19.50	20.60	22.50
10th rib fat, cm	3.07	2.90	2.39
Loin eye area, cm	31.50	31.20	38.40
Percentage of muscling	52.30	52.30	55.70
Dressing percentage	74.60	74.60	74.20

a-Data are means of four replicates of four pigs each. Pigs averaged 22.4 kg initially and the experimental period was 98 d. b-Chromium, 200 ppb. Adapted from: Page, 1991.

Table 3: Effect of source and level of copper on performance of weanling pigs

Item	Control	Cu-lys		$CuSO_4$	
		100	200	100	200
Daily gain, g	364.00	406.00	392.00	391.00	394.00
Daily feed, g	717.00	776.00	745.00	733.00	747.00
Feed:gain ratio	1.97	1.91	1.89	1.88	1.89
Liver Cu, ppm[1]	23.00	33.00	337.00	34.00	272..00

Basal vs all other treatments ($p < .001$); 200ppm of Cu vs 100ppm of Cu (< 0.001). Adapted from: Coffey *et al.* (1994)

1996). The physiochemical factors may also affect nutrient uptake from the intestinal lumen and the incorporation of nutrients into biochemical pathways within the cellular environment (Power and Horgan, 2000). Such factors include the chemical form in which the mineral is ingested, and the amounts and proportions of other dietary components (phytate, phosphate, amino acids, sugars, other metals, etc.) with which it competes and/or interacts metabolically.

Organic iron (Fe) in sows diets: Baby pigs are very susceptible to Fe deficiency anemia because of lack of placental or mammary Fe transfer from the dam to offspring. Pond *et al.* (1961) showed conclusively that whether Fe sources are administered to dams orally or via injection, neither pig stores at birth nor Fe concentration in milk is increased sufficiently to prevent anemia in the offspring. However, with the reported relative availability of chelated or proteinated sources of Fe as 125 - 185% (Henry and Miller, 1995), this has prompted interest in their inclusion and use in sows and piglets diets. According to Ashmead and Graff (1982) iron linked to amino acid increased the transfer of Fe across the placenta and into the embryo. Thus, when provided organic Fe at 200 ppm in the gestation diet significant quantities crossed the placenta and were incorporated into the fetuses. This resulted in significantly reduced mortality as well as heavier piglets at birth and at weaning (Close, 1998). More recently, Close (1999) reported that addition of organic iron to a normal lactation diet fed some 7 days before farrowing and throughout a 26-day lactation, improved feed intake of the sow as well as the weaning weights of the piglets. These indicate that more iron crossed the placenta and transferred into the fetuses which then have higher blood haemoglobin and immunoglobulin levels at birth. This higher immune status and viability resulted to a stronger piglet, consuming more milk and hence, performed better. This observation also provided evidence that organic iron was transported through the body by a different mechanism from normal iron metabolic mechanism (Vandergrifft, 1993).

Organic iron (Fe) in weanling pigs diets: Very little information is available to demonstrate the effects of organic Fe supplementation on the performance of weanling pigs. There was no concrete evidence showing positive response of weanling pigs to organic forms of iron compared with iron sulfate. Lewis *et al.* (1995) reported that the iron in iron-methionine was less bioavailable than the iron in ferrous sulfate but the iron from iron-proteinates was similar to iron in ferrous sulfate (Lewis *et al.*, 1999).

Organic chromium (Cr) in sows diets: There are quite a number of studies indicating positive effect of organic chromium in female reproduction. In experiment conducted by Lindemann *et al.* (1995a) wherein gilts fed diets with 200 ppb Cr from Cr picolinate throughout growth and gestation had larger and heavier litters at birth and at 21 days of age than the controls. Similar experiment was carried out by Campbell (1998) and showed that supplementing the diet with 200 ppb organic chromium fed during gestation and lactation improved fertility through increased litter size and farrowing rate (Table 1). According to Close (1999), the higher number of piglets born alive probably resulted from the action of insulin which influenced follicular development, LH and FSH secretions and hence, increased ovulation rate. Progesterone concentration in the plasma may also change and this affects the action of uterine secretory proteins (uteroferrin and retinol-binding protein) which control embryo survival.

Organic chromium (Cr) in growing-finishing diets: For the past years, efforts are being made to improve carcass quality and organic Cr may be a valuable tool in this respect. In experiments done by Page *et al.* (1991; 1993) in growing/finishing pigs, organic Cr from Cr picolinate significantly increased feed intake, decreased feed efficiency (G/F), reduced 10th rib fat, increased loin eye areas and percentage muscling and reduced serum cholesterol level (Table 2). The work of Lindemann *et al* (1995b) demonstrated a similar response of decreased backfat and increased loin eye area when 200 ppb of Cr from Cr picolinate was fed in diets with 100 or 120% of NRC (1998) lysine requirement for growing-finishing pigs. Similar findings were also reported by Mooney and Cromwell (1995). These beneficial effects of Cr are probably mediated through the action of the growth-promoting hormones which repartition nutrients are in favor of lean rather than fat deposition (Close, 1999).

Positive effects of Cr supplementation were also noted on growth performance of young pigs. Harper *et al.* (1995) examined the effect of adding 200 ppb Cr from Cr picolinate to diets of pigs weaned at 29 days of age. In the first 35 days postweaning, Cr supplementation resulted to an improvement in daily gain ($p < 0.05$) and feed utilization ($p < 0.09$). Wenk, 1994 evaluated multiple forms of Cr such as Cr chloride, Cr yeast and Cr picolinate and reported no performance effects of Cr in the growing period (27-60 kg), but Cr increased growth rates in the finishing period (60-106 kg). The increased growth rate in the finishing period indicated to some extent an improvement on nutrient utilization. This was confirmed when Wang (1995) conducted four nutrient retention experiments to assess the effect of feeding 200 ppb Cr as Cr picolinate on dry matter digestibility and nitrogen balance of growing-finishing pigs. Organic Cr increased the nitrogen absorption ($p < 0.05$), nitrogen retention ($p < 0.10$) and dry matter digestibility ($p < 0.01$). Due to improvement in nitrogen utilization,

Table 4: The growth performance of entire male pigs individually housed and offered diets ad libitum containing either no added copper (control: 20ppm Cu), copper sulphate (150ppm) or organic copper complex(40 ppm)

Feeding period	Parameter	Control	$CuSO_4$	Org.Cu	P-value
Growers	Growth rate, kg/d	0.902	0.957	0.942	0.077
(30-60 kg)	Feed intake, kg/d	1.94	2.05	2.08	0.044
	FCR, feed:gain	2.15	2.16	2.21	0.470
	Faecal Cu, ppm DM	130.00	853.00	275.00	
Finishers	Growth rate, kg/d	0.845	0.871	0.836	0.660
(60-90kg)	Feed intake, kg/d	2.39	2.59	2.65	0.730
	FCR, feed:gain	2.84	2.98	3.02	0.002
	Faecal Cu, ppm DM	108.00	776.00	199.00	

Adapted from: Smits and Henman, 2000.

Table 5: Performance and serum Zn concentration of weanling pigs fed diets with high concentration of zinc (3000 mg/kg) from different sources

Item	ZnO	Zn-met	Zn-lys	$ZnSO_4$
ADG,kg *	0.15a	0.13ab	0.12ab	0.09b
ADFI. kg *	0.26a	0.26a	0.21ab	0.18b
Gain:feed	0.56	0.49	0.58	0.47
Serum concentration, mg/l*	0.98b	1.09b	1.25ab	1.42a

* Means on the same row without common letter differ significantly ($P < 0.05$). Adapted from: Schell and Kornegay, 1996.

organic Cr has been regarded of having the potential of positive environmental impact (Lindemann, 1996).

Organic copper (Cu) in weanling pigs diets: Copper is widely used in the pig industry to promote growth. It is normally added at 100 - 250 ppm to pigs diets in the form of inorganic salt. Although limited research with Cu-amino acids and Cu-proteinates suggesting somewhat greater absorption of Cu than that obtained with copper sulfate ($CuSO_4$) (Baker, 1995), several experiments have been done to evaluate the performance of weanling pigs fed diets supplemented with Cu either as $CuSO_4$ or as organic Cu.

Some of the previous reports have shown that addition of Cu from Cu-lys resulted in improved performance of pigs. Zhou *et al.* (1994) compared $CuSO_4$ with Cu-lys when provided in the diet to weanling piglets over a 24 day period. The piglets on the Cu-lys diet consumed more feeds and had significantly higher growth rates than those fed the $CuSO_4$ diets. Similar results were reported by Coffey *et al.* (1994) who evaluated the efficacy of Cu-lys as growth promotant for weanling pigs (Table 3). Averaged across levels of Cu supplementation, the percentage improvements from Cu-lys additions were greater than those of copper sulphate for growth rate (16.8 vs 11.5%; $P < 0.03$), and feed intake (14.1 vs 8.7%; $P < 0.01$) but not for efficiency of feed utilization (2.2 vs 2.4%). According to Apgar and Kornegay (1996), ADG tended to be higher for pigs fed Cu-lys than pigs fed $CuSO_4$ and at similar levels of feed intake growth rate was 14.3% higher for the pigs fed Cu-lys than $CuSO_4$.

More recently, Smits and Henman (2000) reported that improvement on growth rate was only in the grower stage by increasing feed intake but not at the finishing period (Table 4). However, Close (1999) stated that even though pigs fed diets supplemented with organic copper recorded similar level of performance as those fed diets supplemented with inorganic Cu, there was considerable reduction on coefficient of variation of the growth rate of piglets and a substantial reduction on the amount of copper excretions when organic Cu was provided (Smits and Henman, 2000; Lee *et al.*, 2001a).

Earlier studies done by Kirchgessner and Grassmann (1970), and Grassmann and Kirchgessner (1974) have also shown that absorption rate of Cu in the form of Cu-amino acid complexes was higher than that in the inorganic forms for rats and for cattle (Kincaid *et al.*, 1986) In agreement to this, Du *et al.* (1996) reported that Cu utilization from Cu proteinate and Cu-lys were higher ($P < 0.05$) based on the liver Cu content. Moreso, the rats fed Cu complexes had higher liver Fe or Zn content than the rats fed $CuSO_4$, suggesting that Cu complexes are absorbed via another mechanism that differs from that of inorganic Cu and does not interfere with Fe and Zn.

On the other hand, some studies in which organically bound Cu has been added to swine diets revealed that they are as effective as $CuSO_4$ in improving performance and Cu status in pigs(Bunch *et al.*, 1965; Zoubek *et al.*, 1975; Stansbury *et al.*, 1990; van Heughten and Coffey, 1992; Coffey, *et al.*, 1994; Apgar *et al.*, 1996), in chicks (Baker *et al.*, 1991; Aoyagi and Baker, 1993) and in cattle (Wittenberge *et al.* 1990; Ward *et al.*, 1993). Even at growth-stimulative levels of Cu, $CuSO_4$ the absorption and retention of Cu was similar for both and Cu-lys (Apgar and Kornegay, 1996).

Organic zinc (Zn) in pigs diets: Inorganic zinc supplement in the form of zinc oxide is normally included at high rate (3 kg/ton) in pigs diets due to its pharmacological effects. However, much of this dietary zinc is excreted because the availability of zinc oxide is low (Ammerman *et al.*, 1995). Enhancing therefore, the availability of zinc from sources would reduce the amount of this mineral to be added in animals diets. The use of organically bound zinc such as Zn-lys and Zn-met have received much attention because of their potential of providing readily available zinc. But several researches have been reported with conflicting results. Hahn and Baker (1993) observed no improvement on ADG of weanling pigs fed 3000 ppm Zn from chelated Zn-lys and Zn-met. This report was confirmed by Schell and Kornegay (1996) who investigated the feeding of high concentrations of Zn in pigs to improve postweaning performance and to compare the availability of Zn from several sources when fed to pigs at high concentrations. These previous results showed that the performance of pigs was not generally improved by feeding 3000 mg Zn /kg from any of the Zn sources (Table 5). The serum concentration of Zn was greater ($P < 0.05$) for pigs fed $ZnSO_4$ rather than ZnO and Zn-met. Thus, when used as measurement, the bioavailability of Zn was lowest for ZnO and intermediate for Zn-lys and Zn-met. But when based on plasma concentrations of zinc, equivalent bioavailabilities have been reported for zinc sulfate, zinc-lys and zinc-met (Hahn and Baker, 1993).

Contrary to these reports, Cheng *et al.* (1998) demonstrated that $ZnSO_4$ and Zn-lys complex seemed to be equally effective not only in promoting growth but also in Zn absorption, and tissue stores of young pigs even when diets contained deficient, adequate or slightly more than adequate level of lysine. This was consistent with the previous reports of Kornegay and Thomas (1975); Hill *et al* (1986); Likewise, Wedekind *et al.* (1994); Swinkels, *et al.*, 1996 reported that Zn from both $ZnSO_4$ and Zn-met were equally effective, in contrast to the findings of Wedekind *et al.* (1992) that Zn-met complex was more available than $ZnSO_4$ or ZnO as measured by the Zn content of the tibia of chicks. Ward *et al.* (1996) reported that the growth response of weanling pigs was similar between those fed 250 ppm of zinc from zinc-met complex and 2000 ppm zinc from zinc oxide, which suggests an increase

Table 6: Growth performance and copper (Cu) and zinc (Zn) concentrations in serum and feces of weanling pigs1

Treatment*	A	B	C	D	E	SE
ADG(g)	573.00	571.00	601.00	594.00	597.00	25.06
ADFI(g)	988.00	962.00	934.00	974.00	964.00	46.54
FCR	1.72a	1.68a	1.55b	1.64ab	1.62ab	0.09
Serum(mg/l)						
Cu						
2nd w	1.60b	1.76ab	1.98a	1.75ab	2.02a	0.21
4th w	1.49bc	1.38c	1.70a	1.66ab	1.80a	0.18
Zn						
2nd w	1.44b	1.50ab	1.55ab	1.52ab	1.74a	0.16
4th w	1.64ab	1.60b	1.72ab	1.61b	1.83a	0.13
Feces(mg/kg)						
Cu	1,870.00a	615.00d	1,165.00b	785.00c	1,240.00b	451.90
Zn	1,555.00a	795.00c	1,240.00b	1,155.00b	1,560.00a	297.36

*Treatment: A, $ZnSO_4$ 120ppm, $CuSO_4$ 170ppm; B, ZAC 60ppm,CAC 85ppm; C, ZAC 120ppm, CAC 170ppm; D, ZM 60ppm, CL 85ppm; E, ZM 120ppm, CL 170ppm. 1Values with different superscripts of the same row are significantly different ($p < 0.05$). Adapted from: Lee *et al.*, 2001b.

Table 7: Effect of Se sources and levels fed to sows during late gestation on litter and postpartum milk Se concentration

	Inorganic Se		Organic Se		
Item	0.15	0.30	0.15	0.30	SEM
Colostrum Se ug/mL	0.061a	0.093a	0.131b	0.188c	0.016de
Milk Se, ug/mL					
7 d	0.032f	0.036g	0.077h	0.111i	0.002dj
14 d	0.027f	0.036g	0.072h	0.105i	0.003dj
Litter Se measurements					
7 d					
GSH-Px, units/mL	0.128	0.130	0.109	0.110	0.019
Serum Se, ug/mL	0.015b	0.061ab	0.057b	0.072a	0.005d
14 d					
GSH-Px, units/mL	0.147	0.206	0.215	0.217	0.021
Serum Se, ug/mL	0.072c	0.076c	0.086b	0.104a	0.004dk

a,b,c,Means within each row with different superscripts differ ($p < 0.05$). d-Linear increase ($p < 0.01$) .15 and .30 ppm organic Se level. e-Se level x Se source interaction ($p < 0.01$). f,g,h,i-Means within each row with different superscripts differ ($p < 0.01$). j-Se level x Se source interaction ($p < 0.05$). k-Linear response ($p < 0.01$) .15 and .30 ppm inorganic Se levels. Adapted from: Mahan (2000).

Table 8. Effect of dietary Se sources and levels on growing-finishing pig performances

	Inorganic Se				Organic Se				
Item	0.05	0.10	0.20	0.30	0.05	0.10	0.20	0.30	SEM
Final weight, kga	107.6	104.3	105.5	104.2	106.6	108.0	106.1	106.7	1.4
Daily gain, g									
20 to 55 kg	772	764	780	763	770	782	783	804	20
55 to 105 kg	916	853	880	854	895	900	885	857	15
Overall	852	812	838	813	838	847	837	835	13
Daily feed, g									
20 to 55 kg	1,730	1,691	1,768	1,707	1,715	1,735	1,734	1,774	41
55 to 105 kg	2,695	2,546	2,698	2,523	2,688	2,644	2,628	2,615	57
Overall	2,248	2,147	2,273	2,141	2,232	2,219	2,210	2,228	38
Gain:feed, g/kg									
20 to 55 kg	446	451	445	447	449	451	452	453	6
55 to 105 kg	340	335	326	339	333	340	337	329	6
Overall	379	378	369	380	375	383	379	375	4

aEach mean represents 39 pigs per treatment group with an average initial body weight of 20.4 kg. Adapted from: Mahan, *et al.*, 1999.

in the bioavailability of zinc in zinc-met. More recently, Lee *et al.* (2001a) demonstrated higher ADG in pigs fed diet with 120 ppm Zn from zinc-met than the control group ($ZnSO_4$).

Organic zinc (Zn) and copper (Cu) in pigs diets: Because of the positive response of weanling pigs to the pharmacological levels of Zn and Cu, there has been interest in determining whether Zn and Cu act synergistically. This was first initiated by Smith *et al.* (1997), who evaluated the potential interactive or additive effects of growth-promotional levels of Zn and Cu on weanling pig performance. They observed decreased ADG ($p < 0.01$) and ADFI ($p < 0.05$) in pigs fed the diets with 250 ppm Cu, with or without 3,000 ppm Zn compared with pigs fed either the control diet or the diet with only 3,000 ppm added Zn. The lack of an additive response to Zn and Cu at pharmacological levels could be attributed to Cu:Zn imbalance. In collaboration with this finding Spears *et al.* (1999) showed that the levels of Zn and Cu normally added to pig diets can be greatly reduced without affecting performance from weaning to slaughter. Substantial reduction of Zn and Cu in fecal excretions were obtained by replacing a portion of the reduced inorganic trace minerals in the diet with organic trace minerals. Similar results were also obtained by Lee *et al.* (2001b) wherein the effects of feeding different chelated Zn and Cu sources on growth performance and fecal excretions of weanling pigs were evaluated. Results indicated that the efficacy of chelated Zn and Cu sources at low levels are not statistically different ($p > 0.05$) in terms of growth performance and in maintaining serum concentrations from that of high levels of inorganic Zn and Cu sources. The fecal excretions for Zn and Cu were reduced in pigs fed low level of these minerals using organic sources (Table 6).

Organic selenium (Se) in sows diets: It has been a tradition to supplement swine diets with Se in the form of sodium selenite

particularly in sows diets. A deficiency in Se may lead to problems at farrowing which may include high piglet mortality, lethargic and weak piglets as well as mulberry heart disease. In addition, low Se intake can lead to reduced muscle tone and strength in sows which may eventually prolong the farrowing process with a greater incidence of stillborn piglets (Close, 1998).

With organic Se having more availability of 120 to 150% to sodium selenite (Close,1998), research report of Mahan and Kim (1996) showed the beneficial effects of adding Se-enriched yeast to a gestating diet. First parity gilts fed diets with organic Se had increased milk Se content and when fed .3 ppm Se from Se-enriched yeast source subsequently increased the piglet tissue Se concentrations. In line with these findings, Mahan (2000) evaluated the short-term effects of feeding two dietary Se sources at various levels on the transfer of Se to the dam's milk and nursing pig. Similar responses were observed that organic Se increased milk Se content 2.5 to 3 times that of inorganic source and subsequently increased the nursing pig's serum Se (Table 7), a clear indication that Se is transferred through placental and mammary tissue (Mahan *et al.*, 1977). These results have important implications for the young piglets which are often deficient in Se at weaning, a deficiency that can predispose them to mulberry heart disease (Close, 1998). Hence, pigs of poor Se status may encounter Se deficiency sooner after weaning (Mahan *et al.*, 1975).

Organic selenium (Se) in growing/finishing diets: Inclusion of Se in grower/finisher pig diets is also important in improving the Se status of the various organs in the animal's body as well as the activity of glutathione peroxidase (GSH-Px). GSH-Px functions for the prevention of cell membrane oxidation and a deficiency of Se will therefore leave membrane vulnerable to oxidation and precipitate drip loss in meat (Close, 1999). Mahan *et al.*(1999) evaluated the efficacy of inorganic and organic Se sources for growing/finishing pigs, as measured by performance and various tissue, serum, carcass, and loin quality traits. The performance results of pigs during growing, finishing, and overall experimental period demonstrated no effect ($p > 0.05$) of Se source or level on pig gain, feed intake and gain:feed ratio (Table 8). But both Se source and level positively affected the Se concentration in the various pig tissues at the end of the growing and finishing periods and the magnitude of increase was substantially greater when organic Se source was fed. No carcass measurement benefit resulted from either Se source or level. However, there was a trend for higher drip loss and increased loin paleness; low carcass quality when inorganic Se level increased. These results were consistent with the work of Mahan and Parrett (1996) who demonstrated no growth or feed responses when either inorganic or organic form of Se was added at various levels to growing-finishing cereal grain-based diets for pigs. When organic Se was added there was higher deposition of Se in muscle but with higher GSH-Px activity when the inorganic form of element was provided.

Conclusion: The practical use of organic trace minerals will depend on the performance response, health status of animals and environmental impact. These responses will determine the cost-effectiveness of organic trace minerals in pig production. Positive responses to organic Fe, Cr and Se have been reported in relation to swine reproductive efficiency. Improved carcass quality and increased nitrogen retention are added benefits that could be derived from organic Cr supplementation. However reviews presented revealed no consistent effect of organic sources for Cu and Zn on performance variables and concentrations in serum and soft tissues. Most of the research results demonstrated that organic sources of Cu and Zn at low levels could substantially decrease the concentrations of Cu and Zn excreted compared to inorganic sources at pharmacological levels.

References

AAFCO., 1998. Official Publication of the Association of American Feed Control Officials Incorporated (P. M. Bachman, ed). 237-238.

Ammerman, C. B., D. B. Baker and A. J. Lewis, 1995. Bioavailability of Nutrients for Animals. Academic Press, New York, 367-398.

Apgar, G. A., E. T. Kornegay, M. D. Lindemann and D. R. Notter, 1995. Evaluation of copper sulfate and a copper lysine complex as growth promoters for weanling swine. J. Anim. Sci., 73:2640-2646.

Apgar, G. A. and E. T. Kornegay, 1996. Mineral balance of finishing pigs fed copper sulfate or a copper lysine complex at growth-stimulating levels. J. Anim. Sci., 74:1594-1600.

Ashmead, H. D., 1993. Comparative intestinal absorption and subsequent metabolism of metal amino acid chelates and inorganic metal salts. In: The roles of amino acid chelates in animal nutrition. Noyes Publishers, New Jersey, 306-319.

Ashmead, H. D. and D. J. Graff, 1982. Placental transfer of chelated iron. Proceedings of the International Pig Veterinary Society Congress, Mexico. p: 207.

Ashmead, H. D., D. J. Graff and H. H. Ashmead, 1985. Intestinal absorption of metal ions and chelates. C. C. Thomas, Springfield, Illinois.

Aoyagi, S and D. H. Baker, 1993. Nutritional evaluation of copper-lysine and zinc lysine complexes for chicks. Poult. Sci., 72:165-171.

Baker, D. H., 1995. Copper bioavailability. In: Bioavailability of Nutrients for Animals (Ammerman, C. B., D. H. Baker and A. J. Lewis, eds.). Academic Press. New York. pp: 127-156.

Baker, D. H., J. Odle, M. A. Funk and T. M. Wieland, 1991. Research Note: Bioavailability of copper in cupric oxide, cuprous oxide and in a copper-lysine complex. Poult. Sci., 70:177.

Bunch, R. J., J. T. McCall, V. C. Speer and V. W. Hays, 1965. Copper Supplementation for weanling pigs. J. Anim. Sci., 24:995-1000.

Campbell, R. G., 1998. Chromium and its role in pig production. In: Biotechnology in the Feed Industry. (T. P. Lyons and K. A Jacques, eds.). Nottingham University Press. Nottingham, UK., 229-237.

Cheng, J., E. T. Kornegay and T. Schell. 1998. Influence of dietary lysine on the utilization of zinc from zinc sulphate and zinc-lysine complex by young pigs. J. Anim. Sci., 76:1064-1074.

Close, W. H., 1998. The role of trace mineral proteinates in pig nutrition. In: Biotechnology in the Feed Industry. (T. P. Lyons and K. A. Jacques, eds.). Nottingham University Press. Nottingham, UK., pp: 469-483.

Close, W. H., 1999. Organic minerals for pigs: an update. In: Biotechnology in the Feed Industry. (T. P. Lyons and K. A. Jacques, eds.). Nottingham University Press. Nottingham, UK., pp: 51-60.

Coffey, R. D., G. L. Cromwell and H. J. Monegue, 1994. Efficacy of a copper-lysine complex as a growth promotant for weanling pigs. J. Anim. Sci., 72:2880-2886.

Du, Z., R. W. Hemken, J. A. Jackson and D. S. Trammel, 1996. Utilization of copper in copper proteinate, copper lysine and cupric sulfate using the rat as experimental model. J. Anim. Sci., 74:1657-1663.

Fairweather-Tait, S .J., 1996. Bioavailability of dietary minerals. Biochem. Soc. Trans., 24:775-780.

Graddon, D. P., 1968. An Introduction to Co-ordination Chemistry (2nd edn.). Pergamon Press, Oxford.

Grassmann, E. and M. Kirchgessner, 1974. On the metabolic availability of absorbed copper and iron. In: Trace Element Metabolism in Animals (W. G. Hoekstra, J. W. Suttle, H. E. Ganther and W. Mertz, eds.). University Park Press, Baltimore, MD. pp: 523-526.

Hahn, J. D. and D. H. Baker, 1993. Growth and plasma zinc responses of young pigs fed pharmacological levels of Zinc. J. Anim. Sci., 71:3020-3024.

Harper, A. F., M. D. Lindemann and E. T. Kornegay, 1995. Effect of supplemental dietary chromium on growth performance of weanling swine. J. Anim. Sci., 73(Suppl. 1):194(Abstr.).

Henry, P. R. and E. R. Miller, 1995. Iron availability. In: Bioavailability of Nutrients for Animals (C. B. Ammerman, D. H. Baker and A. S. Lewis, eds.). Academic Press, San Diego, pp: 169-199.

Hill, D. A., E. R. Peo, Jr., A. J. Lewis and J. D. Crenshaw, 1986. Zinc-amino acid complexes for swine. J. Anim. Sci., 63:121-130.

Hynes, M. J. and M. P. Kelly, 1995. Metal ions, Chelates and proteinates. In: Biotechnology in the Feed Industry. (T. P. Lyons and K. A. Jacques, eds.). Nottingham Press, Nottingham, UK., pp: 233-248.

Johnson, P. E., 1989. What can in vitro methods tell us about mineral availability Biological Trace Mineral Research, 19:3-10.

Kincaid, R. L., R. M. Baluwiekel and J. D. Cronrath, 1986. Supplementation of copper proteinate for growing calves fed forages containing molybdenum. J. Dairy Sci., 69:160-163.

Kirchgessner, M. and E. Grassmann, 1970. The dynamics of copper absorption. In: Trace Element Metabolism in Animals (C. F. Mills, ed). Proc. WAAP/IBP Int. Symp. Abeerden, Scotland, pp: 277-287.

Kornegay, E.T. and H. R. Thomas, 1975. Zinc-proteinate supplement studied. Hog Farm Manage, Aug., 50-52.

Lee, S. H., S. C. Choi, B. J. Chae, S. P. Acda and Y. K. Han, 2001a. Effect of feeding different chelated copper and zinc sources on growth performance and fecal excretions of weanling pigs. Asian-Aus. J. Anim. Sci., 14:1616-1620.

Lee, S. H., S. C. Choi, B. J. Chae, J. K. Lee and S. P. Acda, 2001b. Evaluation of metal-amino chelates and complexes at various levels of copper and zinc in weanling pigs and broiler chicks. Asian-Aus. J. Anim. Sci., 14:1734-1740.

Lewis, A. J., P. S. Miller and C. K. Wolverton, 1995. Bioavailability of iron in iron methionine for pigs. J. Anim. Sci., 73(Suppl. 1):172(Abstr.).

Lewis, A. J., H. Y. Chen and P. S. Miller, 1999. Bioavailability of iron in iron proteinate for weanling pigs. J. Anim. Sci., 77(Suppl. 1):61(Abstr.).

Lindemann, M. D., 1996. Organic chromium - the missing link in farm animal nutrition. In: Biotechnology in the Feed Industry. (T. P. Lyons and K. A. Jacques, eds). Nottingham University Press. Nottingham, UK., pp: 299-314.

Lindemann, M. D., A. F. Harper and E. T. Kornegay, 1995a. Further assessments of the effects of supplementation of chromium from chromium picolinate on fecundity in swine. J. Anim. Sci., 73(Suppl 1):185(Abstr.).

Lindemann, M. D., C. M. Wood, A. F. Harper, E. T. Kornegay and R. A. Anderson, 1995b. Dietary chromium picolinate additions improve gain:feed and carcass characteristics in growing-finishing pigs and increase litter size in reproducing sows. J. Anim. Sci., 73:457-465.

Mahan, D. C., A. L. Moxon and J. H. Cline, 1975. Efficacy of supplemental selenium in reproductive diets on sow and progeny serum and tissue selenium values. J. Anim. Sci., 40:624-631.

Mahan, D. C., A. L. Moxon and M. Hubbard, 1977. Efficacy of inorganic selenium supplementation to sow diets on resulting carry-over to their progeny. J. Anim. Sci., 45:738-746.

Mahan, D. C. and Y. Y. Kim, 1996. Effect of inorganic or organic selenium at two levels on reproductive performance and tissue selenium concentrations in first-parity gilts and their progeny. J. Anim. Sci., 74:2711-2718.

Mahan, D. C. and N. A. Parrett, 1996. Evaluating the efficacy of selenium-enriched yeast and sodium selenite on tissue selenium retention and serum glutathione peroxidase activity in grower and finisher swine. J. Anim. Sci., 74:2967-2974.

Mahan, D. C., T. R. Cline and B. Richert, 1999. Effects of dietary levels of selenium-enriched yeast and sodium selenite as selenium sources fed to growing-finishing pigs on performance, tissue selenium, serum glutathione peroxidase activity, carcass characteristics, and loin quality. J. Anim. Sci., 77:2171-2179.

Mahan, D. C., 2000. Effect of organic and inorganic selenium sources and levels on sow colostrum and milk selenium content. J. Anim. Sci., 78:100-105.

Mooney, K. W. and G. L. Cromwell, 1995. Effects of dietary chromium picolinate supplementation on growth, carcass characteristics and accretion rates of carcass tissues in growing-finishing swine. J. Anim. Sci., 73:3351-3357.

Page, T. G., T. L. Ward and L. L. Southern, 1991. Effect of chromium picolinate on growth and carcass characteristics of growing-finishing pigs. J. Anim. Sci., 69(Suppl 1):356(Abstr.).

Page, T. G., L. L. Southern, T. L. Ward and D. L. Thompson, Jr., 1993. Effect of chromium picolinate on growth and serum and carcass traits of growing-finishing pigs. J. Anim. Sci., 71:656-662.

Pond, W. G., R. S. Lowrey, J. H. Maner and J. K. Loosli, 1961. Parenteral iron administration to sows during gestation or lactation. J. Anim. Sci., 20:747.

Power, R. and K. Horgan, 2000. Biological chemistry and absorption of inorganic and organic trace metals. In: Biotechnology in the Feed Industry. (T.P. Lyons and K. A. Jacques, eds.). Nottingham University Press, Nottingham, UK., pp: 277-291.

Schell, T. C. and E. T. Kornegay, 1996. Zinc concentration in tissues and performance of weanling pigs fed pharmacological level of zinc from ZnO, Zn-methionine, Zn-lysine or ZnSO4. J. Anim. Sci., 74: 1584-1593.

Smith, J. W. II, M. D. Tokach, R. D. Goodband, J. L. Nelssen and B. T. Richert, 1997. Effects of the interrelationship between zinc oxide and copper sulfate on growth performance of early-weaned pigs. J. Anim. Sci., 75:1861-1866.

Smits, R. J. and D. J. Henman, 2000. Practical experience with bioplexes in intensive pig production. In: Biotechnology in the Feed Industry. (T.P. Lyons and K. A. Jacques, eds.). Nottingham University Press. Nottingham, UK., pp: 293-300.

Spears, J. W., B. A. Creech and W. L. Flowers, 1999. Reducing copper and zinc in swine waste through dietary manipulation. In: Proc. of the North Carolina Waste Management Symp. North Carolina State University, Raleigh, p:179.

Stansbury, W. F., L. F. Tribble and D. E. Orr, Jr., 1990. Effect of chelated copper sources on performance of nursery and growing pigs. J. Anim. Sci., 68:1318-1322.

Swinkels, J. W. G. M., E. T. Kornegay, W. Zhou, M. D. Lindemann, K. E. Webb, Jr. and M. W. A. Verstegen, 1996. Effectiveness of a zinc amino acid chelate and zinc sulfate in restoring serum and soft tissue zinc concentrations when fed to zinc-depleted pigs. J. Anim. Sci., 74:2420-2430.

Vandergrifft, B., 1993. The role of mineral proteinates in immunity and reproduction. What do we really know about them? In: Biotechnology in the Feed Industry. (T. P. Lyons, ed). Nicholasville, Kentucky, pp: 27-34.

van Heugten, E. and M. T. Coffey, 1992. Efficacy of copper-lysine chelate as growth promotant in weanling swine. J. Anim. Sci., 70(Suppl. 1):18(Abstr.).

Wang, Z., 1995. Influence of supplemental chromium picolinate on nitrogen balance, dry matter digestibility and lean-ness in growing-finishing pigs. M. S. thesis. Virginia Polytechnic Institute and State University.

Ward, J. D., J. W. Spears and E. B. Kegley, 1993. Effect of copper level and source (copper lysine vs copper sulfate) on copper status, performance and immune response in growing steers fed diets with or without supplemental molybdenum and sulfur. J. Anim. Sci., 71:2748-2755.

Ward, T. L., G. L. Asche, G. F. Louis and D. S. Pollmann, 1996. Zinc-methionine improves growth performance of starter pigs. J. Anim. Sci., 74(Suppl. 1):182(Abstr.).

Wedekind, K. J., A. E. Hortin and D. H. Baker, 1992. Methodology for assessing zinc bioavailability: Efficacy estimates for zinc methionine, zinc sulfate, and zinc oxide. J. Anim. Sci., 70:178-187.

Wedekind, K. J., A. J. Lewis, M. A. Giesemann and P. S. Miller, 1994. Bioavailability of zinc from inorganic and organic sources for pigs fed corn-soybean meal diets. J. Anim. Sci., 72:2681-2689.

Wenk, C., 1994. Organic Chromium in growing pigs: observations following a year of use and research in Switzerland. In: Biotechnology in the Feed Industry. (T. P. Lyons and K. A. Jacques, eds). Nottingham University Press, Nottingham, UK., pp: 302-308.

Wittenberg, K. M., R. J. Boila and M. A. Shariff, 1990. Comparison of copper sulfate and copper proteinate as copper sources for copper-depleted steers fed high molybdenum diets. Can. J. Anim. Sci., 70:895-904.

Zhou, W., E. T. Kornegay, H. van Laar, J. W. G. M. Swinkels, E. A. Wong and M. D. Lindemann, 1994. The role of feed consumption and feed efficiency in copper-stimulated growth. J. Anim. Sci., 72:2385-2394.

Zoubek, G. L., E. R. Peo, Jr., B. D. Moser, T. Stahly and P. J. Cunningham, 1975. Effects of source on copper uptake by swine. J. Anim. Sci., 40:880.

Pakistan Journal of Nutrition 1 (1): 31-33, 2002

Energy Balance in Faunated and Defaunated Sheep on a Ration High in Concentrate to Roughage (Good Quality) Ratio

Chandramoni[*1], C. M. Tiwari[2], N. Haque, Murari Lal, S. B. Jadhao[3] and M. Y. Khan
Energy Metabolism and Respiration Calorimetry Laboratory, Animal Nutrition Division,
Indian Veterinary Research Institute, Izatnagar 243 122, (U.P.)
[1] Department of Animal Nutrition, Bihar Veterinary College, Patna 800 014
[2] Department of Animal Nutrition, Rajiv Gandhi College of Veterinary and Animal Sciences,
Kurumbapeth, Pondicherry 600 009
[3] Central Institute of Fisheries Education, 7 Bungalows, Versova, Mumbai - 400 061, India
E-mail: chandramoni@hotmail.com

Abstract: Five faunated and five defaunated sheep were fed diet containing 70:30 concentrate to roughage (oat hay) ratio and energy balances were determined by carbon and nitrogen balance method.Intake and nutritive value of the diet did not differ between faunated and defaunated sheep. Loss of carbon in urine and methane as percent of intake was significantly ($p<0.01$) less in defaunated sheep but energy balance and efficiency of utilisation of ME for maintenance was similar in both faunated and defaunated animals. The energy cost per g protein and fat synthesis in defaunated sheep was 13.9 and 12.3 kcal ME, respectively. It was inferred that high concentrate to roughage ratio and maintenance type roughage like oat hay in ration of defaunated Muzaffarnagari sheep does not yield any additional benefit from energetic point of view, compared to faunated sheep.

Key words: Carbon-nitrogen balance, sheep, defaunation, high concentrate ration, protein and fat synthesis

Introduction

Manipulation of rumen function through the removal of ciliate population (defaunation) appears to have considerable potential in improving ruminant productivity under certain feeding situations (Bird and Leng, 1985). Heat production is affected by defaunation which may be significantly lower (Kreuzer, *et a.*, 1986; Eadie and Gill, 1971; Itabashi *et al.*, 1984) or higher (Whitelaw *et a.l*, 1984) in defaunated sheep as compared to normal animals affecting energy utilisation. The consequent effect of diet induced thermogenesis on carbon; nitrogen and energy balance is little studied. We have earlier reported (Chandramoni *et al.*, 1999) that higher concentrate to roughage ratio in diet of sheep is beneficial to achieve retention of more protein and energy with less methane emission. In this investigation the same technique (C:N balance) have been used to determine the energy balance in two groups, faunated and defaunated, of Muzaffarnagari sheep fed on diet with 30:70 roughage to concentrate ratio.

Materials and Methods

Ten healthy male Muzaffarnagari sheep of about 6-8 months of age were divided into two groups of five each. The animals were dewormed before the start of experiment. One group of sheep was defaunated chemically using a solution of sodium lauryl sulphate at the dose rate of 9-g/100 kg body weight orally by stomach tube for three consecutive days. Before the first dose, 24-hrs fasting was done but water was given *ad libitum*. After 8 hrs of first dose, only 33% of the normal requirement of energy was offered through concentrate. The treatment with sodium lauryl sulphate was repeated two times at intervals of 24 and 48 h from first dose. The presence of protozoa in the rumen was checked every week. The animals were housed in two cemented sheds, which were well ventilated with individual feeding and watering arrangement. Defaunated sheep were kept in a separate shed.

Animals were fed on diet with 30:70 roughage: concentrate ratio as per NRC (1985) at maintenance levels. Oat hay was the roughage used. Concentrate mixture (Crude protein 11.7%, gross energy 4.37 Mcal/kg, DM) contained maize 93, deoiled groundnut cake 3.5, Wheat bran 3.5 parts, respectively. To every 100 kg concentrate mixture 2 kg mineral mixture and 1 kg common salt were added. Animals were shifted to metabolic crate and were adapted for 3 days. Metabolic trial of seven days was conducted after preliminary feeding of a month. During the last two days of the trial, a complete energy balance study was done in an open circuit respiration chamber described by Khan and Joshi (1983). Chamber was maintained at 20-25°C with a relative humidity of 65%. Carbon-dioxide measurement was conducted using modified Sonden apparatus with 100 ml burette. Measurement of methane was done by an infrared gas analyser (Analytical Development Co.Ltd., Hoddesdon, England, Model 300). The estimation of gross energy (GE) of samples was done by Gallenkamp adiabatic bomb calorimeter (CBA 301 series) as per procedure of Gallenkamp manual. Estimation of carbon content of feed, faeces and urine was done by adopting sodalime (self-indicating granule) absorption method given by van Es as described earlier (Chandramoni *et al.*, 1999).

Carbon content of carbon dioxide and methane produced was calculated from the values obtained in respiration calorimetry using factors recommended by Brouwer (1965).

Nitrogen in the samples was analysed by Kjeldahl's method, fat by extracting with petroleum ether (Lab con co.) and crude fibre by successively boiling with dilute acid and alkali.

Estimation of energy balance (EB) was made by the formula of Brouwer (1965) i.e. EB (kcal/d) = 12.387 C - 4.632 N, where C is carbon balance (g) and N is nitrogen balance (g). Body energy content was determined by assuming value of 5.32 kcal /g for protein 9.37 kcal/g for fat. Statistical analysis was done as per Snedecor and Cochran (1967).

Results and Discussion

Chemical composition of oat hay and concentrate mixture used is given elsewhere (Chandramoni *et al.*, 1999) and daily

Table 1: Daily intake of feeds (DM basis) and nutritive value of rations in faunated and defaunated sheep

Particulars	Faunated	Defaunated	SEM
Live weight,W, (kg)	48.1	51.2	1.63
Metabolic bodyweight ($kgW^{0.75}$)	18.2	19.1	0.66
Feed and nutrient intake (g/d)			
Concentrate mixture	840.8	892.4	28.64
Oat hay	263.6	294.1	26.57
Total intake	1104.4	1186.5	29.95
Intake ($g/kg\ W^{0.75}$)	60.68	62.1	1.31
Intake (kg/100kg W)	2.3	2.3	0.16
DP intake (g/d)	78.9	86.7	4.17
DP intake ($g/kgw^{0.75}$)	4.3	4.5	0.16
TDN intake (g/d)	743.0	791.8	23.35
TDN intake ($g\ /kgw^{0.75}$)	40.8	41.4	0.71
Nutritive value			
DP (%)	7.23	7.28	0.25
TDN(%)	68.2	66.7	0.86
DE (Mcal/kg DM)	3.00	2.68	0.08
ME (Mcal/kg DM)	2.71	2.45	0.08

Table 2: Carbon (C) and Nitrogen (N) balances (g/d) in faunated and defaunated sheep

Particulars	Faunated	Defaunated	SEM
C Intake	417.0	447.9	12.9
Faecal-C*	127.7	170.1	7.46
Urinary-C	10.2	9.6	0.35
Carbon dioxide-C*	223.3	216.8	6.5
Methane-C*	10.0	7.03	0.54
Total C balance	45.8	44.4	12.42
Losses of C as % of total intake			
Fecal-C	30.6	38.0	1.61
Urinary-C**	2.4	2.1	0.04
Methane-C**	2.4	1.6	0.13
Carbon dioxide-C*	53.5	48.6	1.75
N-Intake	19.6	20.9	0.55
Fecal-N	6.8	7.1	0.40
Urinary-N	10.5	11.0	0.66
Total N balance	2.3	2.8	0.30
N-Balance ($mg/kg^{0.75}$)	125.3	141.6	14.89
N retained as % of intake	11.7	12.8	1.10
N retained as % of absorbed-N	18.5	19.8	2.3

*P < 0.05, ** P< 0.01

Table 3: Daily energy balance and heat production in faunated and defaunated sheep by carbon nitrogen balance.

Particulars	Faunated	Defaunated	SEM
Protein deposited (g)	14.4	17.5	1.87
Fat deposited (g)	51.2	47.37	15.60
Energy stored as protein (kcal/d)	76.6	93.1	9.08
Energy stored as fat (kcal/d)	480.1	443.9	146.17
Total energy retained (kcal/d)	556.7	537.0	152.81
Heat Production			
kcal/d	2375.1	2381.8	61.35
kcal / kg $W^{0.75}$ /d	130.5	124.7	4.25
Efficiency of ME utilisation for maintenance	51.3	54.7	4.20

intake of these by faunated and defaunated sheep and nutritive value of composite rations is given in Table 1. Digestibility, intake of digestible nutrients and energy (DE and ME) were almost similar in both faunated and defaunated groups resulting in similar nutritive value of composite rations. Similar observation was reported by Rowe *et al.* (1985) in defaunated wethers. This may be due to maintenance level of feeding and quality of roughage (oat hay) used in the experiment.

Carbon and nitrogen balance data obtained in faunated and defaunated groups is presented in Table 2. Fecal carbon was significantly higher ($P<0.05$) and methane carbon was significantly lower ($P<0.05$) in defaunated sheep. However, intake of carbon and carbon loss as CO_2 did not differ between the groups resulting in similar carbon balance. On the other hand, nitrogen balance data show that nitrogen intake and outgo did not differ significantly ($p<0.05$) between groups. No significant difference in the loss of carbon in urine may be due to similar intake of digestible energy. It has been found (Kishan *et al.*, 1986) that level of energy influence the excretion of carbon and nitrogen in the urine and that urinary carbon is positively ($P<0.01$) co-related with DE intake. On the other hand, Khan *et al.* (1986) and Ghosh (1990) reported that intake and excretion of carbon in faeces was dependent on energy intake but urinary, CH_4 and CO_2-C outgo were not affected by energy intake in male buffaloes and crossbred cattle, respectively. In this study, total methane - C as well as methane - C loss as percent of total C intake was significantly low due to lower methane production in defaunated sheep. This is because defaunation reduces methanogenesis to the tune of 30-45% (Jouany *et al.*, 1988). Protozoal activity results in hydrogen gas production and it is used for methane production by methanogenic bacteria (Hungate, 1967) which has got ectosymbiotic relationship with rumen ciliate protozoa (Stumm *et al.*, 1982; Krumholtz *et al.*, 1983). In defaunated sheep, methanogenic bacteria loose their symbiotic partners resulting in reduced methane production.

The loss of carbon as carbon dioxide in defaunated sheep was less (6%) as compared to faunated sheep, which may be due to combined effect of reduced tissue metabolism of host (Kreuzer *et al.*, 1986) and higher carbon dioxide production in the rumen of defaunated sheep (Rowe *et al.*, 1985).

The data on energy retention and heat production as determined by carbon and nitrogen balance method is given in Table 3. It is evident that there was no difference in heat production and energy retention between two groups of sheep. Using FHP value of 53.5 kcal/kg $W^{0.75}$ of sheep (Chandramoni *et al.*, 2000) the efficiency of utilisation of ME for maintenance was estimated as ME intake- (HP-FHP)/ME intake which was found to be 0.513 and 0.547 in faunated and defaunated sheep, respectively. In defaunated sheep, ME for production (ME_p) separated from ME for maintenance (ME_m) was regressed on energy retained as protein (ER_p) and fat (ER_f), which yielded the following equation.

$$Me_p = 2.51\ ER_p + 1.31\ ER_f \quad p<0.01 \quad r^2 = 0.67$$

This gave the efficiency of ME utilisation for protein and fat synthesis, which was 39.8 and 76.3 per cent, respectively. Thus ME required per g of protein and fat synthesis was 13.9 and 12.3 kcal/g. Energetic cost of protein and fat synthesis (per g) in faunated sheep based on 12 observations in the same breed of sheep (Chandramoni *et al.*, 1999) was 14.38 and 11.71 kcal ME, respectively which was similar to defaunated sheep in this study.

It is evident from the present study that high level of concentrate to roughage ratio and good quality roughage like oat hay in ration of defaunated sheep does not yield any additional benefit from energetic point of view compared to faunated sheep because there is no significant positive effect of defaunation on carbon and nitrogen balance i.e. energy balance and efficiency of energy utilisation.

References

Bird, S.H. and R.A. Leng, 1985. Productivity responses to eliminating protozoa from the rumen of sheep. Rev. Rural Sci., 6:109-117.

Brouwer, E., 1965. Report of subcommittee on constants and factors. Proc. 3rd EAAP Symp. on Energy metabolism. Troon, Publ. 11,Academic Press, London, pp: 441-443.

Chandramoni, S.B. Jadhao, C.M.Tiwari and M.Y.Khan, 1999. Carbon and Nitrogen balance studies in Muzaffarnagari sheep fed diets varying in roughage and concentrate ratio. Small Rumin. Res., 31:221-227

Chandramoni, C. M. Tiwari, S. B. Jadhao and M. Y. Khan, 2000. Fasting heat production of Muzaffarnagari sheep, Small Rumin. Res., 36: 43-47

Eadie, J.M. and J. C. Gill., 1971. The effect of the absence of rumen cilliate protozoa on growing lambs fed on a roughage-concentrate diet. Br. J. Nutr., 29 : 155-167.

Ghosh, A., 1990. Studies on energy requirement of adult non-lactating crossbred cows for maintenance. M.V.Sc. Thesis, IVRI, Izatnagar, India.

Hungate,R.E.,1967. Hydrogen as intermediate in the rumen fermentation. Arch. Microbiol., 59:158-167

Itabashi, H., T. Kobayashi, M. Matsumoto, 1984.The effects of rumen cilliate protozoa on energy metabolism and some constituents in rumen fluid and blood plasma of goats. Jpn. J. Zootech. Sci., 55: 248-256.

Jouany, J.P., D. J. Demeyer and J. P. Grain, 1988. Effect of defaunating rumen. Animal Feed Sci. Technol., 21: 229-265.

Khan, M.Y. and D. C. Joshi, 1983. A new simplified open-circuit respiration equipment for sheep-a note. Indian J. Anim.Prod., 15 : 34-36.

Khan, M.Y., J. Kishan, Murari Lal and D. C. Joshi, 1986. Annual Report, IVRI, Izatnagar.

Kishan, J., M. Y. Khan and Murari Lal, 1986. Influence of dietary energy levels on urinary nitrogen and carbon excretions in buffaloes. Indian J. Anim. Nutr., 3 : 197-200.

Kreuzer, M. , M. Kirchgessner, H. L. Muller, 1986. Effect of defaunation on the loss of energy in wethers fed different quantities of cellulose and normal or steam flaked maize starch. Anim. Feed Sci. Technol., 16 : 233-241.

Krumholtz, L. R., O. W. Farsberg, D. M. Veira, 1983. Association of methanogenic bacteria with rumen protozoa. Can. J. Microbiol., 29: 676-680.

NRC., 1985. Nutrients Requirement of sheep, National Research Council, National Aademy of Sciences, Washington D.C.

Rowe, J.B. , A. Davis and A. W. J. Broome, 1985. Quantitative effects of defaunation on rumen fermentation and digestion in sheep. Br. J.Nutr., 54 : 105-119.

Snedecor, G.W. and W. G. Conchran, 1967. Statistical Methods, Iowa State Univ. Press, USA.

Stumm, C. K., H. J. Gijzen, G. D. Vogels, 1982. Association of methanogenic bacteria with ovine rumen ciliates. Br.J. Nutr., 47 : 95-99.

Whitelaw, F.G., J. M. Eadie, L. A. Bruce and W. J. Shand, 1984. Methane formation in faunated and clliate-free cattle and its relationship with rumen VFA proportions. Br. J. Nutr., 52: 261-275.

Pakistan Journal of Nutrition (1) : 34-38, 2002

Urbanisation and Food Selection for South Asian Children

Rubina Hakeem[1], Jane Thomas[2] and Salma H. Badruddin[3],
[1]Department of Food and Nutrition, RLAK Government College of Home Economics, Stadium Road, Karachi-74800, Pakistan, [2]Department of Nutrition and Dietetics, Kings College, London UK, [3]Department of Medicine/Community Health Sciences, The Aga Khan University, Stadium Road, Karachi-74800, Pakistan
Email: hakeem@cyberaccess.com.pk

Abstract: Changes in diet and activity are supposed to be responsible for the increased prevalence of urbanization related diseases. By comparing determinants of food choice among rural and urban south Asians we can assess the impact of urbanization on food selection process and this information can help in planning nutrition education strategies. This study was conducted to compare the determinants of food choice for South Asian children at different levels of urbanization. Among South Asian groups relative impact of males on family food choice and that of parents on children's food choice was more pronounced than it was among British Caucasians (BrC) but the influence decreased with urbanization rank (UR). With urbanization children's likeness for fatty snacks increased significantly. Gender bias in food choice was higher among all south Asian groups as compared to BrC but decreased with urbanization rank. Determinants of food selection for children differ with urbanization status. Influences of children's own preferences increase with UR. Influence of male family members on family food choice and gender bias in food preference decreases with UR.

Key Words: Food selection, food preference, food choice of male and female

Introduction

Demographic transitions accompany variations in dietary habits and health (Harris, 1981; Truswell, 1977; Eaton and Konner, 1988; Vargas, 1990; Popkin, 1994; Omran, 1971; Milio, 1990; Popkin, 1989; Popkin , 1992). Urbanization in less developed countries is exposing people to bilateral nutritional problems (Ge, 1995). Adoption of healthy food and activity habits is the only safeguard against these problems. Behavior modification is less difficult at young ages and an understanding of the determinants of food choice can assist in bringing out this change.

Determinants of food choice for children, however, may vary according to demographic characteristics. In view of increase in the prevalence of diet related disorders with increasing urbanization, it is worthwhile to understand the determinants of food choice at various stages of urbanization. This knowledge could help in planning fruitful strategies for nutrition education and nutrition interventions in various settings.

To explore the possible impact of urbanization on the following aspects of food choice by children and by their parents: what are the foods children like or dislike; what factors effect children's food choice when they choose food themselves; what factors effect food choice in children's families; and to what extent in what ways gender bias may effect food choice by children and by their families.

Materials and Methods

In order to study the association between urbanization and factors effecting food selection following aspects of food habits of six groups of 10-12-year-old school children, representing various urbanization categories was compared.

1.Children's food likes and dislikes
2.Factors effecting children's food choice.
3.Major influence on Food choice at homes
4.Food considered good or bad by parents and children.

Subjects : A total number of 623 10-12-year-old school children belonging to six different groups were asked to identify the perceived importance of various factors influencing their food choice. Three groups represented different levels of affluence and urbanization within the same cultural milieu. These groups included rural Pakistani (RrP, n 100), middle-income urban Pakistani (MIP, n 148) and high income urban Pakistani (HUP, n 159) residing in the province of Punjab in Pakistan. Three further groups of children were drawn from the same geographical area (Slough) in the UK, but with different cultural backgrounds: British Pakistani (BrP, n 110), British Indian-Sikh (BrI, n 72), and British Caucasian (BrC, n 34). Schools were selected on the basis of the catchment population. All 10-12-year-old children from the selected school, who were willing to participate were included in the study. The students completed a questionnaire at school and the questionnaire for mothers was sent to their homes.

Data collection

Likes and dislikes of children : Children were asked to mention three liked, and three most disliked foods. The foods were categorized into five groups. For each food group each child's likeness score was calculated. If the child mentioned one food from any group as liked favorite he got an score of one for preference. Similarly If the child mentioned one food from any group as disliked food he got an score of minus one for aversion. The sum of two scores gave the likeness score. Thus higher showed likeness and vice versa.

Factors effecting children's food choice: Children were asked to indicate on a five-point ranking scale the degree to which certain factors influenced their food choice (higher score meant more importance was given to that factor).

Major influence on Food choice at homes: Schoolchildren and their mothers belonging to six different groups were asked to identify whose likes and dislikes were given the greatest importance when food choices were being made at home.

Food considered good or bad by parents and children: In order to explore these demographic differences in ideas about the

Table 1: Partial Correlation Coefficient between UR and food preference score controlling for sex

Food group	r	n	P
Cereals, bread	-0.0922	(469)	P = 0.045
Fats, cream, fried/fatty snacks	0.4684	(469)	P = 0.000
Fruits & Juices	-0.2445	(469)	P = 0.000
Meat, egg	-0.1988	(469)	P = 0.000
Milk, yogurt, cheese	-0.2069	(469)	P = 0.000
Sweets, sugary drinks	-0.0494	(469)	P = 0.285
Vegetables	0.1854	(469)	P = 0.000

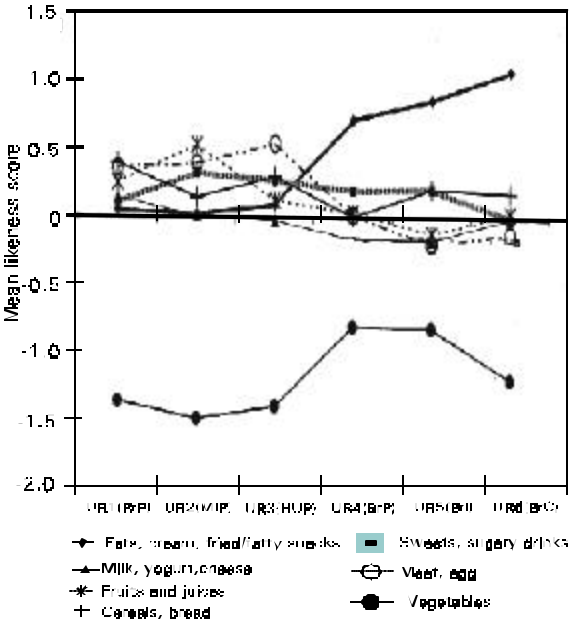


Fig. 1: Mean food preference score according to Urbanisation rank (UR)

appropriateness of foods according to sex, schoolchildren and their mothers were asked to identify any foods which they considered 'good for boys' and 'good for girls' and which foods they considered 'bad' for boys or girls.

Results

Children's food likes and dislikes: Likeness for fatty forms of foods increased steadily and rapidly with urbanization (Fig.1). Preference for meat, milk and fruit was higher at lower URs. Preference for vegetable was lowest in all groups but the likeness was relatively high at higher URs. Preference for sweet food increased sharply from UR1 to 2 and then decreased steadily throughout. Preference for fatty snacks increased significantly and steadily with UR Association between UR and trends in food preferences was statistically significant in most cases (Table 1) .

Factors effecting children's food choice: Comparison of the means of the ranks for each factor according to group indicated that urbanization, (RrP V. MIP), affluence (MIP V. HUP) migration, (HUP V. BrP) culture (BrP V. BrC) and religion (BrP V. BrC and BrI) played an important role in shaping the children's food choices (Fig. 2). Importance of parents and friends decreases with UR and that of taste increases. t appears that with urbanization and probably because of accompanied affluence children become more independent in food choice.

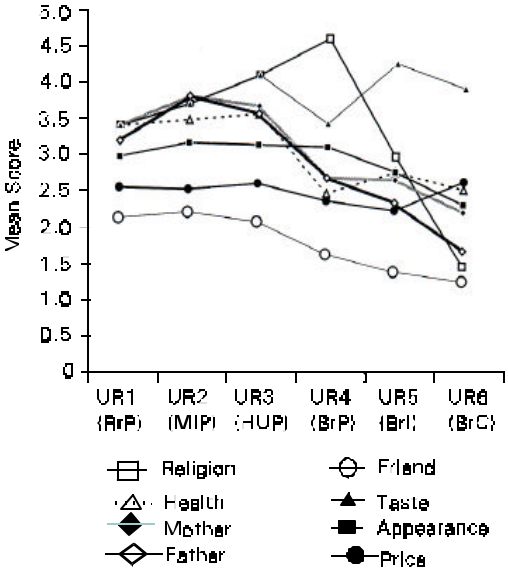


Fig. 2: Importance of various factors on food choice by the six groups of children having different Urbanization status

Major influence on Food choice at homes: Fig. 3a and 3b show that in most of the South Asian groups of parents and children, the percentage of those who considered a male family member to have a major influence on food choice at home was greater than that mentioned by Caucasian parents and children. These results indicate a tendency towards male dominance and lesser possibility of incorporating everyone's food preferences into making food choices in most of the South Asian groups.

Table 2 shows proportion of parents and children who considered any parent or any child to have major influence on family food choice. In all groups, both according to children's perception and according to the parents' perception children had less influence on family food choice as compared to parents. The most often mentioned family members in any group were father, mother or son. In no group daughter was the most often mentioned family member to have major influence on family food choice.

This sex bias may induce lower food intake and could put some vulnerable groups like women and children at a higher risk of malnutrition.

Food considered good or bad by parents and children: In terms of foods considered good for boys or girls by parents and children, preference for milk and meat decreased and preference for vegetable and fruits increased with Urbanization ranks (Table 3 and 4). However, meat and milk were mentioned to be preferred foods more often for boys than for girls by the three group living in Pakistan.

While in the three groups in UK fatty and sugary foods were considered to be bad both for boys and girls the three groups living in Pakistan rarely mentioned such foods to be bad either for boys or girls. No specific pattern of group-wise or gender wise difference was evident within the groups recruited from Pakistan or UK.

Table 2: Children's and their parents' perception regarding the family members whose likes and dislikes are considered most important while selecting food choice at home

	UR1 (RrP)		UR2 (MIP)		UR3 (HUP)		UR4 (BrP)		UR5 (BrI)		UR6 (BrC)	
	%	N	%	N	%	N	%	N	%	N	%	N
Children's view												
Any Parent	68	(54)	55	(32)	61	(60)	76	(74)	54	(34)	64	(16)
Any Child	32	(25)	45	(26)	39	(38)	24	(24)	46	(29)	36	(9)
Parent's view												
Any Parent	85	(51)	90	(74)	97	(34)	94	(60)	85	(33)	94	(15)
Any Child	15	(9)	10	(8)	3	(1)	6%	(4)	15	(6)	6	(1)

Table 3: Foods considered to be good or bad for boys and girls by parents

	UR1 (RrP)	UR2 (MIP)	UR3 (HUP)	UR4 (BrP)	UR5 (BrI)	UR6 (BrC)
Food Good for Boys						
Total responses	% 65	% 60	% 56	% 62	% 40	% 20
Fat	1	0	0	2	0	5
Sugar	0	1	2	0	0	16
Milk	19	30	18	22	26	16
Meat	38	36	46	28	14	11
Fruit	9	10	11	18	23	21
Vegetable*	14	9	13	23	26	26
Cereal	13	7	7	7	11	5
Food Bad for Boys						
Total responses	% 18	% 15	% 16	% 47	% 36	% 21
Fat	5	0	6	49	45	50
Sugar	20	6	11	47	42	29
Milk	5	0	0	0	0	0
Meat	5	0	28	2	11	17
Fruit	0	0	0	0	0	0
Vegetable*	40	13	11	0	0	0
Cereal	10	6	0	2	3	4
Food Good for Girls						
Total responses	% 36	% 54	% 33	% 45	% 33	% 26
Fat	3	2	0	0	0	4
Sugar	3	0	0	0	0	4
Milk	18	22	21	16	30	25
Meat	23	28	44	13	7	8
Fruit	8	9	15	34	27	21
Vegetable*	28	16	9	37	27	33
Cereal	13	9	6	0	10	4
Food Bad for Girls						
Total responses	% 18	% 19	% 9	% 28	% 27	% 15
Fat	5	0	8	48	39	39
Sugar	15	13	0	26	42	39
Milk	5	0	8	0	0	0
Meat	0	30	31	13	16	22
Fruit	0	0	8	3	3	0
Vegetable*	55	9	15	3	0	0
Cereal	5	13	0	6	0	0

* specific vegetables were mentioned like okra, brinjals etc

Discussion

Food likes and dislikes of children from different groups were found to vary. In terms of actual intake, Both fat and sugar intake is found to increase with urbanization (Hakeem *et al.*, 1999). However, according to children's likes and dislikes, with increasing urbanization, behavior modification to lower intake of fatty snacks may be more difficult than decreasing the intake of desserts and sweets. Restrictions on eating favorite foods are found to have negative impacts on food behavior (Fisher and Birch, 1999). Thus a more fruitful strategy could be to modify recipes to decrease fat contents. Children's food choices are usually found to be influenced by parental food preferences (Klesges *et al*., 1991; Feunekes *et al.*, 1998). During adolescence children are assumed to be influenced more by outside world and may adopt new food habits. However we see that this process may not occur at similar ages for various groups of children. The less urbanized children either because of cultural factors or limitation of resources, are under the influence of their parents to a greater degree than the more urbanized groups. Thus, the impact of nutrition education imparted through schools only may not have similar impact in various groups.

Among Pakistani children, Impact of religion on food choices was not found to decrease with urbanization, and those in multicultural environment were most conscious of this factor. This factor needs to be considered in nutrition education.

As children at this age eat most of their meals at home impact of family food on their food choices and health is likely to be

Table 4: Foods considered to be good or bad for boys and girls by children

	UR1 (RrP)		UR2 (MIP)		UR3 (HUP)		UR4 (BrP)		UR5 (BrI)		UR6 (BrC)	
Food Good for Boys												
Total responses	%	46	%	22	%	33	%	22	%	15	%	9
Fat		17		0		3		14		0		0
Sugar		0		5		3		0		0		11
Milk		9		18		9		14		7		0
Meat		26		23		24		9		7		0
Fruit		9		27		0		18		27		44
Vegetable*		22		27		39		41		60		44
Cereal		17		0		15		5		0		0
Food Good Girls												
Total responses	%	21	%	13	%	13	%	20	%	16	%	8
Fat		0		8		8		0		0		0
Sugar		0		0		8		0		0		0
Milk		5		0		15		10		6		13
Meat		19		23		15		5		6		0
Fruit		67		23		8		35		31		38
Vegetable*		5		23		31		40		56		50
Cereal		5		23		15		10		0		0
Food Bad for Boys												
Total responses	%	17		2	%	31	%	27	%	22	%	9
Fat		0		0		3		33		41		56
Sugar		0		0		3		59		55		44
Milk		0		0		6		0		0		0
Meat		35		0		19		4		5		0
Fruit		0		0		3		0		0		0
Vegetable*		53		50		29		4		0		0
Cereal		6		0		10		0		0		0
Food Bad for Girls												
Total responses	%	16	%	4	%	20	%	28	%	19	%	9
Fat		0		0		0		43		37		44
Sugar		6		0		5		50		42		56
Milk		0		0		5		0		0		0
Meat		31		0		35		4		5		0
Fruit		0		0		0		4		5		0
Vegetable*		63		25		20		0		11		0
Cereal		0		0		10		0		0		0

* specific vegetables were mentioned like okra, brinjals etc

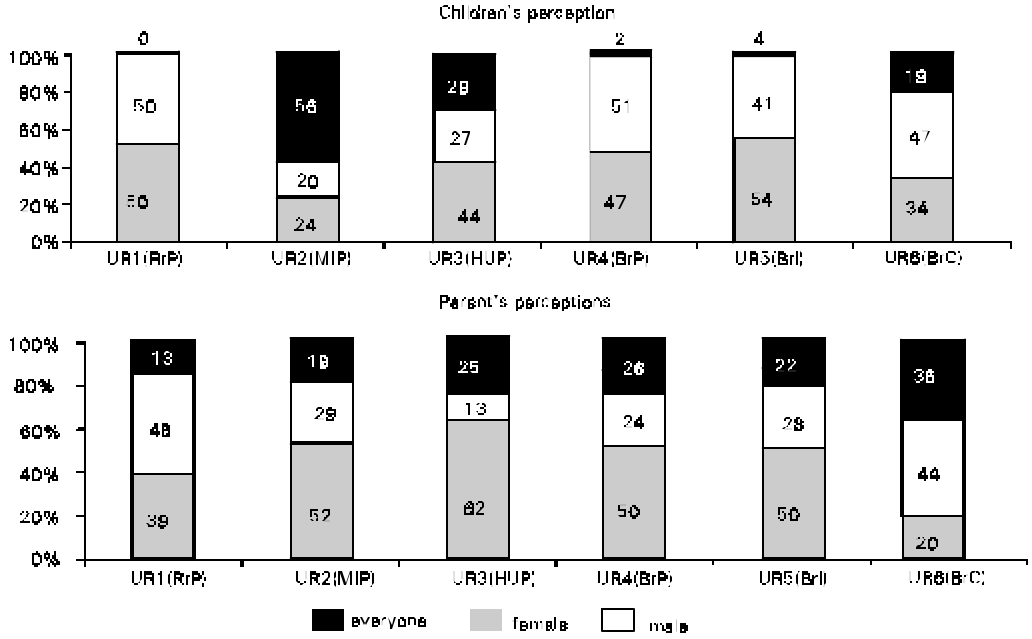


Fig. 3: Perception of major influence on food choice at home by children (c) and parents (p) according to Urbanization status

great. Relative influence of children's preferences on family food choice seems to be low in all sub groups. In family food choice also, in general children's likes and dislikes are not given first preference in the families studied. This finding again indicates that in the age group studied, involvement of families is required for modifying food intake. Indication of gender bias necessitates involvement of males even when women and children nutrition is the concern. It appears that educating only the females may have limited impact on family food choices. Gender bias observed in relative importance of males and females in family food choice and differential preferences for boys and girls needs to be studied further. Nutrition education of males is equally important and crucial for behavior modification within South Asian groups. Further it needs to be explored that differences in food preferences for boys and girls are due to knowledge or due to relative position of males and females in the family. Gender bias favoring males in the distribution of food within households is reported from other countries also(Babu *et al*., 1993; Chakrabarty, 1996; Frongillo and Begin, 1993; Miller, 1997) and this trend should be checked through creating awareness regarding women nutrition.

Socio-demographic factors could effect the process of food selection for and by children in various ways. While nutrition education can influence food preferences of parents and children, understanding of cultural factors can help in designing appropriate nutrition messages and targeting all family members.

Acknowledgement

Dr. R. Hakeem was recipient of Commonwealth scholarship while doing Ph.D. and ISFE Switzerland provided additional financial support. The authors also wish to express thanks to the funding agencies; and the students and staff of the participating schools for their cooperation in this study.

References

Babu S.C., S. Thirumaran and T.C.Mohanam, 1993 . Agricultural productivity, seasonality and gender bias in rural nutrition: empirical evidence from south India. Soc. Sci. Med., 37:1313-1319.

Chakrabarty M., 1996 Gender difference in cereal intake: possible impacts of social group affiliation and season. Anthropol.Anz. 54:355-360.

Eaton, S.B.S. and M. Konner, 1988. The Palaeolithic prescription a program of diet and exercise and a design for living., Harper and Row., New York.

Feunekes G.I., C. de Graaf, S. Meyboom and W.A. van Staveren,1998. Food choice and fat intake of adolescents and adults: associations of intakes within social networks. Prev. Med., 27:645-656.

Frongillo E.A.J. and F. Begin, 1993. Gender bias in food intake favors male preschool Guatemalan children. J Nutr. 123:189-196.

Ge, K., 1995. Dietary Pattern and physical development in China based on the 1992 National Nutrition Survey. Asia Pacific. Asia Pacific J. Clin. Nutr., 4:19-23.

Hakeem R., J. Thomas and S.H. Badruddin, 1999. Rural-urban differences in food and nutrient intake of Pakistani children JPMA, 49:12

Harris, D.R., 1981. The prehistory of human subsistence a speculative outline. In: Food, Nutrition, and evolution food as an environmental factor in the genesis of human variability, Masson, New York. pp: 15-37.

J.O. Fisher and L.L.Birch, 1999 Restricting access to palatable foods affects children's behavioral response, food selection, and intake. Am. J. Clin. Nutr., 69:1264-1272.

Klesges R.C., R.J. Stein, L.H. Eck, T.R. Isbell and L.M.Klesges, 1991. Parental influence on food selection in young children and its relationships to childhood obesity [published erratum appears in Am. J. Clin. Nutr., 1991. Am. J. Clin. Nutr., 53:859-864.

Milio, N. Nutrition policy for food-rich countries a strategic analysis, The Johns Hopkins University Press, Baltimore, M.D.

Miller B.D., 1997. Social class, gender and intrahousehold food allocations to children in South Asia. Soc. Sci. Med. 44:1685-1695.

Popkin, B.M.H., 1989, Food consumption trends of U.S. women. Patterns and determinants between 1977 and 1985. Am. J. Clin. Nutr., 49:1307-1319.

Popkin, B.M.,1994. The Nutrition Transition in low Income Countries An emerging crisis. Nutrition Reviews,52:285-298.

Omran, A.R., 1971. The epidemiologic transition a theory of the epidemiology of population change. Milbank Memorial Fund Quarterly, 49:509-538.

Popkin, B.M. and P.S.P. Haines, 1992. Dietary changes in older Americans, 1977-87. Am. J. Clin. Nutr., 55: 823-830.

Truswell, A. S., 1977. Diet and Nutrition of hunter-gatherers. Amsterdam Elsevier/.Excerpta Medica. Ciba Foundation Symposium 49. Health and disease in tribal societies pp: 1213-1226

Vargas, L.A, 1990. Old and new transitions and Nutrition in Mexico. In: Disease in populations in transition anthropological and epidemiological perspectives., Editors: A.C. Swedlund and G.J. Armelagos. Bergin and Carvey, New York.

Pakistan Journal of Nutrition 1 (1): 39-40, 2002

Potential Intake of Lithium by the Inhabitants of Different Regions in Jordan

M.S.Y. Haddadin, S. Khattari Daniela Caretto, and *R.K.Robinson
Faculty of Graduate Studies, University of Jordan, Amman, Jordan
*School of Food Biosciences, University of Reading, Reading, Berkshire RG6 6AP, England

Abstract: Although it is alleged that lithium is not toxic to humans below an intake of 500 mg day^{-1}, test animals have been affected at levels as low as 50 mg kg^{-1} of feed. This study sought to establish whether fruits grown in the Jordan Valley could accumulate sufficient lithium for consumers to reach a target of 50 mg day^{-1}. The mean soil levels of extractable lithium were 13.1, 20.3 and 25.4 mg kg^{-1} in three different growing regions of the Valley, while the highest levels in citrus fruits were 51.3 mg kg^{-1} in clementines (*Citrus mitis*) and 49.4 mg kg^{-1} in the lemon (*Citrus limon*). As the latter values were on a dry weight basis, the risk of lithium toxicity would appear to be minimal but, equally important, deficiency syndromes should not be manifest either.

Key words: lithium, Jordan Valley, citrus fruits

Introduction

Lithium is widely distributed in soils around the world, and a general estimate puts the level in the earth's crust at 50-65 mg kg^{-1}. It is present in soils mainly as a component of silicates and, for this reason, sandstone soils are especially rich in lithium. By contrast, soils with excessive levels of organic matter tend to be low in lithium (3-4 mg kg^{-1}), and these differing concentrations between the soils are reflected in the levels in adjacent reservoirs. Consequently, local drinking water and, perhaps, crops subject to irrigation will contain concentrations of lithium typical of a given region.

Equally important is the fact that plants differ in their ability to accumulate lithium. Cereals and cereal-based products contain very little lithium (1 mg kg^{-1}), but most vegetables (dry weight) contain > 1 mg kg^{-1} (Anke, 1993; Bibak *et al.*, 1999); fruits like apples or lemons are cited as having around 1.4 mg kg^{-1} of lithium. The vegetative parts of plants often retain higher concentrations, and hence the level in milk tends to vary depending on the type of fodder or silage consumed. The low values in these common foods are in accord with published data on lithium intakes in Europe, with levels of 0.1 - 0.6 mg day^{-1} being recorded at different sites in Germany; as lithium is readily soluble, regional differences in the concentrations of lithium in drinking water may explain the observed variations (Anke, 1993).

However, other studies have suggested that normal intakes of lithium may reach 3.4 mg day^{-1}, but it is probable that none of these estimates take account of local factors, such as the availability of a balanced diet or, conversely, dependence on one or two locally-grown food items. Whether higher than average intakes would have any nutritional significance is not clear, for it is suggested that an intake of 500 mg day^{-1} would be needed to raise the level of blood serum lithium in humans to close to the toxic level of 1.6 meq l^{-1} (Anke, 1993). Thus, although lithium appears to be essential for the full activity of enzymes associated with both glycolysis and nitrogen metabolism, levels above the toxic threshold have been linked with a range of disorders ranging from thyroid changes to skeletal damage. The risk of this type of damage is a cause for concern mainly with patients being treated for mental disorders with daily doses of lithium in the range of 160 - 300 mg day^{-1}, but some degree of toxicity cannot be ruled out at much lower levels of intake.

Thus, studies with a range of animals have shown adverse effects at quite modest dose rates. Chickens, for example, showed reduced feed consumption, live weight gain and egg production when lithium was added to feed at the rate of 50 mg kg^{-1}, and rats fed a lithium-rich diet gave birth to lighter off-spring which, in turn, developed more slowly.

Obviously it is unwise to assume that data obtained from animal trials applies to humans but, assuming that the dry matter intake for an adult human is 500 g, then there could be scope for some populations to achieve intakes of lithium day^{-1} approaching the critical values for some animals. In practice, the risks of encountering levels of 50 mg kg^{-1} of lithium in a normal diet are extremely low but, given that litle information was available about the levels of soil lithium in the Jordan Valley which supplies the citizens of Jordan with most of their fruits and vegetables, a study was undertaken to:

assess the lithium levels in the surface soils of the Jordan Valley;
determine the lithium concentrations in the edible portions of some crops grown in the Valley; and
see if a correlation could be established between the levels of soil lithium and the concentrations in fruits reaching the consumer.

Materials and Methods

Three different regions of the Jordan Valley were chosen on the basis of their location, land usage/cultivation practices and sources of water for irrigation, and these were designated as: Area (1) from South Shuna to Amman; Area (2) from Ardha to Kraima; and Area (3) from Kraima to Addassyah. In each area, farms were selected that grew citrus crops or miscellaneous vegetables, and composite soil samples (covering a depth from 0 - 30 cm) were taken from selected locations on each farm. Each sample of soil was then air-dried, passed through a sieve (2 mm openings) and oven-dried to a constant weight over 3 days at < 50°C.

The total organic matter was determined by the procedure outlined by Allison (1965) and pH in a slurry of soil in water (1 : 1, w/v). Exchangeable lithium was extracted from the soil as described by Knudsen *et al.* (1982) using 0.1 N ammonium acetate at pH 7.0. A sample of air-dried soil (10 g) was placed in a plastic bottle and 15 ml of ammonium acetate were added. The bottle was shaken for 10 min on a slow-speed 'wrist-action' shaker and then centrifuged at 12,000 rpm for 20 min to obtain a clear supernatant. This extraction procedure was repeated twice more on the same soil sample, and the supernatants were then bulked and made up to 100 ml with distilled water. After filtration through a membrane filter, the lithium concentration was derived with a Flamephotometer (AOAC, 1990). A standard curve using solutions of 0.00 - 10.0 mg l^{-1} lithium was prepared in order to calculate the concentrations of lithium in the different extracts.

The species of plant sampled are given in Table 2. A composite leaf sample was obtained from each citrus orchard (ten/species) by taking one or two fully expanded leaves from a number of trees; at the same time, one fruit was selected at random from the same trees. The leaves were subsequently washed in distilled water, air-dried for 1 day, and then oven-dried to constant weight at < 50°C. The dry leaves were ground in a Moulinex Mill (Moulinex, Paris, France) so as to pass through a 40 mesh sieve. The fruits were cut into small pieces, placed in Petri dishes and oven-dried to constant weight at < 50°C.

For digestion of the plant samples, 0.25 g of finely ground

Table 1: Average values for total available lithium (mg kg^{-1}) at various locations within the Areas indicated.Location Number Total Extractable Lithium

	Area 1	Area 2	Area 3
	11.2	45.0	24.4
	12.7	11.2	22.4
	10.2	19.8	18.4
	12.2	19.8	20.8
	12.7	18.3	20.3
	11.2	18.3	20.3
	11.2	19.3	23.3
	12.2	18.8	19.8
	12.7	17.7	25.8
	11.7	17.7	37.9
	12.7	23.3	40.4
	11.7	22.4	30.4
	16.2	20.8	-
	14.2	11.7	-
	16.7	-	-
	20.4	-	-
Mean	13.1	20.3	25.4

The locations cited above refer to individual farms, and where more than one soil sample was taken on a farm, the 'location' figure is a mean for all the samples for that farm.

Table 2: The species of plant tested for lithium, together with details of the number of individual plants/species examined and the origin of the test materials; all figures for lithium as mg kg^{-1} of dried tissue

Plant	Origin	Range	Mean	No. of samples
Citrus mitis (clementine)	fruit	13.6-70.1	51.3	10
	leaves	58.8-205.6	143.5	10
Citrus paradisi (bomali)	fruit	13.7-25.0	20.7	10
	leaves	13.7-228.2	130.8	10
Citrus limon (lemon)	fruit	36.2-58.8	49.4	10
	leaves	70.1-171.7	134.5	10
Citrus reticulata (mandarin)	leaves	25.0-126.6	93.04	3
Corchorus olitorius (Mallow)	leaves	92.7-160.5	139.0	4
Capsicum annuum (red pepper)	leaves	194.3-318.5	256.4	2
Hibiscus esculenta (okra)	leaves		171.4	1
Sorghum bicolor	leaves		58.8	1
Trifolium alexandrinum (berseem)	leaves		183.0	1
Vitis vinifera (grape)	leaves		70.1	1

material was placed in a digestion tube along with 3 ml of concentrated sulphuric acid. The temperature of this mxixture was brought to 100°C, and the digestion process allowed to proceed for 1 hour. After cooling to 25°C, 3 ml of concentrated perchloric acid were added to the digest. The tubes were then returned to the digestion unit and, when the temperature of the liquid reached 250°C, digestion was allowed to proceed until a clear solution was obtained (~ 4 hours). The cooled digests were transferred to volumetric flasks (25 ml) and, after rinsing the tubes with deionised water, the volumes were adjusted to the mark. The total lithium concentrations in each extract were determined as before, and the same procedure was applied to sub-samples of finely-grounds fruits.

The results were analysed for L.S.D using the General Linear Models procedure of SAS as outlined by Steel and Torrie (1980).

Results and Discussion

Soils on the all the farms were calcareous with high pH. In the first Area (16 samples), the pH ranged from 7.5 - 8.1, in the second Area (14 samples) the lowest pH was 7.6 and the highest 8.0, while in the third Area (12 samples) the pH ranged from 7.5 to 7.8. The similarity in pH values suggests that there should be no effect of pH on the general status of lithium in the different soils. The total organic matter contents were low at 0.22 - 2.50 g kg^{-1} in Area (1), 0.65 - 2.50 g kg^{-1} in Area (2) and 0.95 - 2.70 g kg^{-1} in Area (3). Overall, the results for pH and organic matter tend to mirror the values reported by Khattari (1990), and hence it seems likely that the sites selected for the analysis of lithium were typical of the regions in question. However, the low level of organic matter could be relevant in this context, because it is 'peaty' soils that tend to have low levels of lithium.

The values in Table 1 show that the lithium concentrations in the Jordanian soils ranged from 10.2 to 44.9 mg kg^{-1}, and these high values reflect the nature of the parent rock which is rich in alkaline elements; these elements, in turn, accumulate in the arid soil due to the lack of leaching. The comparison between Areas is of interest as well, as there is a clear gradient from the south (Area 1) to the north (Area 3) of the Jordan Valley. Statistical analysis of the data showed a significant correlation between location and total amount of extractable lithium ($P < 0.05$).

The concentrations of lithium in the different species of plant are shown in Table 2, and some of the values were extremely high compared with the figures (~ 1.4 mg kg^{-1} of dry matter) reported by Anke *et al.* (1993) for fruits from the citrus family. Thus, assuming that the clementine has a moisture content of 87.5% (Holland *et al.*, 1991), then a rough conversion of the dry weight value to a wet weight figure gives a mean value for lithium of 0.63 mg *per* fruit. Given that a consumer could easily eat two or three clementines at one sitting, a lithium intake of ~ 2.0 mg could easily arise just from 'snacking'; a glass of lemon juice would further add to the intake.

The vegetative tissues retained, as expected, much higher levels of lithium, and it was unfortunate that no vegetative crops were available for analysis; none of the leaves studied are eaten. It may be, of course, that accumulation in the leaves is typical only of certain genera, for it has been suggested that the accumulation of lithium in leaves of citrus trees is a mechanism for removal from the body of the tree. However, in soils with little leaching, it may be that much of the lithium is simply taken-up again during the subsequent growing season, and this potential for recycling might explain why the levels of lithium in plants growing in Area 1 were similar to those in Area 3 (data not shown). Certainly some correlation might have been expected between the concentrations in the soils and the levels found in the plants, but no such connection could be established. Equally curious was the apparent absence of lithium damage to the citrus trees, because Aldrich *et al.* (1951) suggested that leaf damage could be initiated at concentrations as low as 1 - 4 mg kg^{-1} of fresh tissue; the reasons for this apparent contrast were not investigated.

Conclusion: The high silicate content of soils in the Jordan Valley means that lithium is readily available to plants and, as a consequence, quite high levels build-up in the vegetative tissues of a number of plants. Much lower levels accumulated in the edible portions of citrus crops and, although the levels were higher than those reported by other workers, there appeared to be little hazard for the average consumer of citrus fruits.

References

Aldrich, D.G., A.P. Vanselow and G.R. Bradford, 1951. Lithium toxicity in citrus. Soil Science, 71: 291-295.

Allison, L.E., 1965. Organic carbon. In Methods of Soil Analysis, ed. C.A.Black, pp:1035-1048. Wisconsin: American Society of Agronomy.

Anke, M., 1993. Lithium. In Encyclopaedia of Food Science, Food Technology and Nutrition. eds. R. Macrae, R.K.Robinson & M.Sadler, pp: 2779-2782. London: Academic Press.

AOAC., 1990. Official Methods of Analysis. Volume 1, 15th. Edition AOAC, Arlington, USA.

Bibak, A., S. Sturup, L. Knudsen and V. Gundersen, 1999. Concentrations of 63 Elements in Cabbage and Sprouts in Denmark. Commun. Soil Sci. Plant Anal., 30: 2409-2418.

Holland, B., A.A. Welch, I.D. Unwin, D.H. Buss, A.A. Paul and D.A.T. Southgate, 1991. McCance and Widdowson's The Composition of Foods. The Royal Society of Chemistry, London.

Khattari, S.K., 1990. Heavy metals in soils of the Jordan Valley.Dirasat, 17B: 35-48.

Knudsen, D., G.A. Peterson and P.F. Pratt, 1982 . Lithium, sodium and potassium. In: Methods of Soil Analysis. Ed. Page, A.L. Soil Science Society of America, Madison, Wisconsin.

Steel, G.D. and J.M. Torrie, 1980. Principles and Procedures of Statistics. Tokyo: McGraw-Hill Book Company.

Pakistan Journal of Nutrition 1 (1): 41-42, 2002

Determination of Pesticides Residues in Selected varieties of Mango

Saqib Hussain, Tariq Masud and Karam Ahad*
Department of Food Technology, University of Arid Agriculture, Rawalpindi,
* Toxicology Research Center, NARC, Pakistan

Abstract: The present study was undertaken to access the residue of commonly used pesticides viz Cypermethrin, Methamedophos, Monocrotophos, Cyfluthrin, Dialdrin and Methyl Parathian respectively in three varieties of Mango being collected from the grower fields in Multan division. The samples were treated with organic solvent Cyclohexane and ethylacetate (1:1), cleaned on Gel Permeation Chromatograph (GPC) and analyzed on auto system Gas Chromatograph (GC) with electron capture detector (ECD). All the samples were found to be contaminated with a degree of variation of pesticides residue studied. However, all the samples were within permissible limits being set by FAO/WHO with reference to public health.

Key Words: Pesticides, residue, mango varieties

Introduction

Mango (*Mangifera indica*), a member of family Anacardiaceae, is known as King of fruits. Its popularity is mainly due to its excellent flavour, delicious taste and high nutritive value. It is the choiscest fruit of the sub-continent. Its original home is belived to be south Asia where it has been cultivated for the last four thousand years (Salunkhe and Desai, 1984). It is now an important fruit of the tropic and mild sub-tropical parts of the world like Pakistan, India, South China and Malaya. It is a rich source of vitamin A and vitamin C. Ripe pulp of mangoes provides 74 Kcal of energy per 100 grams of edible portion. Its general composition is, moisture (79.2 - 82%) total soluble solids (12.9 - 20.8%) total sugars (10-17.3%), non-reducing sugars (7.27 - 12.35%), ash content (0.49 - 0.58%) and crude protien (0.38 - 0.62%) on fresh weight basis. Agriculture sector contribute 25% to GDP in which the share of fruits and vegetables is upto Rs. 5353.6 millions (Anonymous 1999). For more quantity and cosmetic quality of these commodities our farmers rely on pesticides. About 27% of the pesticides being consumed in the country are used on fruits and vegetables. Here mango is planted over an area of about 83300 hectares producing about 883700 tones of fruits (Ijaz and Inayat, 1997). Its cultivation is on the increase and vast tracts of land in Multan, Bahawalpur, Khairpur and Hyderabad divisions have been planted with Mangoes. Here about 250 varieties are known. Few important verieties of mango are Dusehri, Aphanso, Chaunsa, Anwar Retual and Sindhri. About 86 species of mango insect pests have been recorded in the country (Giani, 1968). Of these, fruit flies, mango mealy bug, scale insects, and mango hoppers causes more damage to this crop. To minimize the economic losses caused by these noxious insects, fungi, weeds etc., various Insecticides, Fungicides and Herbicides are used over this fruit on a massive scale. When applied improperly, residues of some of these pesticides can remain on foods and as such can pose a significant hazard to human health.

There is great demand for Pakistani mangoes in the country as well as in abroad. This delicious fruit is highly appreciated in many of the European, Middle Eastern and Far-Eastern Countries, thus opening tremendous opportunities for its exports and fetch premium prices in the world markets. Today's market demands for not only the quality of agricultural produce but also the safety and environment friendly production practices. In the year 2004 when the rules and regulations of WTO will be promulgated and implemented, the FAO/WHO Codex Alimentarus Commission standards for pesticide residues will act as reference points for Sanitary and Phytosanitary (SPS) measures. Thus certification with regard to residue limits, good agriculture practices (GAP) and environment friendly production processing will be a prerequisite for agricultural produces. To cope with the contemporary international market there is a dire need to carry out systematic research for determination of pesticide residues in different fruits.

Keeping in view of this subject, this study was designed to determine Pesticide residues in different varieties of mango fruits collected from Multan division with special reference to public health significance according to FAO/WHO Codex Alimentarius Commission.

Material and Methods

Collection of sample : The samples were collected from the fields' farmers of Multan division. Samples were taken from randomly selected tree. The samples were from different parts of canopy. After collecting samples, about 1 kg sample was separated and sealed in polythene bag.

Extraction and clean-up: Chopped 1 Kg of the mango and mixed thoroughly. A subsample of 25 gm was taken out and blended with 50 ml of acetone, 50 gm of anhydrous sodium sulphate and 50 ml of a mixture of cyclohexane and ethylacetate (1:1). Allowed the mixture to stand for some time till a clear supernatant is formed. Took 30 ml out of the supernatant into a round bottom flask. A few drops of 10% propandiol in ethylacetate and about 4-6 glass beads were added. The solvent was evaporated to dryness at 40 °C under vacuum and nitrogen stream in rotavapor. The contents were reconstituted in 6 ml of cychlohexane and ethylacetate (1:1) and then passed through highflow super cells. Applied 2 ml of this sample on Gel Permeation Chromatographic (GPC) column for further cleanup. After passing through GPC column, the samples were dried under vacuum and reconstituted in 1ml ethylacetate for analysis on Gas Chromatograph (GC).

Instrumental analysis: All the extraction steps mentioned above and the clean up on Gel permeation chromatography (GPC) were standardized and checked for optimum behaviour and quantitative recoveries. Perkin Elmer Autosystem Gas Chromatograph equipped with electron capture detector (ECD-Ni^{63}) and capillary column was used through out the study under specific operational conditions (temperature programming).

Table 1: Quantitative analysis of residues in different varieties of mango (mg/Kg)

Pesticides	Insect/Pest	MRLs	Sample I (Dusehri)	Sample II (Chaunsa)	Sample III(Sindhri)
Cypermethrin	Tip Borer	0.50	0.25	0.38	0.19
Methamedophos	Mango hopper	1.00	0.16	0.41	0.36
Monocrotophos	Fruit fly	1.00	0.46	0.25	0.69
Cyfluthrin	Mealy bug	0.50	0.24	0.18	0.39
Dialdrin	Termites	2.00	-	-	0.56
Methyl Parathian	Thrips	1.00	0.15	-	-

(-) Stands for not detected. Source: FAO/WHO Codex Alimentarius Commission (1993).

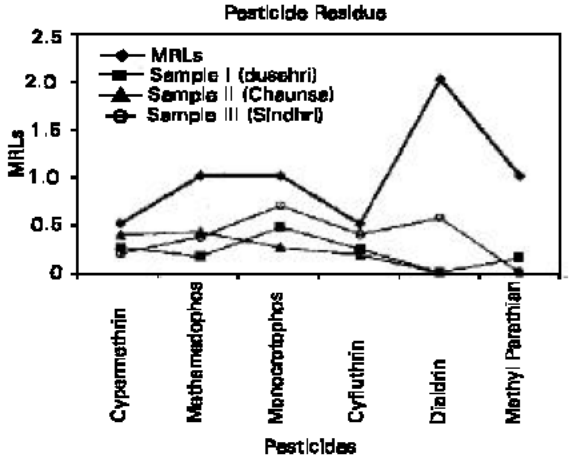


Fig. 1: Residue limits in Mango

Residues of insecticides in the samples were identified on the basis of their respective retention times, quantified on the basis of respective peak areas, reported on the basis of sample weight and are expressed as mg/Kg. Since percent recoveries were optimum, therefore no corrections were made while calculating the concentrations in the samples.

Results and Discussion

A number of chemicals are in use today in the production of agricultural commodities. They are essential to modern agriculture. Pesticides are chemicals developed and produced for use in the control of agricultural and Public health pests. Contamination of fruits and Vegetables may results from treatment as well as form conditions such as improper use of pesticide from preceding treatments in the soil and cross contamination. Prevention of health risks, including toxicological risks, due to food intake is central in food safety policy (Miller, 1987).

In the present investigation an attempt has been made to determine the pesticide residues in mango samples collected from the fields of farmers in Multan division. In this, study three varieties of mango samples, sample I (Dusehri), sample II (Chaunsa) and sample III (Sindhri) were analyzed for 6 pesticides namely Cypermethrin, Methamedophos, Monocrotophos, Cyfluthrin, Dialdrin and Methyl Parathian. It was observed that all the samples were contaminated with pesticides. MRLs (Maximum Residual Limits) of different pesticides are presented in Table I., which are taken form FAO/WHO Codex Alimentarius Commission (1993). Since the residual levels of all pesticides in the samples were within the permissible limits being set by FAO/WHO Codex Alimentarius Commission, (1993) hence are harmless and can safely be used for human consumption.

The result further revealed that concentration of Cypermethin and Methamedophos in Sample II (Chaunsa) was comparatively higher as compared to other two samples. Whereas concentration of Monocrotophos and Cyfluthrin were higher in Sample III (Sindhri) as compared to other tested samples. Dialdrin and Methyl Parathian are only detected in sample III (Sindhri) and sample I (Dusehri) respectively. The variation of pesticide residues among the different varieties of mango is mainly due to use of different pesticide in different concentrations in different localities depending upon the pest attack. These observations are in accordance to the finding of (Rup and Dhanaraj 1989).

The result of present investigation further supports the findings of the previous surveys conducted in Pakistan by Masud and Hassan (1992). They reported the residues of organochlorine, organophosphate and pyrethroid insecticides in fruit and vegetable samples, collected from retail market of Karachi. Moreover these results negate the findings of Masud and Akhtar (1997) who monitored samples for fruit, vegetable, oil, milk and water brought from Gadoon Amazai. All the samples were free from pesticides residues. The possible reason for this difference may be that pesticides in that area are being used in accordance with good agricultural practice

Based on these observations it may be assumed that though all the assumed samples of mango varieties are within the permissible range of MRLs proposed by FAO/WHO and may no pose a serious threat to public health. In this regard a recent published work of Perveen and Masud (2001) revealed that 85% of blood samples were found to contain residue of tested pesticide. However this situation is particularly worrying in view of lack of reliable data on the long-term consequences of exposure to pesticides. It is therefore suggested that continuous monitoring systems of pesticides is needed of all food commodities in order to protect the end user for the indiscriminate use of pesticides.

References

Anonymous, 1999., Economic Survey of Pakistan, 1999-2000. Economic Affairs Advisors Wing, Finance Division, Government of Pakistan, Islamabad.

FAO/WHO., 1993 Codex Alimentarius Commission, Codex Committee on Pesticide Residues, Rome, Supplement 1, Vol. 2

Giani, M. A. ,1968. A Treatise to Horticulture, Lahore.

Ijaz, A. and M. Inayat., 1997. Post Harvest handling of mangoes. Brochure of mango and summer fruits exposition. The Horticultural Foundation of Pakistan, Islamabad. p: 33-36.

Masud. S. Z. and N. Hassan, 1992. Pesticide residues in food stuffs in Pakistan, organochlorine, organophosphorus and pyrethroid insecticide in fruit and vegetable. Pak.J.Sci.Ind.Res., 35:499-504.

Miller, K. 1987., Toxicological Aspects of Food. Elseviers Applied Science, London.

Masud,S.Z. and S.Akhtar.,1997. Pesticides in the biosphere of Pakistan. A review. Papa bulletin:11-16.

Parveen Z. and S.Z. Masud., 2001. Studies on pesticide residues in human blood. Pak.J.Sci.Ind.Res., 44 : 137-141.

Rup Lal, P. and S. Dhanaraj, 1989. Residues of Organochlorine Insecticides in Delhi Vegetable, Environmental Contamination and Toxicology, New York.

Salunkhe, D. K. and B. B. Desai., 1984. Post Harvest Biotechnology of Fruits, Vol. 1. CRC Press, Florida. p: 85.

Pakistan Journal of Nutrition 1(1) : 43-48, 2002

Isolation, Purification and Characterization of Fatty-Acid-Binding Protein from Milk Fat Globule Membrane: Effect of Bovine Growth Hormone Treatment

Vitaly L. Spitsberg[1] and R. C. Gorewit[2],
[1]BioVita Technologies, Inc., Ithaca, NY, [2]Lactation Physiology Laboratory,
Cornell University, Ithaca, NY 14853

Abstract: Fatty-acid-binding protein (FABP) was purified from bovine milk fat globule membrane (MFGM) by ion-exchange chromatography on DEAE-Sepharose and by gel-filtration on Sephadex G-50. Purified FABP was similar to bovine mammary gland heart (H)-type FABP/ mammary derived growth inhibitor (MDGI). It inhibited growth of mammary epithelial cells at nanogram concentrations, had a relative molecular mass of 15 kDa, as determined by SDS-PAGE, had two isoforms with pI around 5.0 and cross-reacted with antibody to mammary gland H-FABP. The content of FABP in MFGM, obtained from growth hormone (GH)-treated cows, was not essentially different from that of MFGM obtained from untreated cows. However, the level of *in vitro* phosphorylated FABP of MFGM, obtained from GH-treated cows, was diminished in comparison to the sample of MFGM, obtained from untreated cows. The role of the insulin receptor in the phosphorylation of FABP in mammary gland secretory epithelial cells is suggested.

Key words: Milk fat, globule membrane, bovine growth, protein

Introduction

The role of fatty-acid-binding protein (FABP) in fatty acid transport and lipid metabolism is well known (Bass, 1988; Spener *et al.*, 1985). However, recently accumulated experimental findings have allowed us to suggest that FABP can also play a role in regulation of cell proliferation and differentiation, at least, in the mammary gland (Grosse and Langen, 1990; Bohmer *et al.*, 1984; Bohmer *et al.*, 1987; Yang *et al.*, 1994).

It has been established that bovine mammary gland contains a 14.5 kDa protein that inhibits the growth of bovine mammary gland cells and some breast cancer cell lines. This protein was purified and named mammary derived growth inhibitor (MDGI) (Bohmer *et al.*, 1985; Bohmer *et al.*, 1987; Grosse and Langen, 1990; Grosse *et al.*, 1991). Direct protein sequence analysis of MDGI revealed 95 % homology to bovine H- FABP (Bohmer *et al.*, 1987). However, the sequence of MDGI, deduced from the presumably cloned bovine MDGI cDNA, was identical to bovine H-FABP (Grosse *et al.*, 1991). Despite this last finding and the fact that recombinant H-FABP had a similar cell growth inhibiting effect as MDGI (Yang *et al.*, 1994), bovine mammary gland FABP, as isolated and purified according to the protocol of Bohmer *et al.* (1985) or to the improved procedure of Grosse *et al.* (1991), was still called MDGI, suggesting the existence of a specific message for MDGI.

Recent studies by Specht *et al.* (1996); Borchers *et al.* (1997) helped resolve the controversy of the primary structure of MDGI. These studies demonstrated that the bovine mammary gland contains at least two types of FABP, namely, H-FABP and adipocyte (A)-type FABP. Comparison of the sequences of H-FABP and A-FABP allowed researchers to conclude that the direct protein sequencing analysis of MDGI, reported previously, had led to the erroneous identification of amino acid residues in, at least, 7 positions. This was a result of the presence of contaminating amounts of the co-purified A-FABP in the analyzed MDGI preparation. However, this research group (Borchers *et al.*, 1997) demonstrated that H-FABP, but not A-FABP, was involved in growth inhibition and differentiation in the mammary cell, and was localized in secretory epithelial cells of mammary gland tissue. A-FABP was found only in myoepithelial cells of the mammary gland. Therefore, based on the study of Grosse's research group (Grosse *et al.*, 1991) and Specht *et al.* (1996) and Borchers *et al.* (1997), we have designated the bovine mammary gland 15 kDa FABP with cell growth inhibitory property as bovine mammary gland H-FABP/MDGI. FABPs related to bovine MDGI were also identified in mammary gland of humans (Shi *et al.*, 1998) and mice (Binas *et al.*, 1992; Bansal and Medina, 1993; Treuner *et al.*, 1994).

The inhibitory action of H-FABP, or MDGI, on cell proliferation can be mimicked by synthetic peptides related to the 11-amino acid C-terminus of this protein (Grosse *et al.*, 1991; Yang *et al.*, 1994). This C-terminus has structural homology to Type I repeat of thrombospondin (TSP) (Spitsberg *et al.*, 1995). It is thought that the Type I repeat domain of TSP is responsible for the physiological effects of TSP through its binding to surface glycoprotein CD36 (Greenwalt *et al.*, 1992). Glycoprotein CD36 is significantly expressed in epithelial cells of the lactating mammary gland (Greenwalt *et al.*, 1992). It is suggested that the cellular action of FABP/MDGI, as a differentiation factor, can be exerted through an apocrinic loop mechanism (Brandt *et al.*, 1988), i.e. through the secretion by mammary gland cells of FABP/MDGI into extracellular spaces with subsequent binding of FABP/MDGI to CD36 (Spitsberg *et al.*, 1995). This would then lead to the triggering of specific signal transduction pathway(s) involved in cell differentiation. Recent studies also suggested that FABP/MDGI can function as a tumor suppressor gene product (Huynh *et al.*, 1996).

FABP, similar to mammary gland FABP/MDGI, has been found in bovine milk fat globule membrane (MFGM) (Brandt *et al.*, 1988). However, this membrane-associated FABP was not isolated from MFGM in its intact form, and, therefore, its physicochemical nature and biological activity has not been defined.

In this work we report the purification, characterization and biological activity of FABP from bovine MFGM. Amino acid analysis, SDS/PAGE, isolectrofocusing, Western blotting, two-dimensional electrophoresis (2D-E) and inhibitory cell growth assay demonstrated the similarity of FABP from MFGM to H-FABP/MDGI of bovine mammary gland tissue. In this work, we also showed that treatment of cows with bovine growth hormone (GH) appears to influence the level of *in vitro* FABP phosphorylation within the MFGM.

Materials and Methods

Milk from non-hormone treated and bovine recombinant growth hormone (rGH, Posilac, Monsanto, St.Louis, MO, USA) treated cows was obtained from the Cornell University Dairy Teaching and Research farm. Milk from hormone-treated cows was collected from those cows which had received injections of rGH (500 mg of Somatribove zinc) every two weeks over a five month period. MFGM was prepared according to Spitsberg *et al.*(1995). Rabbit polyclonal antibodies to bovine mammary gland-derived FABP/MDGI were obtained according to Spitsberg *et al.* (1995). DEAE-Sepharose CL-6B and Sephadex G-50 (fine) were from Sigma Chemical Co. (St.Louis, MO, USA). Precast polyacrylamide slabs for isoeletrofocusing (Ampholine PAGplate, pH 4.0 - 6.5; A=5%,

C = 3%) were from Pharmacia (Uppsala, Sweden). Ultrafiltration membranes (pore size, 0.45 im) were from Bio-Rad Laboratories (Hercules, CA, USA). Gamma-^{32}P-ATP (3000-6000 Ci/mmol) was purchased from Amersham, Inc. All other reagents were high purity grade and were purchased from various vendors.

Isolation and purification of FABP from bovine MFGM: All purification procedures were carried out at 2-4 °C. MFGM was obtained from composite (bulk tank) milk according to the protocol of Spitsberg *et al.* (1995). The MFGM was suspended in 50 mM imidazole buffer (pH 8.0) at about 10 mg/ml. This suspension was ultrasonicated by four strikes (each of 15 sec duration) at maximum output. After that, â-mercaptoethanol was added to the suspension to a concentration of 15-20 mM. The suspension was mixed for 10-15 min and was subjected to centrifugation at 80,000 x g for 1 h. The resulting supernatant was applied to a DEAE-Sepharose column (2 x 15 cm) equilibrated with 50 mM imidazole buffer, pH 8.0 (IB). The FABP fraction was eluted from the column by IB, containing 50 mM NaCl. The FABP fraction was concentrated by ultrafiltration with membrane UM1 (molecular weight cut off < 3 kDa) to about 5 ml, and this protein solution was applied to a Sephadex G-50 column (90 x 2.5 cm; Vo = 150 ml), equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl (TBS-buffer). The eluted protein fraction/peak with $K_d = 1.6$ (Fig. 1) was collected and concentrated by ultrafiltration, and stored at -70 °C. The isolation and purification of FABP from MFGM was repeated three times using the protocol described above. The average yield of FABP was approximately 400 μg of protein per isolation procedure.

Isolation and purification of bovine mammary gland FABP/MDGI: Bovine mammary gland FABP/MDGI was prepared by the protocol, designated as Method B, of Grosse *et al.* (1991). This method is more advanced then the method previously published by Bohmer *et al.* (1985). In Method B, the following improvements were made: the concentration of reducing agent, beta-mercaptoethanol, in the buffer, used for gel-filtration and ion-exchange chromatography, was increased from 10^{-4} M to 10^{-3} M, at the same time 10% glycerol was added to this buffer. The volume of the DEAE-Sepharose column was increased from 2 ml to 26 ml. The buffer, containing increased amount of beta-mercaptoethanol and glycerol, provides conditions sufficient to prevent unnecessary aggregation of proteins during purification. The increased amount of the ion-exchanger provides a better condition for separation of the different types of FABP present in mammary gland tissue.

Amino acid analysis: Amino acid analysis of isolated and purified FABP from MFGM and bovine mammary gland tissue was done by the standard protocol at the Biotechnology Facilities of Cornell University.

Isoelectrofocusing: Isoelectrofocusing was done according to the procedure recommended by the vendor (Pharmacia) with precast polyacrylamide slabs, containing ampholines to support the pH gradient from pH 4.0 to pH 6.5. Protein (2-3 μg) was applied to each lane. After isoelectrofocusing, the slabs were fixed using 40% methanol, containing 7% acetic acid, for 16 h. The slabs were then processed for silver staining according to the procedure of Gooderham (1984).

Cell growth inhibitory activity of FABP derived from MFGM: The determination of bilogical activity, i.e. inhibition of cell growth, of MFGM-derived FABP was done essentially as described by Spitsberg *et al.* (1995). MAC-T cells and primary mammary gland epithelial cells were used in these experiments. The experiments were repeated four times and the standard mean error (SME) was about ± 5%.

Protein phosphorylation: *In vitro* protein phosphorylation of MFGM proteins was done according to Spitsberg and Gorewit (1997). Samples of MFGM were prepared from the milk of cows treated with recombinant bovine GH and from milk of non-treated cows (controls), as indicated by Spitsberg *et al.* (1995). The examining of *in vitro* phosphorylation of 15 kDa protein within bovine MFGM was done in three different series of the experiments. In the first series we analyzed five samples of MFGM obtained from GH-treated cows and five samples from non-hormone treated cows. In the second series, three samples from the GH-treated cows and one sample of MFGM from non-hormone treated cow were analyzed; and we, also, analyzed two samples of MFGM prepared from the bulk milk, obtained from one of the local dairy farms, which was using injections of recombinant GH (Posilac, Monsanto) for increased milk production.

Other procedures: SDS/PAGE and Western immunoblotting were performed as described by Spitsberg *et al.* (1995). Non-denatured (12%) polyacrylamide gel electrophoresis was done with the buffer system used for SDS/PAGE, with no addition of SDS to the buffer and gel.

Two-dimensional electrophoresis (2D-E) was done according to Anderson (1991). After 2D-E the gel slabs were subjected to Western immunoblotting (Spitsberg *et al.*, 1995).

Protein concentration was determined by Bradford's method (Bradford, 1976). Densitometric analysis of the autoradiograms was done with the Saggita Color LE scanner (Qtronix) and with the image analysis program, developed by Molecular Dynamics (Sunnyvale, CA, USA).

Results

Isolation, purification and physicochemical properties of FABP from MFGM: The protocol described above for the isolation and purification of FABP from MFGM allowed us to obtain 300-500 μg of highly pure FABP from MFGM, obtained from 3 l of milk. The high purity of FABP was confirmed by the resolution of a single band after SDS-PAGE electrophoresis and Western blotting (Fig. 2A and B). Though the purification procedure employed in this work was quite similar to that used previously to purify of FABP/MDGI from mammary gland tissue (Grosse *et al.*, 1991), the ultrasonication and addition of â-mercaptoethanol (15-20 mM) to the extraction solution were necessary for the maximum solubilization of FABP from MFGM. Attempts to improve the yield of FABP from MFGM by inclusion of Triton X-100 into extraction buffer were not successful.

The SDS-PAGE analysis of purified FABP from MFGM showed the presence of only one protein with a relative molecular mass of 15 kDa, identical to that of mammary gland FABP /MDGI (Fig. 2A). Western immuno-blotting analysis of FABP showed that FABP from MFGM was immunologically similar to mammary gland FABP/MDGI (Fig. 2B). Like FABP/MDGI from mammary gland, the purified FABP from MFGM had two components, fast and slow, when it was analyzed by PAGE at non-denatured conditions (Fig. 2C). It is likely that the appearance of these two components was a result of the existence of two isoforms of FABP (Fig. 3). It is unlikely that the slow and fast components were a result of the nonspecific self-aggregation of FABP (Fournier and Rahim, 1983), since the aggregation would lead to the appearance of numerous bands rather then only two bands, detected with different preparations of MFGM FABP and mammary gland FABP/MDGI. One of the isoforms of FABP from MFGM had a pI = 5.20, and the other had a pI = 5.35. These isoforms, appeared as a result of the charge heterogeneity of the same protein, were similar to isoforms of FABP/MDGI from mammary gland (Fig. 3, lanes 2 and 3 versus lane 1). In an additional experiments done by 2D-E, in which 8 M urea was used in the first dimension (isoelectrofocusing), only two isoforms of the purified FABP from MFGM were detected at pH around 5.0. Similarly, only two isoforms at pH around 5.0 were found in purified mammary gland FABP, analyzed by 2D-E. Therefore, the 2D-E analysis of MFGM FABP and mammary gland FABP, purified by the protocol as it is

Table 1: Inhibition (in %) of MAC-T and primary bovine mammary epithelial cells with various doses of MFGM-derived FABP/MDGI. Data are based on four experiments performed in triplicate. The mean standard error was ±5.0.

Concentration, ng/ml	MAC-T cells (%)	Primary epithelial cells (%)
0.1	28.0	30.0
1.0	57.0	60.0
10	71.0	71.0
100	87.0	90.0

indicated in the Methods, did not reveal any noticeable amount of 15 kDa band related to the A-FABP (Specht *et al.* 1996). The different numbers in pI for two isoforms of FABP, reported in this study, and for bovine mammary gland H-FABP, reported by Specht *et al.* (1996), can be explained by an inaccuracies in determination of pH gradient in isoelectrofocusing experiments rather than by differences in the nature of the investigated FABP's. What is essential in both cases, is that the two isoforms of FABP in this study and Specht's study had quite similar differences in their pI=s, namely, 0.15 units in our study, and 0.2 units in study of Specht *et al.* (1996), and these two isoforms were positioned at pH around 5.0. The possibility that MFGM FABP or FABP/MDGI, purified in our study, could represent bovine brain (B)- type of FABP, which has a high homology (90-95%) with bovine H-FABP (Schoentgen *et al.*, 1989), can be excluded, since the B-FABP would have a pI around 6.0. Because the 11-amino acid C-terminus of B-FABP is quite different from that of H-FABP, it is unlikely that B-FABP would have cell growth inhibitory properties. On similar grounds, keratinocyte/epidermal lipid-binding protein with 48% homology to H-FABP and bovine MDGI (Krieg *et al.*, 1993) can not represent the MFGM FABP studied in this work.

In our work it was also found that the amino acid composition of purified MFGM FABP was quite similar to that of purified FABP/MDGI and was not significantly differed from the amino acid composition derived from the published bovine mammary gland H-FABP amino acid sequence (Borchers *et al.*, 1997).

The analytical parameters of MFGM FABP and bovine mammary gland FABP/ MDGI, purified by the Method B (Grosse *et al.*, 1991), provided evidence that both preparations of FABP analyzed in our work belong to the H-type of FABP, i.e. they are identical to H-FABP, described by Borchers *et al.* (1997).

Cell growth inhibitory activity of FABP derived from MFGM : The examination of growth inhibitory activity of FABP from MFGM showed that this protein had biological activity quite similar to FABP prepared from mammary gland (Grosse *et al.*, 1991; Spitsberg *et al.*, 1995). A activity was exerted in the concentration range 1-10 ng/ml (Table 1).

***In vitro* phosphorylated FABP** : The level of *in vitro* phosphorylation of FABP or 15 kDa-protein (Spitsberg and Gorewit, 1997) within MFGM obtained from cows, treated with recombinant bovine growth hormone, were examined in this work. Fig. 4, Panel B, represents the autoradiogram of the *in vitro* phosphorylation of MFGM proteins obtained from the raw milk of GH-treated cows and from untreated cows. The level of phosphorylation of FABP, i.e. the densitometric density (DD) of the 15 kDa bands of the autoradiogram (Fig.4, Panel B) is presented in Fig 5. The fig. 4 and 5 clearly demonstrated that the *in vitro* labeling of 15 kDa protein of all five samples of MFGM, obtained from the GH-treated cows, was less than in the samples of MFGM obtained from non-treated cows. The labeling of 15 kDa protein was especially reduced in the samples of MFGM analyzed in lanes 2 and 6 in Fig.4B. The phosphorylation of other major bands of MFGM, such as the 66 kDa and 51-52 kDa bands, was not markedly affected by GH treatment. We found that the ratio of the DD of the 66 kDa band to the DD of the 15 kDa band was practically constant when it was measured in five different samples of MFGM obtained from non-hormone-treated cows. Therefore, this ratio can serve as an indicator of the status of the phosphorylation level of 15 kDa protein. The lesser phosphorylation of 15 kDa protein/ FABP in MFGM could be explained by diminished content of FABP in MFGM obtained from hormone-treated animals. However, the amount of 15 kDa protein/FABP within MFGM, obtained from control and GH-treated cows, was quite similar in both types of MFGM, as determined by SDS-PAGE analysis (Fig. 4A). The possible reduction of the *in vitro* phosphorylation of 15 kDa protein within of MFGM, obtained from the GH-treated cows, was confirmed in the additional experiments with three individual samples of MFGM from GH-treated cows and with two samples of MFGM, prepared from the bulk milk of one of the local dairy farms, using GH for the increased production of milk.

Discussion

Previously it has been shown that bovine MFGM contains FABP which is related to bovine FABP/MDGI (Brandt *et al.*, 1988). However, in this study MFGM FABP was isolated from SDS-polyacrylamide gels, i.e. in a denatured form. Therefore, FABP obtained in this way is not suitable for examining its biological activity. Moreover, the biochemical characteristics of MFGM FABP were unknown. We felt it important to determine if MFGM associated FABP is structurally and functionally similar to FABP/MDGI. MFGM, as it is known, consists mostly of apical plasma membrane elements of secretory epithelial cells of the lactating mammary gland (Kanno, 1990). The discovery of FABP/MDGI in this membrane may support the suggestion of the involvement of FABP/MDGI in cell growth and differentiation through an apocrinic loop mechanism (Brandt *et al.*, 1988).

In our work, we presented evidence that FABP, associated with the bovine MFGM, is identical to the soluble, cytosolic form of H-FABP or MDGI of bovine mammary gland tissue (Grosse *et al.*, 1991). Firstly, we demonstrated that purified 15 kDa protein from bovine MFGM and FABP/MDGI from mammary gland tissue (Grosse *et al.* 1991; Spitsberg *et al.*, 1995) had similar biological activities. Both FABPs were able to suppress growth of mammary-derived cells at nanomolar concentrations. Secondly, the identity of the isolated 15 kDa protein from MFGM and FABP/MDGI was confirmed by analysis of the macromolecular species of these proteins by SDS-PAGE, non-denaturing PAGE, isoelectrofocusing, Western immunoblotting and 2D-E. Since, in bovine mammary gland, only two types of FABP, namely, H-FABP, localized in secretory epithelial cells, and A-FABP, localized only in myoepithelial cells, were clearly identified, we feel strongly that the isolated and purified 15 kDa protein from MFGM is FABP of heart-type, and can be referred to MFGM-associated H-FABP/MDGI.

The identification of H-FABP/MDGI associated with MFGM, representing the apical plasma membrane of secretory epithelial cells of mammary gland, opens a new avenue for studying the expression and biochemical modification of MFGM associated H-FABP/MDGI in the mammary gland under various physiological conditions.

We analyzed whether GH-treatment of cows can effect the content of H-FABP/MDGI within the MFGM and/or the level of *in vitro* phosphorylation of MFGM-associated H- FABP / MDGI (Spitsberg and Gorewit, 1997). This work showed that the level of phosphorylation of 15 kDa FABP, within the MFGM obtained from GH-treated animals was effected by GH-treatment. The level of phosphorylation was less than that for MFGM obtained from non-treated animals. The content of H-FABP/MDGI of MFGM was not effected by prolonged GH-treatment of cows, although the effect of GH-treatment on total expression of H-FABP/MDGI in mammary gland tissue can not be ruled out. Here, it is worthy to mention the recent *in vitro* finding of Huynh and Beamer (1998) that GH and insulin-like growth factor 1 (IGF-1) can suppres of MDGI gene expression.

The observed reduced level of *in vitro* phosphorylation of FABP within the MFGM, obtained from GH-treated cows, can be

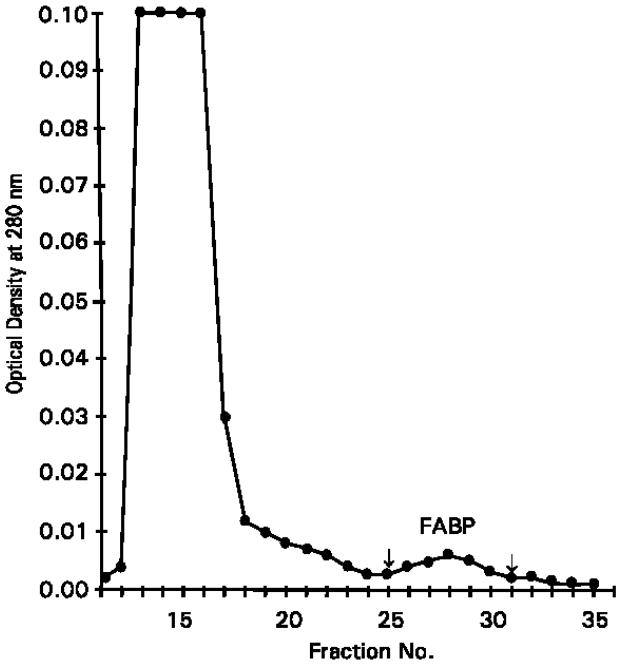


Fig.1: Elution profile of the MFGM-FABP containing fraction, obtained from DEAE-Sepharose, on Sephadex G-50 (90x2.5 cm). Equilibration buffer: 50 mM Tris-HCl, pH 7.4, containing 140 mM NaCl. The protein peak with $V_e/V_o = 1.5\text{-}1.6$ ($V_o = 150$ml; $V_e = 240$ ml) was collected and analyzed by SDS-PAGE, Western immunoblotting and cell growth inhibitory assay (see Methods). The first 10 fractions were collected at 14.5 ml/tube and subsequent fractions were collected at 5.3 ml/tube. The triangle pointers show the V_o (left) and V_e (right).

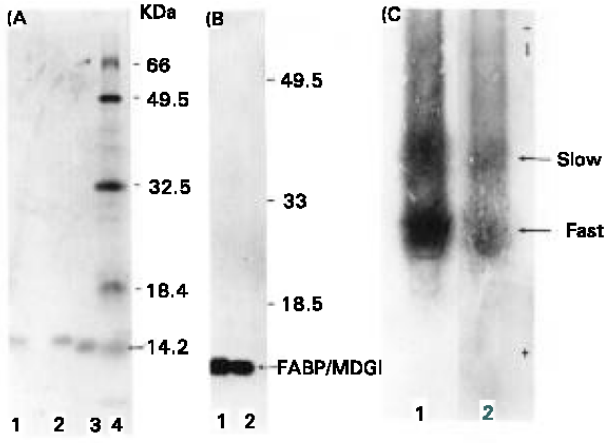


Fig. 2: Analysis of purified FABP from MFGM by PAGE and Western immunoblotting. Panel A: (12%) SDS-PAGE; lane 1- 100 ng of bovine mammary gland FABP; lane 2- 125 ng of bovine MFGM-FABP; lane 3- 100 ng á-lactalbumin (14.2 kDa); lane 4 - protein molecular weight standards. The gel was stained with silver (Gooderham, 1984). Panel B: Western immunoblotting analysis of purified bovine MFGM-FABP. The proteins were separated by 12% SDS-PAGE and transfered to nitrocellulose membrane. Lane 1- about 100 ng of bovine mammary gland FABP; lane 2- about 100 ng of MFGM-FABP. Primary antibody was anti-bovine mammary gland FABP (Spitsberg *et al.*, 1995). Secondary antiboby was conjugated goat anti-rabbit IgG-HRPO. The blott was developed with 4-chloro-1-naphthol and H_2O_2. Panel C: Western immunoblotting analysis after the separation of the proteins in nondenaturing (12%) PAGE. Lane 1: 100 ng of mammary gland FABP; 2- 100 ng of MFGM-FABP. Antibodies and detection were as it is described in panel B.

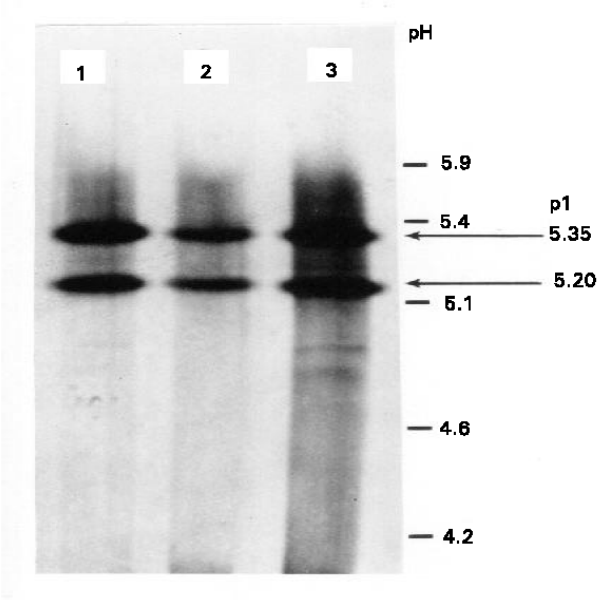


Fig.3: Analysis of FABP by isoelectrofocusing. Lane 1: 3 μg of bovine mammary gland FABP; lane 2- 1.8 μg of MFGM-FABP; lane 3- 3.0 μg of MFGM-FABP. The gel was stained by silver.

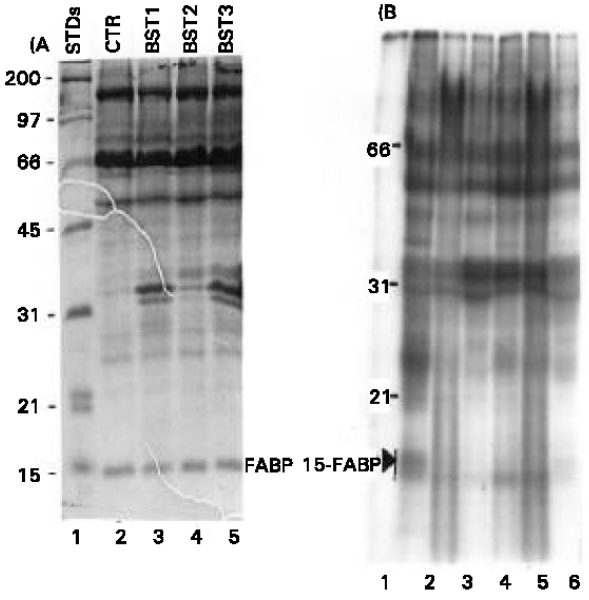


Fig.4: Analysis of *in vitro* ^{32}P-labeled MFGM proteins. Panel A. (12%) SDS-PAGE of bovine MFGM, obtained from GH-treated and un-treated cows. Lane 1: MW (kDa) standards; Lane 2: 50 μg of MFGM obtained from milk of non-hormone-treated cow [(CTR control)]; lanes 3-5: 50 μg of MFGM obtained from three individual samples of milk of GH-treated cows (BST1, BST2, BST3). The gel was stained by Coomassie blue R-250. The 15 kDa protein, previously identified only as FABP (Brandt *et al.*, 1988; Spitsberg *et al.*, 1995; Spitsberg and Gorewit, 1997) is present in these samples of MFGM in equal amount. Panel B. Autoradiogram of (12%) SDS-PAGE of the *in vitro* ^{32}P-labeled MFGM proteins, obtained from GH-treated and non-hormone-treated cows. 50 μg of proteins of MFGM were *in vitro* phosphorylated and subjected to SDS-PAGE (see Methods). Lane 1: MFGM from non-treated cow (control); lanes 2-6: individual MFGM from five GH-treated cows. The labelling pattern of other samples of MFGM from four untreated cows were similar to that shown in lane 1. Note that the *in vitro* labeling of 66 kDa protein (butyrophilin) (Spitsberg and Gorewit, 1997) was quite similar in all analyzed samples of MFGM.

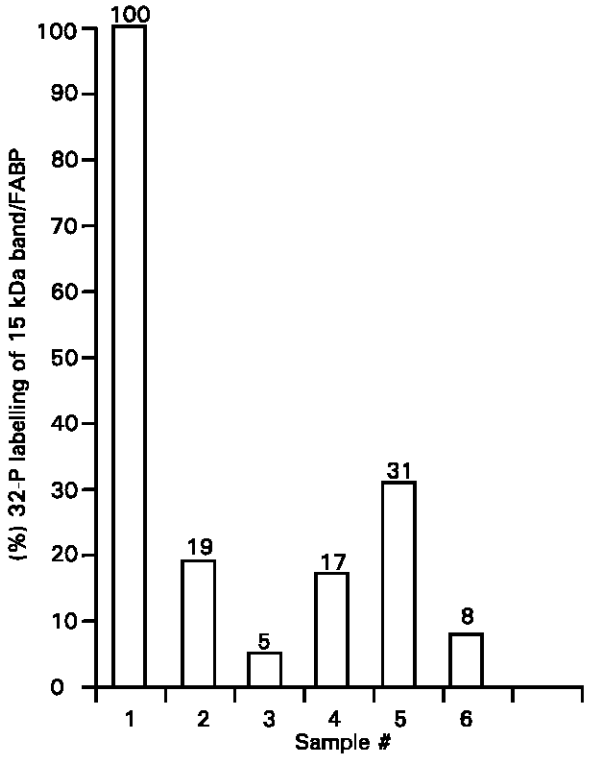


Fig. 5: Graph of the densitometric analysis of the autoradiogram in Fig. 4, Panel B. The graph demonstrates the decreased labeling of 15 kDa protein/FABP in five individual samples of MFGM obtained from GH-treated cows. The level of labelling of 15 kDa protein/FABP within control MFGM was taken as 100%.

interpreted as a result of diminished content or diminished activity of the phosphorylating FABP protein kinase in this membrane. One of the candidates for this kinase is the beta-subunit of insulin receptor since this polypeptide was detected in MFGM by Western immunoblotting (Spitsberg and Gorewit, 1997). In addition, the specific insulin-binding sites in bovine MFGM have been also reported (Smith *et al.*, 1987). In our previous work, we demonstrated that a tyrosine residue of the 15 kDa protein of bovine primary epithelial cells is phosphorylated and it reacted positively with FABP-antibody (Spitsberg *et al.*, 1994). There is evidence that FABP in mouse mammary gland epithelial cells is phosphorylated by the insulin receptor (Nielsen and Spener, 1993; Nielsen *et al.*, 1994). There are a few recent publications indicating the relationships between GH and insulin receptor (Balbis *et al.*, 1996, 1992; Leenanuruksa and McDowell, 1988). Balbis *et al.* (1996, 1992) demonstrated that overexpression of bovine GH in transgenic mice is associated with down regulation of hepatic insulin receptor. In other work (Leenanuruksa and McDowell, 1988), injection of recombinant bovine GH into aloxan-diabetic-insulin-stabilized ewes led to increased plasma glucose, which was decreased by a double-dosed insulin infusion.

If the phosphorylation of FABP takes place through the involvement of the beta-subunit of the insulin receptor, the association of FABP with membrane should occur on the cytoplasmic side of the plasma membrane and consequently on the cytoplasmc side of MFGM since MFGM represents the portion of plasma membrane encircling the secreted lipid droplets. The analysis of immunoprecipitates formed after the addition of anti-FABP or anti-CD36 to the solubilized proteins of MFGM showed that one of the candidates for the binding of FABP to the plasma membrane is the cytoplasmic domain of CD36 (Spitsberg *et al.*, 1995). Further study will be needed to show if the complex of FABP with CD36 is also associated with the beta-subunit of insulin receptor.

The significance of the finding that the phosphorylation of FABP within the MFGM is under hormonal regulation may have a practical application. Our preliminary work (Spitsberg and Gorewit, unpublished data) indicates that there is a correlation between the level of phosphorylation of FABP within the MFGM and the level of milk production by individual animals. We suggest the degree of *in vitro* phosphorylation of FABP within the MFGM can serve as an indirect sign as to whether or not the animals were given biologically effecations doses by GH. Therefore, the analysis of the *in vitro* phosphorylation of individual samples of MFGM could provide an understanding as to why some cows do not respond to the injection of GH with significantly elevated milk production.

Acknowledgment

We appreciate Dr. E. Matitashvili for determination of the biological activity of MFGM-FABP.

References

Anderson, L. ,1991. Two-dimensional electrophoresis: Operation of the Iso-Dalt System. Large Scale. Biology Press, Washington , DC .

Bansal, M.P. and D. Medina, 1993. Expression of fatty acid-binding proteins in the developing mouse mammary gland. Biochemical and Biophysical Research Communications, 191: 61-69.

Balbis, A., A. Bartke and D. Turyn, 1996. Overexpression of bovine growth hormone in transgenic mice is associated with changes in hepatic insulin receptors and in their kinase activity. Life Science, 59: 1363-1371.

Balbis, A., J.M. Dellacha, A. Bartke and D. Turyn, 1992. Down regulation of masked and unmasked insulin receptors in the liver of transgenic mice expressing bovine growth hormone gene. Life Science, 51: 771-778.

Bass, N.M.,1988. The cellular fatty acid binding proteins: aspects of structure, regulation and function. International Review of Cytology, 111: 143-185.

Binas, B., E. Spitzer, B. Zschiesche, B. Erdmann, A. Kurtz, T. Muller, C. Niemen, W. Blenau and R. Grosse, 1992. Hormonal induction of functional differentiation and mammary-derived growth inhibitor expression in cultured mouse mammary gland explants. *In vitro* Cellular and Developmental Biology, 28A: 625-634.

Bohmer, F.-D., W. Lehmann, H. Schmidt, P. Langen and R. Grosse, 1984. Purification of a growth inhibitor for Ehrlich ascites mammary carcinoma cells from bovine mammary gland. Experimental and Cellular Research, 150: 466-476.

Bohmer, F.-D., W. Lehmann, F. Noll, R. Samtleben, P. Langen and R. Grosse, 1985. Specific neutralizing antiserum against a polypeptide growth inhibitor for mammary cells purified from bovine mammary gland. Biochimica et Biophysica Acta, 846: 145-154.

Bohmer, F.-D., R. Kraft, A. Otto, C. Wernstedt, A. Kurtz, K. Muller, G. Rohde, G. Etzold, P. Langen, C.-H. Heldin and R. Grosse, 1987. Identification of a polypeptide growth inhibitor from bovine mammary gland . MDGI sequence homology to fatty acid and retinoid binding proteins. (1987) J. Biol. Chem., 262: 15137-15143.

Borchers, T., C. Hohoff, C. Buhlmann and F. Spener 1997. Heart-type fatty acid binding protein-involvement in growth inhibition and differentiation. Prostaglandins, Leukotrienes and Essential Fatty Acids, 57: 77-84.

Bradford, M.M., 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72: 248-254.

Brandt, R., M. Papperle, A. Otto, R. Kraft, F.-D. Boehmer and R. Grosse 1988. A 13-kDa protein purified from milk fat globule membrane is closely related to a mammary-derived growth inhibitor. Biochemistry, 27: 1420-1425.

Chen, N.Y., W. Y. Chen and J. J. Kopchick, 1997. Liver and kidney growth hormone (GH) receptors are differently in diabetic GH and GH antagonist transgenic mice. Endocrinology, 138: 1988-1994.

Fournier, N. and M. H. Rahim, 1983. Self-aggregation, a new property of cardiac fatty acid-binding protein. Predictable influence on energy production in the heart. J. Biol. Chem., 258: 2929-2933.

Gooderham, K., 1984. High-sensitivity silver staining of proteins following polyacrylamide gel electrophoresis. In: Methods in Molecular Biology. Vol. 1, Proteins. Edited by J.M. Walker ed., pp: 113-118. Humane Press, Clifton, NJ.

Greenwalt, D. E., R.H. Lipsky, R.H. DeKenhause, H. Ikeda, N. N. Tandon and G.A. Jamieson, 1992. Membrane glycoprotein CD36 : A review its role in adherence, signal transduction, and transfusion medicine, Blood, 80: 1105-1115.

Grosse, R., F.D. Boehmer, P. Langen, W. Lehmann, M. Mieth and G. Wallukat, 1991. Purification, biological assay and immunoassay of mammary-derived growth inhibitor. Methods in Enzymology, 198, 425-440.

Grosse, R and P. Langen, 1990. Mammary derived growth inhibitor. In Handbook of Experimental Pharmacology 95/II. M. Sporn and A. Roberts, editors. Springer Verlag, Heidelberg. pp: 249-265.

Huynh, H. , L. Alpert and M. Pollak, 1996. Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasma is associated with epigenetic changes. Cancer Research, 56: 4865-4870.

Huynh, H. and W. Beamer, 1998. Suppression of mammary-derived growth inhibitor gene expression by growth hormone and insulin-like growth factor 1. International J. oncology, 13: 577-582.

Kanno, C., 1990. Secretory membranes of the lactating mammary gland. Protoplasma, 159: 184-208.

Krieg, P., S. Feil, G. Furstenberger and G. T. Bowden, 1993. Tumor-specific overexpression of a novel keratinocyte lipid-binding protein. J. biol. Chem., 268: 17362-17369.

Leenanuruksa, D. and G. H. McDowell, 1988. Control of glucose homeostasis in lactating ewes use of the alloxan-diabetic-insulin-stabilized ewe to study effects of insulin and growth hormone. Australian J. Biol. Sci., 41: 421-434.

Nielsen, S. U. and F. Spener, 1993. Fatty acid-binding protein from rat heart is phosphorylated on Tyr-19 in response to insulin stimulation. J. Lipid Res., 34: 1355-1366.

Nielsen, S. U., R. Rump, P. Hojrup, P. Roepstroff and F. Spener, 1994. Differentiational regulation and phosphorylation of the fatty acid -binding protein from rat mammary epithelial cells. Biochimica et Biophysica Acta, 1211: 169-197.

Schoentgen, F., G. Pignete, L. M. Bonnano and P. Jolles, 1989. Fatty-acid-binding protein from bovine brain. Amino acid sequence and some properties. European. J. Biochem. 185: 35-40.

Shi, Y. E., J. Ni, G. Xiao, E. Liu, A. Fuchs, G. Yu, J. Su, J. M. Cosgrove, L. Xing, M. Zhang, J. Li, B.B. Aggarwal, A. Meager and R. Gentz, 1998. Antitumor activity of the novel human breast cancer growth inhibitor, mammary-derived inhibitor-related gene, MRG. Cancer Research, 57: 3084-3091.

Smith, D.H., K. L. Roehring, D.L. Palmquist and F.L. Schanbacker, 1987. Effect of dietary fat and lactation status on insulin binding to bovine milk fat globule membranes. J. Dairy Sci., 70: 1537-1562.

Specht, B., N. Bartezko, C. Hohoff, H. Kuhl, R. Franke, T. Borchers and F. Spener, 1996. Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. J. Bio.l Chem., 271: 19943-19949.

Spener, F., T. Borchers and M. Mukhejea, 1985. On the role of fatty acid-binding protein in fatty transport and metabolism. FEBS Letters, 244: 1-5.

Spitsberg, V. L. and R. C. Gorewit, 1997. *In vitro* phosphorylated bovine milk fat globule membrane proteins. J. Nutr. Biochem., 8: 181-189.

Spitsberg, V. L. , E. Matitashvili and R. C. Gorewit, 1994. Bovine mammary cells contain a low molecular weight phosphorylated protein that is immunoreactive to MDGI/FABP. J. Dairy Sci., 77 (Suppl.1), 75.

Spitsberg, V. L., E. Matitashvili and R. C. Gorewit, 1995. Association and co-expression of fatty-acid-binding protein and glycoprotein CD36 in the bovine mammary gland. European J. Biochem., 230: 872-878.

Treuner, M., C.A. Kozak, D. Gallahan, R. Grosse and T. Muller, 1994. Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor heart-fatty acid-binding protein. Gene (Amst.) 147, 237-242, 1993

Yang, Y., E. Spitzer, N. Kenny, W. Zschiescho, M. Li, A. Krominga, , T. Muller, F. Speher, A. Lezius, J. Veerkamp, G. Smith, D. Salomon and R. Grosse, 1994. Members of fatty acid binding protein family are differentiation factors for the mammary gland. J. Cell Bio., 127: 1097-1109.

Yang, C. W., G. E. Striker, W. Y. Chen, J. J. Kopchick and L. J. Striker, 1997. Differential expression of glomerular extracellular matrix and growth factor mRNA in rapid and slowly progressive glomerulosclerosis: Studies in mice transgenic for native or mutated growth hormone. Laboratory Investigation, 76: 467-476.

Abbreviations: MFGM - milk fat globule membrane; H-FABP - heart-type fatty acid binding protein; A-FABP - adipocyte-type fatty acid binding protein; MDGI - mammary derived growth inhibitor; GH - growth hormone; BST - bovine somatotropin, SDS - sodium dodecylsulfate; PAGE- polyacrylamide gel electrophoresis.

Pakistan Journal of Nutrition 1(1) : 49-52, 2002

Effects of Estrogen and Prolactin on Bovine BRCA1 Gene Expression

[1]Yongji Chung and R.C.Gorewit
[1]Department of Pathology, Northwestern University, Evanston Hospital Evanston, IL 60201
Department of Animal Science, Lactation Physiology Laboratory, Cornell University Ithaca, New York 14853

Abstract : Primary cultures of bovine mammary secretory epithelial cells and spontaneously transformed mammary secretory epithelial cells were treated with estradiol 17-ß or prolactin separately and in combination with both hormones. Both estradiol and prolactin induced BRCA1 expression in the cells. Hormone induced expression of BRCA1 was highest in transformed cells. Moreover, the induction of BRCA1 gene expression was highest when both estrogen and prolactin were in the culture medium. Gel shift assays showed that STAT5a antibody bound to a 20-bp double stranded oligonucleotide, containing a potential STAT5 response element (5'-acagtttctaaggaacactg-3', 3'-tgtcaaagattccttgtgac-5'). This is the first report showing that bovine mammary cells, in culture, are responsive to estrogen and prolactin in inducing expression of bovine BRCA1 and that BRCA1 may contain a STAT5 response element directing expression of the bovine BRCA1 gene. Further work is needed to examine the presence of estrogen response element(s) in estradiol induced BRCA1 expression. One must determine if PRL induced BRCA1 induction occurs after transfection of STAT5 -/- cells with STAT5a and STAT5a mutant constructs. This will give the strongest evidence of the direct involvement of a STAT5–BRCA1 signaling pathway.

Key words: BRCA1, estrogen, prolactin, mammary cells

Introduction

Prolactin and estrogen influence mammary cell growth and differentiation (Tucker, 1974). Serum concentrations of these hormones increase during gestation and prolactin is believed to be involved in the initiation of stage two lactogenesis (Tucker, 1974). Both hormones and their receptors have been implicated in breast cancer and its metastases (Wennbo and Tornell, 2000). The BRCA1 (breast ovarian cancer susceptibility protein) gene encodes a 220 kDa secretory protein with tumor suppressor like properties (Chen *et al.*, 1996; Miki *et al.*, 1994; Nevalainen *et al.*, 1996). The protein contains a short sequence shared by granins, proteins in various types of secretory vesicles whose function(s) is not yet clear (Jensen *et al.*, 1996). Mutation in the BRCA1 gene is found in about two-thirds of heritable breast carcinomas and in an un-estimated number of ovarian cancers (Futreal *et al.*, 1994).

BRCA1 protein has been localized in both the nucleus and cytoplasm of cells (Jensen *et al.*, 1996). Its localization appears to be related to the type of cell and whether it is normal or of neoplastic origin. We have shown that BRCA1 is differentially expressed in bovine mammary tissue throughout its development and involution (Chung and Gorewit, 2001). We have also shown that the protein is present in milk from lactating cattle, suggesting that it can be a secreted protein during lactation (Chung and Gorewit, 2002).

It has been recently shown that estrogen and prolactin up-regulate (BRCA1) expression in human breast cancer cells (Favy *et al.*, 1999). Although it has been shown that BRCA1 is expressed in normal and malignant breast tissue, very little is known about the mechanism(s) whereby prolactin regulates its expression.

STAT family proteins (Signal Transducers and Activators of Transcription) are transcription factors that have been found in the interferon system (Schindler and Darnell, 1995). They interact with gamma interferon via the GAS element (Heim *et al.*, 1995; Schindler and Darnell, 1995). At present, eight different STATs have been molecularly cloned (Heim *et al.*, 1995). STAT proteins have common features, including SIC and SH3 domains that are important in protein-protein interactions.

STAT proteins bind to cytokine receptor via interactions between phosphorylated tyrosine on the receptor and the SH2 domain of the STAT (Heim *et al.*, 1995). STAT binding to receptors is activated by JAK (Janus kinase) (Schindler and Darnell, 1995). Activated STATs form homodimers or heterodimers and translocate into the nucleus, where they bind to their specific target sequences and control gene expression.

STAT5 was initially identified as mammary gland factor (MGF) regulated by prolactin (Raught *et al.*, 1994). In the mammary epithelium, although STATI, STAT3, STAT5A, STAT5B and STAT6 are present in a latent form, only STAT5A and STAT5B are activated. This selective activation of STAT5 by prolactin was also observed in COS-7 cells co-transfected with the long form of the mouse prolactin receptor (PRL-R) and expression vectors for STATI, STAT3, STAT5 and STAT6 (Mayr *et al.*, 1998).

Prolactin receptor (PRLR) is a member of the cytokine/growth hormone/PRL receptor super family (Goffin *et al.*, 1999). The mechanism through which PRLR modulates STAT5 tyrosine phosphorylation, nuclear translocation, and DNA binding has been analyzed in HC11 cells, a mammary epithelial cell line, and 293-LA cells, a human kidney cell line stably over-expressing Jak2 kinase (Samir and Suhad, 1998). Wartmann *et al.*, 1996 have shown that in HC11 cells, STAT5 is specifically activated by PRL treatment, demonstrating that STAT5 is a physiological substrate downstream of the PRLR.

The pathways connecting extra cellular signals with the regulation of transcription factor activity are being investigated in detail. Considerable progress has been made in the description of the mechanism of action of steroid hormones and cytokines. Steroid hormones associate intra-cellularly with latent receptor molecules, cause the dissociation of masking proteins, the dimerization of receptors, and their binding to specific hormone response elements in the promoters of target genes (Beato *et al.*, 1996). It is well known that estrogen up regulates the expression of mammary gland prolactin receptors (Cassy *et al.*, 2000; Nevalainen *et al.*, 1996; Ormandy *et al.*, 1997).

Cytokines also activate latent transcription factors (STATs), but act through an enzymatic mechanism. For cytokines, tyrosine kinases associated with the trans-membrane cytokine receptors phosphorylate STAT molecules (Romagnolo *et al.*, 1998). The phosphorylated monomers dimerize and assume specific DNA binding ability. Both classes of transcription factors bind to different response elements and regulate different target genes and both signals, cytokines and steroid hormones, can affect growth differentiation and homeostasis of different cell types (Wartmann *et al.*, 1996).

Wyszomierski *et al.*, 2001 have recently shown that STAT5 functionally interacts with members of the steroid receptor family. They found that glucocorticoid receptor, mineralocorticoid receptor and progesterone receptor synergize with STAT5 in the induction of the transcription from the beta-casein gene promoter. The estrogen receptor decreased STAT5 mediated induction and the androgen receptor had no effect. Conversely, STAT5

negatively interfered with glucocorticoid receptor. The estrogen receptor induced transcription from an estrogen response element-containing promoter.
The objectives of the work described in this report were to determine if estrogen and prolactin could increase expression of BRCA1 in bovine mammary cells and explore the potential role of STAT5 in the process.

Materials and Methods

Cell Culture: Bovine primary mammary epithelial cells were obtained as described by (Zavizion *et al.*, 1992). Cells were cultured in medium containing a 1:1 (v/v) mixture of RPMI-1640 and Dulbecco's Modified Eagle Medium (Life Technologies, Inc) supplemented with 12% fetal bovine serum, penicillin (100 IU/ml), and streptomycin (100 ng/ml) at 37 C in 5 % CO_2. Media were changed everyday the first week. After that, media were changed 2 to 3 times/week. Cells were passed before confluence using a 0.05 % trypsin solution (Life Technologies, Inc.) to maintain experimental cultures. A bovine mammary epithelial cell line (BME) was also cultured under the same culture conditions as primary epithelial cell (Zavizion *et al.*, 1992).

Effects of estrogen and prolactin on BRCA1 induction: In order to determine the effect of estrogen and prolactin on induction of BRCAl expression, primary cultures of bovine mammary epithelial cells and BME cell line cultures were treated with estradiol 17-ß or prolactin or a combination of estradiol 17-ß and prolactin and assayed for BRCA1 expression by RNAse protection assay (RPA). Cells (2×10^6) were plated in 100 mm dishes and left to grow to confluency. Before hormone treatment, cells were serum starved for 24h and media were changed without serum twice. Cells were then treated with E2 (0.5 ng/ml) or PRL (200 ng/ml) or a combination of E2 and PRL (.5ng/ml E2 and 200 ng/ml PRL). After 1 hour, medium was discarded and cells were lysed by Tri reagent LS (Molecular Research Center) to isolate total RNA using the manufacture's recommendation on assay procedures.

Ribonuclease Protection Assay (RPA): Total BRCA1 transcript levels were measured using a ribonuclease protection assay and a Multi-NPA hybridization procedure (Ambion, Inc.). The template to generate a ribo-probe was prepared by PCR from bovine BRCA1 cDNA. BRCA1 primer pair, forward (+85/+104): 5'tctgctctgggtaagttca 3' and backward (+640/+620): 5'atttaggtgacactatagaatgatgttgtatccgctgctt 3' was used for preparation of template. The reverse primer contained the SP6 RNA polymerase-binding site to make antisense ribo-probe.
A 500 nt biotin labeled antisense ribo-probe was synthesized by MAXI Script (Ambion) according to the manufacturer's recommendations. The synthesized probe was gel purified and hybridized with 15 :g of total RNA to protect a fragment of the expected length. After nuclease digestion, the protected fragment was electrophoresed and transferred to nylon membrane by electro-transfer. Protected fragments were detected by Ambion's Bright star biodetection kit. BRCA1 mRNA was quantified by Photo Image IS 1000 with arbitrary units corrected for the expression of the control, actin mRNA (BRCA1/Actin).

Gel shift assays: Bovine mammary epithelial cell line (BME) was cultured until confluency, serum starved for 24h and media were changed without serum twice. The cells were then treated with estrogen (0.2, 0.8 ng/ml) or prolactin (100, 200 ng/ml) or a combination of estrogen (0.2 ng/ml) and prolactin (100 ng/ml) for 1 hour. BME Nuclear extracts were prepared according to the method of Lemkin *et al.*, 2000. A 20-bp double stranded oligonucleotide, containing potential STAT5 response element, (5'-acagtttctaaggaacactg-3', 3'-tgtcaaagattccttgtgac-5') was synthesized based on the human BRCA1 promoter sequence (NID: gl 147602). Oligonucleotide was purified by polyacrylamide gel electrophoresis, and equimolar quantities of complementary strands were annealed in buffer (10 mM Tris, pH 8.0, lmM EDTA, 10 mM NaCL) by heating at lOO °C for 5 min and cooling to room temperature. Annealed oligonucleotide pairs were 3' end labeled with biotin-dATP by terminal transferase (Life Technologies, Inc.). Ten nanograms of nuclear extracts were pre-incubated for 10 min in a buffer containing 20 mM HEPES, pH 7.9,50 mM NaCl, l mM $MgCl_2$, l mM dithiothretol, l mM EDTA, l ng of poly (dA-dT)-poly (dA-dT), and 10 % glycerol. Probes (15 ftnol) were then added and the incubation continued at room temperature for 15 min.
In experiments to identify the protein component of the protein-DNA complexes (super shift assay), antibodies raised against STAT5a, were purchased from Santa Cruz Biotechnology (CA). Antibodies were incubated with the nuclear extracts for 15 min at room temperature before addition of the probe. Protein-DNA complexes were separated on a 5% nondenaturing polyacrylamide gel (38:1, Acrylamide-bisacrylamide; 2 % glycerol; 22 mM Tris-borate, 0.5 mM EDTA, pH 8.3) at 15 mA (l h at 4 °C). Gels were transferred onto nylon membrane and cross linked and DNA-protein antibody complexes were detected by Ambion's CDP-star biotin detection kit.

Results and Discussion

Effects of erstrogen and prolactin on BRCA1 induction: The bovine mammary epithelial cell line (BME) was more responsive to E2 and PRL, within an hour of incubation, than primary epithelial cells (Fig.1) for BRCA1 gene induction. Estradiol 17-ß and PRL induced significant increases in BRCA1 expression in BME cells. The combination of these two hormones showed an additive effect, which was two times the increase compared to BRCA1 gene induction seen in the control (Fig.1).
Marquis *et al.*, 1995; Gudas *et al.*, 1995 reported up-regulation of BRCA1 by ovarian hormones, 17B-estradiol and progesterone (P) in ovariectomized mice and in breast cancer cells. More recently, Favy *et al.*, 1999 reported prolactin (PRL)-dependent up-regulation of BRCA1 expression in human breast cancer cells. However, molecular mechanisms explaining how E2, and PRL up regulate BRCA1 are unknown.
In previous studies, breast cancer cells were incubated with hormones 18 or 24 h before analysis of BRCA1 mRNA expression. However, our cells were incubated for only one hour with these hormones. The BME cells showed high sensitivity within an hour to E2 and PRL for BRCA1 gene induction. The E2 and PRL combination induced additive BRCA1 mRNA expression. In BME cells, BRCA1 responded to mammotropic hormones like an early response gene. The primary epithelial cells did not show the same degree of response.
This result posed two questions. Why was the response different in these two different cells and do these hormones directly effect BRCA1 gene induction? To answer the first question, let us consider primary characteristics of these two cells. BME cells are spontaneously mutated cells. We believe this cell line is much more proliferative than primary epithelial cells in response to E2 and PRL. This characteristic may have resulted in the difference of BRCA1 induction seen, even though its response was within an hour. Therefore, we speculate that protective feedback mechanisms of cells induce the growth regulator, BRCA1.

Gel shift assays : We selected BME cells for the gel shift assay because the BME cells were more responsive to E2 and PRL individually, than primary epithelial cells. We used oligo nucleotides of potential STAT5 response elements for the assay. A potential GAS/STAT5 binding element was incubated with nuclear extract and resulted in bands on membrane (Fig. 2). Hormone treated cells showed more intense bands than untreated control cells. Moreover, the band intensity was sensitive to PRL concentration (Fig. 2). Super-shift assays showed reactions of STAT5a antibody with BRCA1. Shifted bands were seen compared to controls without STAT5a antibody (Fig. 2).
The best-known functions of PRL in the mammary gland are its ability to induce lobuloalveolar growth (Wennbo and Tornell, 2000) and to stimulate postpartum lactogenesis (Wennbo and Tornell,

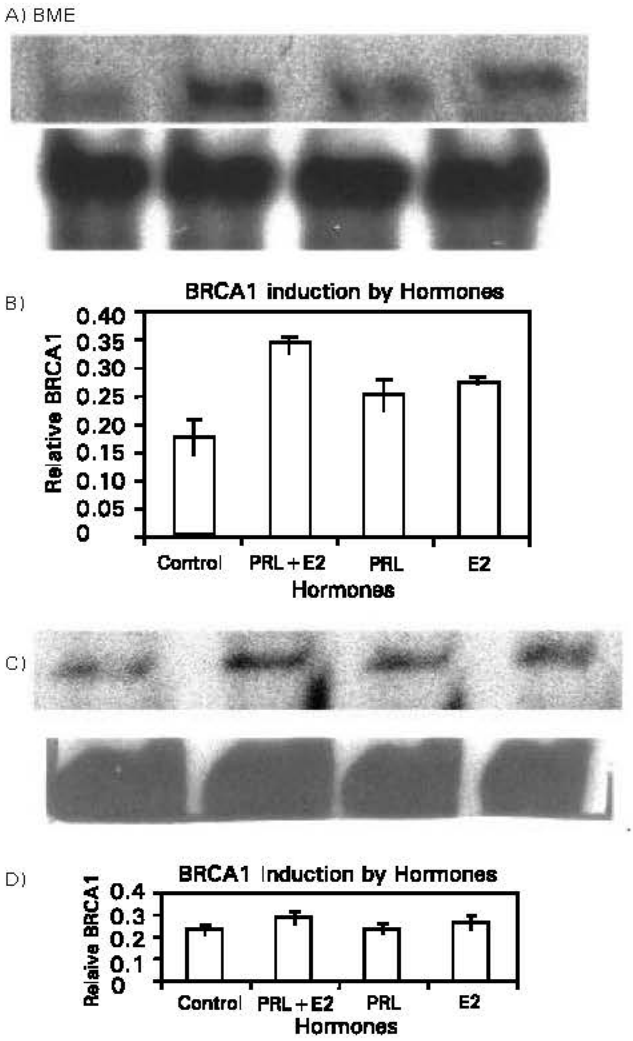


Fig. 1: BRCA1 gene expression in cultured bovine mammary epithelial cells. An RNAse protection assay was performed for bovine BRCA1 gene induction within 1 h of incubation with E2 (0.5 ng/ml) or PRL (200 ng/ml) or a combination of E2 and PRL (.5ng/ml E2 and 200 ng/ml PRL) in BME cells (A) and primary cultured bovine mammary epithelial cells (C) using biotin anti-sense probes of BRCA1 and ß-actin. (B) and (D) quantification of BRCA1 mRNA expression is shown in (A) and (C), respectively. Relative BRCA1 mRNA expression to ß-actin mRNA expression is also shown. Experiments were done in triplicate and means and standard errors of the means are shown as bars on graphs. The radiographs are examples of the replicates.

2000) . These properties are mediated through the activation of genes involved in many growth control and differentiation. STAT5 knockout mice show poor mammary gland development (Lemkin *et al.*, 2000). Further, Yang *et al.*, 2000 recently showed that STAT5 is differentially expressed throughout various stages of lactation in cows, thus suggesting that the STAT system is involved in the regulation of mammary gland growth and differentiation. The expression of STAT5 was identical to the expression patterns that were found for bovine mammary gland BRCA1 (Chung and Gorewit, 2001).

In our experiments, the potential GAS (-177/-160) element showed a shifted band with STAT5a antibody. Therefore, there is a

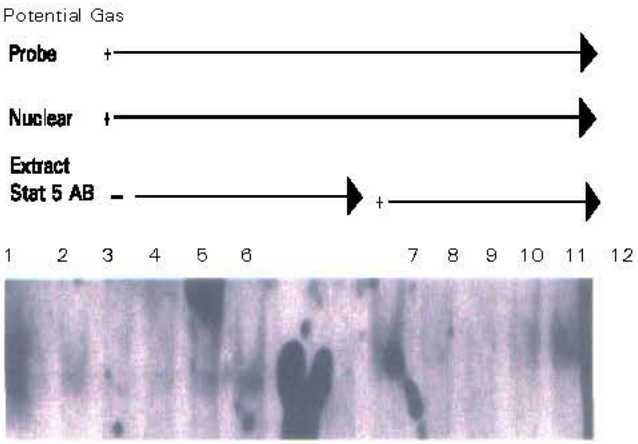


Fig. 2: Identification of a GAS(-290/-309) oligonucleotide in the BRCA1 promoter. The potential GAS biotin labeled oligonucleotide was incubated with nuclear extract. Nuclear extracts from control cells (lane 1), nuclear extracts from cells treated with 100 ng/ml PRL (lane 2) and 10 ng/ml PRL (lane 3). Lane 4 represents extracts treated with 0.2ng/ml E2. Lanes 5 and 6 represent extracts treated with a combination of 100 ng/ml PRL + 0.2 ng/ml of E2. Lanes 7-12 represent extracts incubated with STAT5a antibody alone. Experiments were done in triplicate and the radiograph is an example of the replicates.

possibility that the PRL-STAT5 pathway may be a direct BRCA1 regulator. To confirm a PRL-STAT5-BRCA1 pathway, it is necessary to perform further studies. One must determine if PRL induced BRCA1 induction occurs after transfection of STAT5 -/- cells with STAT5 and STAT5 mutant constructs. This will give the strongest evidence of the direct involvement of a STAT5 -BRCA1- signaling pathway. Further work is needed to determine if the estrogen receptor response element is involved in the estrogen induced up regulation of BRCA1 in bovine mammary cells.

Estrogen and prolactin enhance expression of the bovine BRCA1 gene in cultured bovine mammary secretory epithelial cells. The combination of estrogen and prolactin enhance BRAC1 above that seen for either hormone given alone. Antibody to STAT5a binds to an element that binds to the BRCA1 gene suggesting that a STAT 5 signaling system may be involved in BRCA1 gene expression in bovine mammary epithelial cells.

References

Ali Samir and Ali-Suhad., 1998. Prolactin receptor regulates STAT5 tyrosine phosphorylation andnuclear translocation by two separate pathways. J. Biol. Chem., 273: 7709-7716

Beato, M., S. Chavez and M. Truss, 1996. Transcriptional regulation by steroid hormones. Steroids, 61: 240-251.

Cassy, S., M.Charlier, L. Belair, M.Guillomot, K.Laud and J. Djiane, 2000.Increase in prolactin receptor (PRL-R) mRNA level in the mammary gland after hormonal induction of lactation in virgin ewes. Domestic Animal Endocrinology, 18: 41-55.

Chen, Y., A.A. Farmer, C.F Chen, D.C. Jones, P.L. Chen and W.H. Lee, 1996. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner [published erratum appears in Cancer Res., 1996 Sep. 1;56(17):4074] Cancer Res., 56:3168-72.

Chung, Y. and R.C.Gorewit, 2002. Cloning and expression of bovine BRCA1. Pak. J. Nutr.(in press).

Favy, D., A. Rio-Pascale, Jean-Claude Maurizis, Yves-Jean Bignon, B. Gallon, G. Bernard and J.Dominique, 1999. Prolactin-dependent up-regulation of BRCA1 expression in human breast cancer cell lines. Biochemical and Biophysical Research Communications. 258 (2). May, 10: 284-291.

Futreal, P.A., Q. Liu, D. Shattuck-Eidens, C.J. Cochran, K. Harshman, S. Tavtigian, L.M. Bennett, A. Haugen-Strano, J. Swensen, Y. Miki, K. Eddington, M. McClure, C. Frye, J. W Weaver-Feldhaus, Z. Ding, P. Gholami, L. Söderkvist, A., Kamb, and R.W. Wiseman, 1994. BRCA1 mutations in primary breast and ovarian carcinomas. Science, 266:120-122.

Goffin, V., N. Binart, C. Lacroix-Philippe, B. Bo., C.F. Bole, M. Edery, B.K. Lucas, P. Touraine, A. Pezet, R. Maaskant, C. Pichard, C. Helloco, N. Baran, H. Favre, A.A. Bernichtei, C. Ormandy and Paul Kelly, 1999. Should prolactin be reconsidered as a therapeutic target in human breast cancer?. From the molecular biology of prolactin and its receptor to lessons learned from knockout mice models. . Genetic Analysis Biomolecular Engineering, 15 :189-201.

Gudas-J. M., H. Nguyen, L. Tao and K.H. Cowan, 1995 Hormone-dependent regulation of BRCA1 in human breast cancer cells. Cancer Research, 55: 4561-4565.

Heim, Markus. I. M. H. Kerr, G.R. Stark and J. E. Darnell, 1995. Contribution of STAT SH2 Groups to Specific Interferon Signal the Jak-STAT Pathway. Science, 267: 1347-1349.

Jensen R.A., M.E. Thompson, T.L. Jetton, C.I. Szabo, R. van der Meer, B.Helou, S.Tronick, M.C. King, D.L. Page, and J.T. Holt, 1996. BRCA1 is secreted and exhibits properties of a granin. Nat. Genet., 12: 303-308.

Lemkin, P., F. Thornwall, C. Gregory, K. D. Walton and L. Hennighausen, 2000. Nucleic Acids Research, 28: 154452-4459.

Marquis S.T., J.V. Rajan, B. A. Wynshaw, J. Xu, G.Y. Yin, K.J Abel, B.L.Weber and L.A. Chodosh, 1995.The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues Nat. Genet., 11: 17-26.

Mayr, S., T. Welte, M. Windegger, J. Lechner, P. May, C. Heinrich, F. Horn and W. Doppler, 1998. Selective coupling of STAT factors to the mouse prolactin receptor. European J. Biochem., 258: 784-793.

Miki, J. D. Swensen, E. Shattuck, P.A. Futreal, K. Harshman, S. Tavtigian, Q. Liu, C. Cochran, L.M. Bennett and W. Ding, 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA-1. Science, 266: 66-71.

Nevalainen, M. T., E. M. Valve P.M. Ingleton, nand P.L.Harkonen, 1996. Expression and hormone regulation of prolactin receptor in rat dorsal and lateral proSTATe. Endocrinology, 137: 3078-3088.

Ormandy C.J., A. Camus, J. Barra, D. Damotte, B. Lucas, H. Buteau, M. Edery, N. Brousse, C. Babinet, N. Binart and P.A.Kelly, 1997. Null mutation of the prolactin receptor genes produces multiple reproductive defects in the mouse. Genes and Development, 11 : 167-178.

Raught, B., B. Khursheed, A. Kazansky and Jeffrey Rosen, 1994 YY1 represses beta-casein gene expression by preventing the format of a lactation-associated complex. Molecular and Cellular Biology, 14 : 1752-1763.

Romagnolo, D. A. Annab and T.E. Thompson, 1998. Estrogen upregulation of BRCA1 expression with no effect on localization. Mol. Carcinogenesis, 22: 102-109.

Schindler, C. and J. E. Darnell.,1995. Transcriptional responses to polypeptide ligands: The JAK-STAT pathway. Annu. Rev. Biochem., 64, 621-651.

Tucker H. A., 1974. General endocrinological control of lactation. Lactation Vol. I. B.L. Larson and V.R. Smith, ed. Academic Press, Inc. New York, New York.

Wartmann, M., N. Cella, P. Hofer, B. Groner, X. Liu, L. Hennighausen and N.E. Hynes, 1996. J. Biol. Chem., 271: 31863-31868.

Wennbo, H. and J. Tornell, 2000.The role of prolactin and growth hormone in breast cancer. Oncogene, 19:1072-1076.

Wyszomierski, S.L. and J.M. Rosen, 2001. Cooperative effects of STAT5 (signal transducer and activator of transcription 5) and C/EBP beta (CCAAT/enhancer-binding protein- beta) on beta-casein gene transcription are mediated by the glucocorticoid receptor. Molecular Endocrinology, 15 : 228-240.

Yang, J., J.J. Kennelly and V. E. Baracos, 2000. Physiological levels of STAT5 DNA binding activity and protein in bovine mammary gland. J. Animal Sci., 78: 3126-3134.

Zavizion, B., Politis, I, and R.C. Gorewit. 1992. Bovine mammary myoepithial cells 2. Interaction with epethelial cells in vitro. J. Dairy Sci., 75: 3381-3393.

Abreviation key: BME = bovine mammary epithelial cells, BRCA1 = breast ovarian cancer susceptibility protein one, E2 = estradiol 17-ß , PRL = prolactin, STAT = signal transducer and activator of transcription, PRLR = prolactin receptor, RPA = RNase protection assay, RNA = ribonucleic acid, PCR = polymerase chain reaction, GAS = interferon gamma activated site, JAK = Janus kinase.

Pakistan Journal of Nutrition 1(1) : 53-55, 2002

Expression of Bovine Mammary Gland SMAD 4 and its Relationship to BRCA1

[1]Yongji Chung and R.C.Gorewit
[1]Department of Pathology, Northwestern University, Evanston Hospital Evanston, IL 60201
Lactation Physiology Laboratory, Department of Animal Science, Cornell University,
Ithaca, New York 14853

Abstract: Little is known about the role of TGF-ß in ruminant mammary growth and development. The literature suggests that TGF-ß could play an active role in influencing early mammary development and involution. SMADs are proteins that function as intracellular signaling effectors for the TGF-ß super family of polypeptides. We cloned bovine SMAD 4 and examined its mRNA expression in the mammary gland at various developmental stages. Sequencing analysis showed that bovine SMAD 4 had 96 and 94% homology to human and rat SMAD 4, respectively. Therefore, SMAD 4 appeared to be a very conserved gene in these species. SMAD 4 mRNA expression was highest in early pregnancy and involution, and then decreased in late pregnancy and maintained a low level through lactation. We speculate that SMAD 4 is involved in induction of regulatory genes involved in mammary gland growth and apoptosis. BRCA1 is a breast and ovarian cancer-specific tumor suppressor protein, with properties of a transcription factor involved in cell cycle regulation, DNA repair and apoptosis. BRCA1 gene expression is induced by steroid hormones and its pattern of mRNA expression mimicks that for TGF-ß and SMAD 4. We thought it of interest to determine if various doses of TGF- ß would induce BRCA1 gene expression. TGF- $ß_1$ treated bovine mammary cells showed a dose dependent increase in BRCA1 mRNA expression compared to control treatments. Our results suggest that TGF-ß1, SMAD 4 and BRCA1 may play important roles in regulating ruminant mammary cell proliferation, differentiation, and involution or apoptosis.

Key words: SMAD 4, BRCA1, mammary, development

Introduction

TGF-ß regulates ductal growth and morphogenesis in the mouse mammary gland (Daniel *et al.*, 1989; Silberstein and Daniel, 1987). The growth factor is a potent inhibitor of mammary development prior to puberty, but does not influence mammary development during pregnancy in mice (Daniel *et al.*, 1989; Robinson *et al.*, 1991). In cattle, very little is known about the role of TGF- ß in mammary growth and development.

Plaut, 1993 examined receptor binding of TGF-ß1 to bovine mammary membranes from pubertal and lactating animals. Specific binding of TGF-ß1 was higher during the prepubertal and pubertal periods than during lactation, suggesting that the growth factor could play an active role in influencing early mammary development. Plath *et al.*, 1997 showed expression of TGF- ß1 during mammogenesis, lactogenesis, galactopoiesis and involution in bovine mammary gland. TGF- ß1 expression was higher during mammogenesis of virgin heifers and involution than during lactogenesis and galactopiesis.

Woodward *et al.*, 1993 characterized TGF- ß receptors and their autoregulation, and the growth response to TGF- ß1 and TGF- ß2 in cultured bovine mammary epithelium (MAC-T) and fibroblasts. Affinity labelling studies revealed that fibroblast and epithelial cells contained type I, II, and III (betaglycan) receptors, with the type III receptor being the predominant binding component. Pre-incubation of MAC-T cells with 50 pM TGF- ß1 or TGF- ß2 markedly downregulated TGF-beta receptors.

Proliferative response was measured using both total DNA and 3H-thymidine incorporation. Both TGF-beta isoforms inhibited MAC-T and fibroblast proliferation. Inhibition was reversible as shown by return of cellular proliferation to control levels following TGF-beta removal. Although growth inhibition was not transient as culture of MAC-T cells in TGF-beta resulted in sustained inhibition of proliferation for at least 144 h. Although it appears that TGF-ß is involved in the regulation of mammary growth and development, the mechanism(s) of its action is unknown.

SMADs are a class of proteins that function as intracellular signaling effectors for the TGF-ß superfamily of secreted polypeptides. SMADs received their name as a contraction of the names of the *C. elegans* Sma and Drosophila Mad, the first identified members of this class of signaling effectors. In all vertebrate cells studied, TGF- ß signals through sequential activation of two cell surface receptor serine-threonine kinases, that phosphorylate SMAD 2 and/or SMAD 3 (Zhang *et al.*, 1996; Zhang-Ying *et al.*, 1998). Phosphorylated SMAD 2 or SMAD 3, together with SMAD 4, translocate into the nucleus and associate with other transcription factors, leading to the activation of specific gene transcription (Derynck-Rik *et al.*, 1998; Heldin *et al.*, 1997; Labbe *et al.*, 1998; Lagna *et al.*, 1996; Liu *et al.*, 1997).

BRCA1 is a breast and ovarian cancer-specific tumor suppressor protein, with properties of a transcription factor involved in cell cycle regulation, DNA repair and apoptosis (Chapman and Verma, 1996; Paterson, 1998). BRCA1 inhibits proliferation of breast cancer cells (Paterson, 1998). We have recently shown that BRCA1 is differentially expressed in normal bovine mammary tissue. Moreover, the profile of expression is similar to that of TGFb1 expression . BRCA1 mRNA expression was examined over developmental stages from biopsied mammary tissue by RNase protection assay . The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded.

Since it has been shown in the literature that TGF-B1 is differentially expressed at various stages of mammary development, lactation and involution, we thought it of interest to determine if bovine mammary gland SMAD4, the intracellular signaling effector for the TGF-ß superfamily was also differentially expressed in the same manner. In an additional experiment, we determined if TGFb1 was capable of inducing transcription of BRCA1 in cultured bovine mammary cells..

Materials and Methods

Cloning SMAD4 : Total RNA was isolated from 0.5g of mammary tissue of non-lactating cows obtained from the slaughterhouse. Tissue was homogenized by a Polytron in Tri reagent (Molecular Research Center) and the manufacturer's recommendations were followed with regard to homogenization of tissue. Total RNA (1μg) was used for reverse transcription (RT) with a cycle kit (Invitrogen). RT procedures were according to manufacturer's recommendations and 2/10 of RT product (4μl) was used for PCR amplification. The PCR steps were performed as follows: 95 °C for 3 min, then 30 cycles of amplification (95 °C × 30 sec, 55 °C

```
bSMAD4  (1)     tgtgaatccatatcactacgaacgagttgtgtcacctggaattgatctct
hSMAD4  (509)   tgtgaatccatatacactacgaacgagttgtatcacctggaattgatctct
rSMAD4  (509)   tgtgaacccatatcactatgagcgggttgtctcacctggaattgatctct
bSMAD4  (51)    caggattaacactgcagagtaatgctccaccaagtatgttggtgaaggat
hSMAD4  (559)   caggattaacactgcagagtaatgctccatcaagtatgatggtgaaggat
rSMAD4  (559)   ctggattaacactgcagagtaatgctccaccaagtatgttagtgaaggat
bSMAD4  (101)   gaatatgttcatg
hSMAD4  (609)   gaatatgtgcatg
rSMAD4  (609)   gaatatgttcatg

Bovine  SMAD4   1-113    (113 bps)   Homology
Human   SMAD4   509-621  (113bps)    96 %
Rat     SMAD4   509-621  (113 bps)   94%
```

Fig. 1: Partial sequence of bovine SMAD4 cDNA and sequence comparisons with human and rat (Identical bases are underlined). Percent homologies between species are also shown.

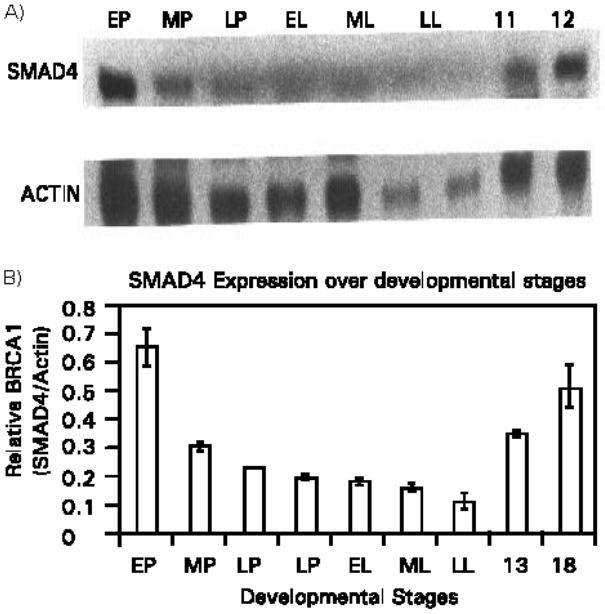


Fig. 2: Bovine mammary gland SMAD4 expression over various developmental stages. (A) RNase protection assay (RPA) was used to quantify bovine SMAD4 mRNA expression. Each lane contains 15μg of total RNA extracted from the bovine mammary gland. (B) Quantification of Relative SMAD4 mRNA expression to ß-actin mRNA expression is shown. EP (Early Pregnancy): 3 months; MP (Mid Pregnancy): 5 months; LP (Late Pregnancy): 6 months; LP (Late Pregnancy): 8 months; EL (Early Lactation): 3 months; ML (Mid Lactation): 6 months; LL (Late Lactation): 9 months; 6wk Involution, and 8wk Involution. Representative radiographs are shown in A. Means and standard errors are shown for three separate electrophoretic runs of three animals at each developmental stage in B.

×60 sec, 72 °C × 1 min), ending with 72 °C ×3 min. Taq polymerase (Gibco) was used for amplification. The primer set used for SMAD4 was 5′ gtgtgaatccatatcactac 3′/ 5′ aacgatggctgtccctcaaa 3′. The amplified product (15 kb) was cloned using the TA-Cloning Kit (Invitrogen). The procedures used were according to the manufacture's instructions. The cloned element was sequenced at the Cornell University sequencing facility.

Differential expression of bovine mammary SMAD4 :

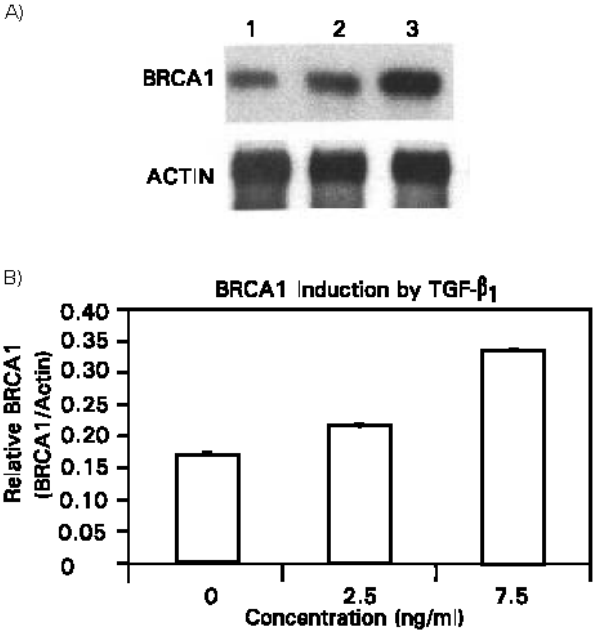


Fig. 3: BRCA1 induction by TGF-ß$_1$ in BME cells. (A) RNase protection assay for analysis of bovine BRCA1 mRNA induction within 1 hr by TGF-ß$_1$. Lane 1: Control, Lane 2: 3.76 ng/ml of TGF-ß$_1$, Lane 3: 14.3 ng/ml of TGF-ß$_1$) Lanes 1-3 contain 15 μg of total RNA isolated from cultured BME cells. (B) Quantification of BRCA1 mRNA expression showed in (A). Relative BRCA1 mRNA expression to ß-actin mRNA expression is shown.

Mammary Biopsy: Mammary tissue was obtained by biopsy from conscious female Holstein cattle in the following physiological stages (primiparous and three and eight months pregnant; multiparous and lactating for 3-5 months (early lactation); multiparous and lactating for 6-8 months (mid lactation); multiparous and lactating for 9-11 months (late lactation); and multiparous and undergoing mammary involution (three, six, and eight weeks). Three animals were biopsied per each developmental stage.

Animals were brought into a surgery suite and the point of tissue excision was anesthetized with lidocane. Five ml lidocane was also injected around the excision site. Approximately 10 grams of tissue was removed containing parenchymal elements. Blood vessels were cauterized or sutured to prevent further bleeding after biopsy removal. The excision site was closed with self-dissolving suture. Animals were given an injection of penicillin and returned to the Cornell University dairy farm.

Ribonuclease Protection Assay (RPA): Total RNA was extracted using Tri reagent (Molecular Research Center), as decsribed by the supplier. The SMAD4 Primer set was 5′ gtgtgaatccatatcactac 3′/ 5′atttaggtgacactatagaaaacgatggctgtccctcaaa3′. The reverse primer contained the SP6 RNA polymerase binding site (underlined) to make antisense ribo-probe. Approximate 150 nt biotin labeled SMAD4 antisense ribo-probes were synthesized by MAXI Script (Ambion) according to the manufacturer's recommendations. The synthesized probes were gel purified and hybridized with 20 μg of total RNA to protect a fragment of the expected length. After nuclease digestion, the protected fragment was eletrophoresed, transferred to nylon membrane by electro-transfer, and detected by Ambion's Bright Star Bio-Detection Kit SMAD4 mRNA was quantified by Photo Image IS1000 with arbitrary units corrected for the expression of the control, actin mRNA (SMAD4 /Actin).

BRCA1 induction by TGF- ß: In order to determine the effect of TGF- ß on the induction of BRCA1 expression BME cells were treated with TGF- $ß_1$ and RPA was performed. Cells ($2x10^6$) were plated in 100 mm dishes and grown to confluency. Before hormone treatment, cells were serum starved for 24h and media was changed without serum twice. Cells were then treated with various concentrations of TGF- $ß_1$ (0, 3.76, 14.3 ng/ml). After 2h, medium was discarded and cells were lysed by Tri reagent LS (Molecular Research Center) to isolate total RNA. Fifteen microgram of isolated total RNA was used for the RNase protection assay, as described above.

Results and Discussion

Cloning of SMAD4 : We cloned a partial cDNA of bovine SMAD4 by RT-PCR and confirmed it by DNA sequencing (Fig. 1). Sequencing analysis showed that it has 96 % homology to the human and 94 % to the rat SMAD4. Therefore, SMAD4 appeared to be a very conserved gene in these species. All PCR primers used for molecular cloning of SMAD4 were based on human sequences.

Differential expression of bovine mammary SMAD4 : SMAD4 mRNA expression was high in early pregnancy and involution, and then decreased in late pregnancy and maintained a low level through lactation (Fig. 2). There is a great degree of cell proliferation during early pregnancy and massive tissue remodeling occurring in the mammary gland during involution. Therefore, we speculate that SMAD4 is involved in induction of growth and apoptosis regulatory genes in these developmental stages with SMAD2, or 3.

It is not known exactly what factors are involved in regulation of SMAD4 gene induction. In general, TGF- ß binding to its receptor induces phosphorylation of SMAD2, 3 and phosphorylated SMAD2, 3 recruit SMAD4 to generate a complex (Derynck-Rik, 1998). However, there is no report that TGF-ß induces up-regulation of SMAD4 in the mammary gland. Our data suggests that SMAD 4 is constitutively expressed over satges of mammary development and involution.

BRCA1 induction by TGF- ß: To address if TGF-ß can induce BRCA1 gene induction *in vitro*, we treated BME cells with TGF- $ß_1$ and then performed RPA to observe BRCA1 induction (Fig. 3). TGF- $ß_1$ treated cells showed a dose dependent increases of BRCA1 induction compared to control treatments.

Transcription factors that cooperate with SMAD proteins, to regulate transcription of certain genes, include FAST-1, a winged-helix transcription factor, which mediates activin induction of the Mix.2 gene during embryonic frog development , c-Jun, c-Fos, ATF2, and vitamin D receptor, which interact with phosphorylated SMAD3 to mediate TGF- ß-induced transcription of various genes (Zhang-Ying, 1998).

Zawel *et al.*, 1998 identified a palindromic SMAD binding element. GTCTAGAC, by selecting for SMAD3 and SMADd4 binding sequences from a pool of random oligonucleotides. The three dimensional structure of the SMAD3 MH1 domain (N domain) indicates that an MH1 monomer binds precisely to a 4-bp sequence, -AGAC- (Labbe *et al.*, 1998; Zhang-Ying *et al.*, 1998). This AGAC SMAD binding element (SBE) should appear on average once every 256 bp in the genome ($1:4^4$). Thus, most genes that contain binding sites for SMAD partner transcription factor, such as FAST-1, AP-1, and transcription factor uE3 (TFE3) , will have SBEs in their promoters. Not all genes with binding sites for such transcription factors are transcriptionally responsive to TGF- ß. Therefore, it is unclear what controls the specificity of TGF-ß signaling at the transcription level.

The human BRCA1 promoter has several copies of -AGAC-element. However, we have not seen either palindromic repeats or direct tandem repeats of AGAC, used as a response elements for SMAD3/4 complex binding, in BRCA1 promoter sequences. Although this AGAC single repeat would expect to show weak binding to SMAD3/4, we can not neglect the possibility of these AGAC single repeats for BRCA1 gene induction. Needless to say, more studies are necessary to investigate the TGF-ß effect on regulation of BRCA1 gene induction.

Our studies are the first to describe gene expression for SMAD 4 through various stages of mammary gland development and involution. Expression patterns in the bovine were identical to those for TGF-B and BRCA1 during the same physiological stages. TGF-B1 induced expression of BRCA1 in cultured bovine mammary cells, thus, suggesting that TGF-B and its SMAD signaling system may be involved in the regulation of BRCA1 gene expression. Further studies are necessary to define the synergistic role of these factors in normal and neoplastic mammary gland growth, differentiation, and involution.

References

Chapman, M.S. and I. M. Verma, 1996. Transcriptional activation by BRCA1.Nature, 382: 678-679.

Daniel, C.W., G.B. Silberstein, K.Van Horn, P.Strickland and S. Robinson, 1989. TGF-β1- induced inhibition of mouse mammary ductal growth: developmental specificity and characterization. Dev. Biol., 135:20.

Derynck-Rik. Zhang-Ying. Feng-Xin-Hua, 1998. Smads: Transcriptional activators of TGF-beta responses. Cell 95(6). Dec. 11: 737-740.

Heldin, C.H., K. Miyazono, T. D. Peter, 1997. TGF-beta signalling from cell membrane to nucleus through SMAD proteins. Nature, 390 : 465-471.

Labbe, E., C. Silvestri, P. A. Hoodless, J. L. Wrana, L. Attisano, 1998. SMAD2 and SMAD3 positively and negatively regulate TGFbeta-dependent transcription through the forkhead DNA-binding protein FAST2. Molecular Cell, 2: 109-112.

Lagna, G., A. Hata, B. A. Hemmati, J. Massague, 1996. Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. Nature, 383 : 832-836.

Liu, F., C. Pouponnot, J. Massague, 1997. Dual role of the SMAD4/DPC4 tumor suppressor in TGFbeta-inducible transcriptional complexes. Genes & Development, 11: 3157-3167.

Paterson J.W. ,1998. BRCA1: a review of structure and putative functions. Dis Markers., Feb., 13: 261-274.

Plath, A.,R., Elinspanier, F.Peters, F. Sinowatz and D. Schams, 1997. Expression of transforming growth factors alpha and beta-1 messenger RNA in the bovine mammary gland during different stages of development and lactation. J. Endocrinol., 155:501.

Plaut, K., 1993 Role of epidermal growth factor and transforming growrth factors in mammary development and lactation. J. Dairy Sci., 76:1526

Robinson, S.D.,G.B. Silberstein, A.B. Roberts, K.C. Flanders, and C.W. Daniels, 1991. Regulated expression of and growth inhibitory effects of transforming growth factor-β isoforms in mouse mammary gland development (CAMB) 113:867.

Silberstein, G. B. and C.W. Daniel, 1987. Reversible inhibition of mammary gland growth by transforming growth factor-β. Science, 237:291.

Woodward, T.E., W.E. Beal and R.M. Akers, 1993. Cell interactions in initiation of mammary epithelial proliferation by estradiol and progesterone in prepubertal heifers. J. Endocrinol., 136:149.

Zawel-Leigh, Le-Dai-Jia., P. Buckhaults, Zhou-Shibin, W. Kenneth Kinzler, Bert Vogelstein and. Scott E. Kern, 1998. Human Smad3 and Smad4 are sequence specific transcription activators. Molecular Cell, 1: 611-617.

Zhang, Y., X. H. Feng, R. Y. Wu and R. Derynck, 1996, Receptor-associated Mad homologues synergize as effectors of the TGF-beta response. Nature, 383, 168-172.

Zhang-Ying. Feng-Xin-Hua and Derynck-Rik, 1998. SMAD3 and SMAD4 cooperate with c-jun/c-fos to mediate TGF-beta- induced transcription. Nature, 394: 909-913.

Abreviation key: TGF = transforming growth factor beta, BME = bovine mammary epithelial cells, BRCA1 = breast ovarian cancer susceptibility protein 1, SMADS = signaling effectors.

Pakistan Journal of Nutrition 1(1) : 56-63, 2002

Cloning and Expression of Bovine BRCA1

[1]Yongji Chung and R.C.Gorewit
[1]Department of Pathology, Northwestern University, Evanston Hospital, Evanston, IL 60201
Lactation Physiology Laboratory, Department of Animal Science, Cornell University, Ithaca, New York 14853

Abstract: Breast ovarian cancer susceptibility (BRCA) proteins appear to be involved in cell cycle regulation, DNA repair or genome integrity and induction of apoptosis in a variety of cells from humans and laboratory animals. The BRCA gene and protein have not been identified in cattle. The pattern of BRCA1 gene expression during normal mammary gland development and involution has not been examined in detail in any mammalian species. Therefore, the purpose of the present study was to clone the BRCA1 gene in Holstein dairy cattle and determine if the BRCA1 gene is differentially expressed through various stages of mammary gland development. We also localized immunoreactive BRCA1 protein in bovine mammary cells and milk fat globule membrane. Bovine BRCA1 cDNA was highly conserved to the human. Five hundred base pairs of exon 11 (+3384/ + 3866, human BRCA1 cDNA position) and the C-terminus 1 kb were identical to the human. Seven hundred base pairs of the N-terminus, which contain two ring domains, showed 90 % homology to human BRCA1. In bovine tissues, the degree of BRCA1 gene expression, from highest to lowest , was as follows: liver, spleen, mammary tissues and kidney. The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary gland BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded. Immunohistochemical studies showed that immunoreactive BRCA1 was localized in the nucleus and cytoplasm of mammary epithelial cells from lactating cows. It was not present in myoepithelial cells. The protein was also localized in the milk fat globule membranes. Our data suggest that BRCA1 is involved in bovine mammary gland development and/or differentiation, is specifically localized in secretary epithelial cells and is likely a secreted protein during normal lactation.

Key words: BRCA1, mammary, development, gene expression, cow, cells, involution

Introduction

Breast ovarian cancer susceptibility (BRCA) genes and their expressed proteins are currently being studied in human cells and tissues, as well as laboratory animals (Chen *et al.*, 1996; Futreal *et al.*, 1994; Hall *et al.*, 1990; Jensen *et al.*, 1996; Marquis *et al.*, 1995; Miki *et al.*, 1994; Paterson, 1998). These proteins appear to be involved in cell cycle regulation; thereby influencing tissue growth and /or differentiation (Chen *et al.*, 1996; Marquis *et al.*, 1995; Rajan *et al.*, 1996; Ruffner and Verma, 1997). They have also been implicated in DNA repair or genome integrity and induction of apoptosis in a variety of cells (Paterson, 1998; Shao *et al.*, 1996). Moreover, the BRCA1 gene has been identified as a tumor suppressor gene (Paterson, 1998). The gene is mutated in sporadic breast cancer and there are also inherited mutant forms of the gene that show a familial predisposition to cancer (Futreal *et al.*, 1994; Hall *et al.*, 1990; Miki *et al.*, 1994).

The BRCA1 gene encodes a 190-220 kDa protein consisting of 1,863 amino acids (Chen *et al.*, 1996; Paterson, 1998). The protein has sequence homology and biochemical analogy to the granin protein family (Jensen *et al.*, 1996). Both BRCA1 and the granins, are localized in secretory vesicles and are secreted by a regulated pathway. They are posttranslationally glycosylated, and are responsive to hormones (Romagnolo *et al.*, 1998).

Two putative nuclear localization signals and a potential ring-finger motif suggest that the BRCA1 protein is a nuclear protein. Chen *et al.*,1996 showed that BRCA1 was a nuclear phospo-protein in normal and non-breast /ovarian cancer cells, while in breast/ ovarian cancer cells BRCA1 was exclusively localized in the cytoplasm. They suggested that abnormalities of nuclear transport in sporadic tumors might influence BRCA1 cellular localization. Scully *et al.*, 1996; Ruffner and Verma 1997, however, reported that BRCA1 was predominantly localized in the nuclei of normal as well as breast /ovarian cancer cells.

It is very difficult to obtain non malignant or "normal" human breast tissue from females over various stages of mammary development, lactation and involution for studies focusing on differential gene expression. Therefore, the pattern of BRCA1 gene expression in normal mammary tissue, over various stages of development, has not been examined in detail. Cattle, however are useful for studies that are designed to describe changes in gene expression throughout various physiological states. They are convenient for multiple tissue biopsy and blood sampling. Furthermore, much of what we know about mammary gland biology has been derived from cattle and other ruminants. The purpose of the present study was to clone the BRCA1 gene in Holstein dairy cattle and determine if the BRCA1 gene is differentially expressed through various developmental stages of the mammary gland. We also localized immunoreactive BRCA1 protein in mammary cells and milk fat globule membrane.

Materials and Methods

Cloning and Sequencing of Bovine BRCA1: Total RNA was isolated from 0.5 g of mammary tissue of a non-lactating cow obtained from the slaughter house. Tissue was homogenized by a Polytron homogenizer in Tri reagent (Molecular Research Center) according to the manufacturer's recommendations. Total RNA (1 μg) was used for reverse transcription (RT) with a Cycle Kit (Invitrogen). RT procedures were according to manufacturer's recommendations, and 1/5 of the RT product (4 μg) was used for PCR amplification. The PCR steps were performed as follows: 95°C for 3 min, then 30 cycles of amplification (95°C X 30 sec, 52°C X 60 sec, 68°C X 4 min.), ending with 68 °C X 8 min. Advanced Taq polymerase (Clontech) was used for amplification. The amplified products, 5.5 kb and 2.1 kb, were cloned into Topo-XL Cloning Kit (Invitrogen). The procedures used were according to the manufacturer's instruction. The cloned elements were sequenced at the Cornell University sequencing facility. All primers used are shown in Table 1.

Northern blot analysis: For Northern blot analysis, 15 μg of total RNA was mixed with formaldehyde loading buffer (40mM Mops pH 7, 10 mM sodium acetate, 1mM EDTA, 50 % formamide, 5% formaldehyde), and loaded on a denatured agarose gel (40 mM Mops pH 7, 10 mM sodium acetate, 1 mM EDTA, 5% formaldehyde, 1% agarose). After electrophoresis, samples were transferred onto nylon membranes (Gene Screen; Dupont New England Nuclear) and UV crossed-linked. Prehybridization, hybridization, and washing steps were performed, as described by Sambrook *et al.*, 1989.

Briefly, the 5' region of BRCA1 cDNA, 1.2kb, was used as a

Table 1: Primers used to amplify various regions of bovine BRCA1 cDNA by PCR

Name of Primer pairs[a]	Position[b]
85104S/0414A	+85/+5669
YJ1S/YJ2A	+225/+809
85104S/yj2A	+85/+809
792S/4214A	+792/+4214
LAIIS/925IIA	+3384/+3847
LAIS/925IIA	+2757/+3847
RG2S/0614A	+4661/+5584

[a]All primers were 20 nucleotides in length. S for sense strand and A for antisense strand. [b]Primer position is based on the human BRCA1 cDNA sequence. NID is g555931.

template to make a cDNA probe. The cDNA probe was labeled with P^{32}-dATP by a random primer DNA labeling kit (Life Technologies, Inc.). Prehybridization was carried out in hybridization buffer (6X SSPE, 5X Danhart's solution, 0.1 % SDS, 50% formamide, and 100 μg/ml Salmon sperm DNA) for 1 hour, and $1x10^6$ cpm/ml of the labeled probe were added and incubated over night at 42 °C. The membrane was washed twice with 2x SSPE/0.1 % SDS for 30 min at 65 °C, and 1x SSPE /0.1 % SDS for 1 hour at 65 °C prior to autoradiography exposure.

Western Blot Analysis: Bovine mammary tissue from a lactating cow (.5 g) was homoginized in a Polytron with 2 ml of 0.25 M sucrose containing 0.1 M Tris-HCl (pH 7.4) and the following protease inhibitors: 1mM PMSF, 25 μg/ml leupeptin, 25 μg/ml apotinin, 1 mM benzamidine and 10 μg/ml soybean trypsin inhibitor (Sigma Chemical Co.). The homogenates were then centrifuged at 36,500 rpm for 20 min at 4 °C. After centrifugation, the supernatant was collected and quantified for protein concentration using the Bradford method (Biorad). The supernatant (120 μg) was run on a 5% SDS-PAGE, transferred to a PVDF membrane, the blot was blocked with phosphate-buffered saline (PBS, pH 7.2) containing 3% BSA and 0.05% Tween-20. BRCA1 immunoblots were performed using the BRCA1-8F7 monoclonal antibody (GeneTex), or BRCA1-Ab3 (Oncogene Research), diluted 1: 50 in 1% BSA in PBS. Binding was visualized by incubating the membrane with a horseradish peroxidase conjugated rabbit anti-mouse antibody (Sigma). The immunocomplexes were detected by color development with 4-chloro-1-naphtol (Sigma) and hydrogen peroxide, or LumiGLO chemiluminescent substrate (KPL).

Distribution of BRCA1 in bovine tissues

Ribonuclease Protection Assay (RPA): The expression patterns of BRCA1 mRNA in various bovine tissues and in mammary tissue across various physiological stages and involution (see below) were measured by RPA and a Multi-NPA hybridization procedure (Ambion). Liver, spleen, kidney, and mammary gland (.5g of each tissue) were isolated from three non pregnant cows in the fifth and seventh month of lactation. Briefly, total RNA was extracted from liver spleen kidney and mammary tissue using Tri reagent (Molecular Research Center), as described by the supplier. The template to generate a ribo-probe was prepared by PCR from bovine BRCA1 cDNA. BRCA1 primer pair, forward (+85/+104): 5'tctgctctgggtaagttca 3' and backward (+640/+620): 5'atttaggtgacactatagaatgatgttgtatccgctgctt 3' was used for preparation of template. The reverse primer contains the SP6 RNA polymerase binding site (underlined in the above sequence) to make antisense ribo-probe. A 500 nt biotin labeled antisense ribo-probe was synthesized by MAXI Script (Ambion) according to the manufacturer's recommendations. The synthesized probe was gel purified and hybridized with 15 μg of total RNA to protect a fragment of the expected length. After nuclease digestion, the protected fragment was electrophoresed and transferred to a nylon membrane by electro-transfer. Protected fragments were detected by Ambion's Bright star bio-detection kit. BRCA1 mRNA was quantified by Photo Image IS1000 with arbitrary units corrected for the expression of the control, actin mRNA (BRCA1/Actin).

Differential expression of BRCA1 in mammary tissue

Mammary biopsy: Mammary tissue was obtained by biopsy from conscious female Holstein cattle in the following physiological stages (primiparous and three and eight months pregnant; multiparous and lactating for 3-5 months (early lactation); multiparous and lactating for 6-8 months (mid lactation); multiparous and lactating for 9-11 months (late lactation); and multiparous and undergoing mammary involution (three, six, and eight weeks). Three animals were biopsied per each developmental stage.

Animals were brought into a surgery suite and the point of tissue excision was anesthetized with lidocane. Five ml lidocane was also injected around the excision site. Approximately 10 grams of tissue was removed containing parenchymal elements. Blood vessels were cauterized or sutured to prevent further bleeding after biopsy removal. The excision site was closed with self-dissolving suture. Animals were given an injection of penicillin and returned to the Cornell University dairy farm.

Total RNA was extracted from the mammary biopsy samples according the procedures described above. The expression patterns of BRCA1 mRNA in mammary tissue across various physiological stages and involution were measured by RPA and a Multi-NPA hybridization procedure (Ambion) as described above.

Identification of BRCA1 in mammary tissue, milk fat globule membrane and MCF-7 breast cancer cells

Mammary tissue: Mammary tissue (25 grams) was obtained from a lactating Holstein cow at slaughter. The tissue was cut into 10 mm3 sections, fixed with Bouin's fixative and embedded in paraffin and blocked. After cutting serial sections (6 μm), they were mounted on plain glass slides. Slides were deparaffinized through a series of xylene baths and then rehydrated. Finally, slides were placed in a Microprobe holder. The slides were dipped and blotted one time in 0.5% hydrogen peroxide in methanol, and then dipped and left for 10 minutes to block endogenous peroxidase activity. Slides were incubated with normal blocking serum (goat) for 10 min at room temperature in a humid chamber and then blotted. The slides were incubated with 1: 50, 1:200 affinity purified rabbit polyclonal antibody to BRCA1 (I-20, Santa Cruz Biotechnology) or 1: 50, 1:200 mouse monoclonal antibody to BRCA1 (Oncogene Science) in a humid chamber for 2 hours at 37 °C. Non-immune serum was used for control slides. After incubation, biotinylated secondary anti-rabbit or anti-mouse IgG antibody (Zymed) were applied to slides for 20 min at room temperature in a humid chamber. After incubation and washing slides were incubated again with streptavidin/peroxidase conjugate for 10 min at room temperature in a humid chamber. Slides were removed from the Microprobe holder and incubated (30 S) with chromogen/substrate solution (Zymed) at room temperature . Slides were then washed in tap water and counterstained with Gill's #1 hematoxylin for 10-30 seconds. The slides were washed in tap water and blotted. Permount was added to each slide and the cover slips were attached. Staining was visualized using a binocular microscope.

Isolated MFGM: Milk fat globule membranes (MFGM) were prepared as described by Spitsberg *et al.*,1997 with minor modifications. Milk was obtained from a healthy mastitis free cow in early lactation. Cream was isolated by centrifugation of milk at 3,000 x g for 50 min. It was then suspended in two volumes of 50 nmol/L Tris-HCL, pH 7.5, containing 0.15 mmol/L NaCl (TBS), and homogenized in a Waring blender for 1 min. The homogenate was centrifuged at 100,000 x g for 90 min at 4 °C. The MFGM pellet was washed once and resuspended in a small volume of TBS, followed by recentrifugation at 100,000 x g for 90 min and stored in a -70 °C freezer. No somatic cells were found in the MFGM, as determined by light microscopy.

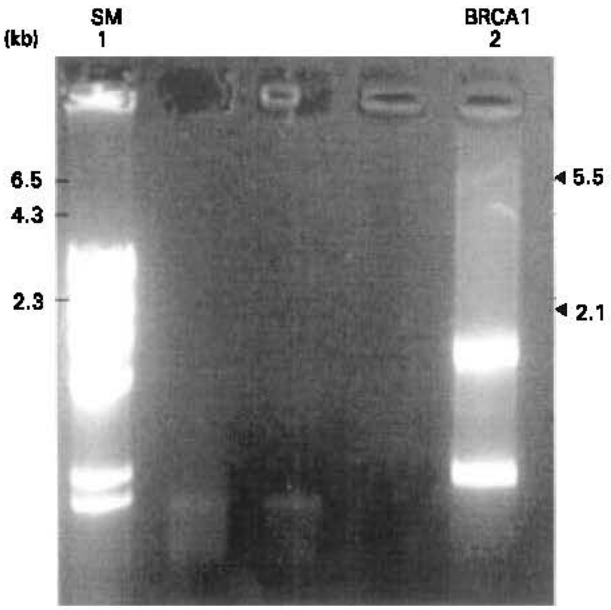


Fig. 1: PCR Amplification of Bovine BRCA1 cDNA. Left lane 1: A HindIII DNA size marker. Right lane 2: Amplified bovine BRCA1 cDNAs. The top band represents 5.5 kb. The bottom band (2.1 kb) is an alternating splicing variant of BRCA1.

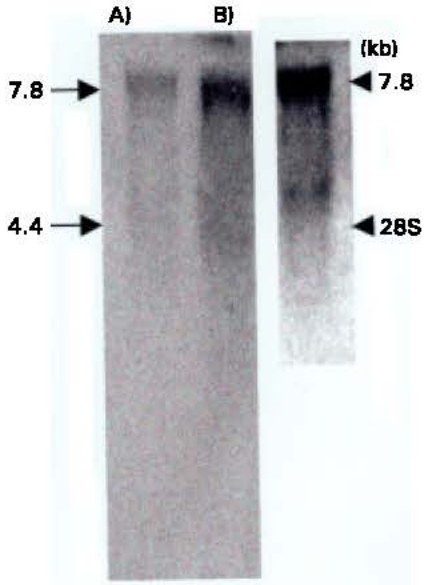


Fig.2: Northern blot analysis of bovine BRCA1. (A) Each lane contains 10 and 20 μg, respectively of total RNA extracted from a lactating bovine mammary gland. Arrows indicate an approximate 7.8 kb transcript of BRCA1 and the 28S position. (B) 20 μg of total RNA was blotted and hybridized with the N-terminus probe of bovine BRCA1. The top arrow indicates the 7.8 kb transcript and the bottom arrow shows the 4.4 kb splicing variant transcript.

Microscope slides were coated with L-lysine and left for 10 minutes to dry. MFGM pellets were then smeared on the slides as thin as possible. After fixation of the MFGM coated slides, in 3.7% formalin in PBS (pH 7.4) for 30 min, they were washed in PBS, 3 x for 5 min. The slides were treated with PBS + 0.1% Triton-X-100 for 5 min to permeabilize the membranes and the slides were dried . Immunostaining was carried out as described above for mammary tissue.

MCF-7 breast cancer cells: BRCA1 is expressed in MCF-7 human breast cancer cells (Paterson, 1998). Therefore, we isolated an MCF-7 total cell lysate, as a positive control for experiments identifying BRCA1 in bovine tissues and MFGM. MCF-7 cells were cultured and protein extracts were prepared using cell lysis buffer (Promega). MFGM was prepared as described above. MCF-7 protein extracts (100 μg) and MFGM (200 μg) were electrophoresed on a 5% SDS-PAGE, transferred to a PVDF membrane. The blot was blocked with phosphate-buffered saline (PBS, pH 7.2) containing 3% BSA and 0.05% Tween-20. The BRCA1 immunoblot was performed using BRCA1-17F8 monoclonal antibody (GeneTex), BRCA1-Ab3 (Oncogene), I-20 (Santa Cruz biotech) diluted 1: 50 in 1% BSA in PBS. Binding was visualized by reacting with a horseradish peroxidase conjugated secondary antibody (Sigma). The immunocomplexes were detected by peroxide, or LumiGLO chemiluminescent substrate (KPL).

Results and Discussion

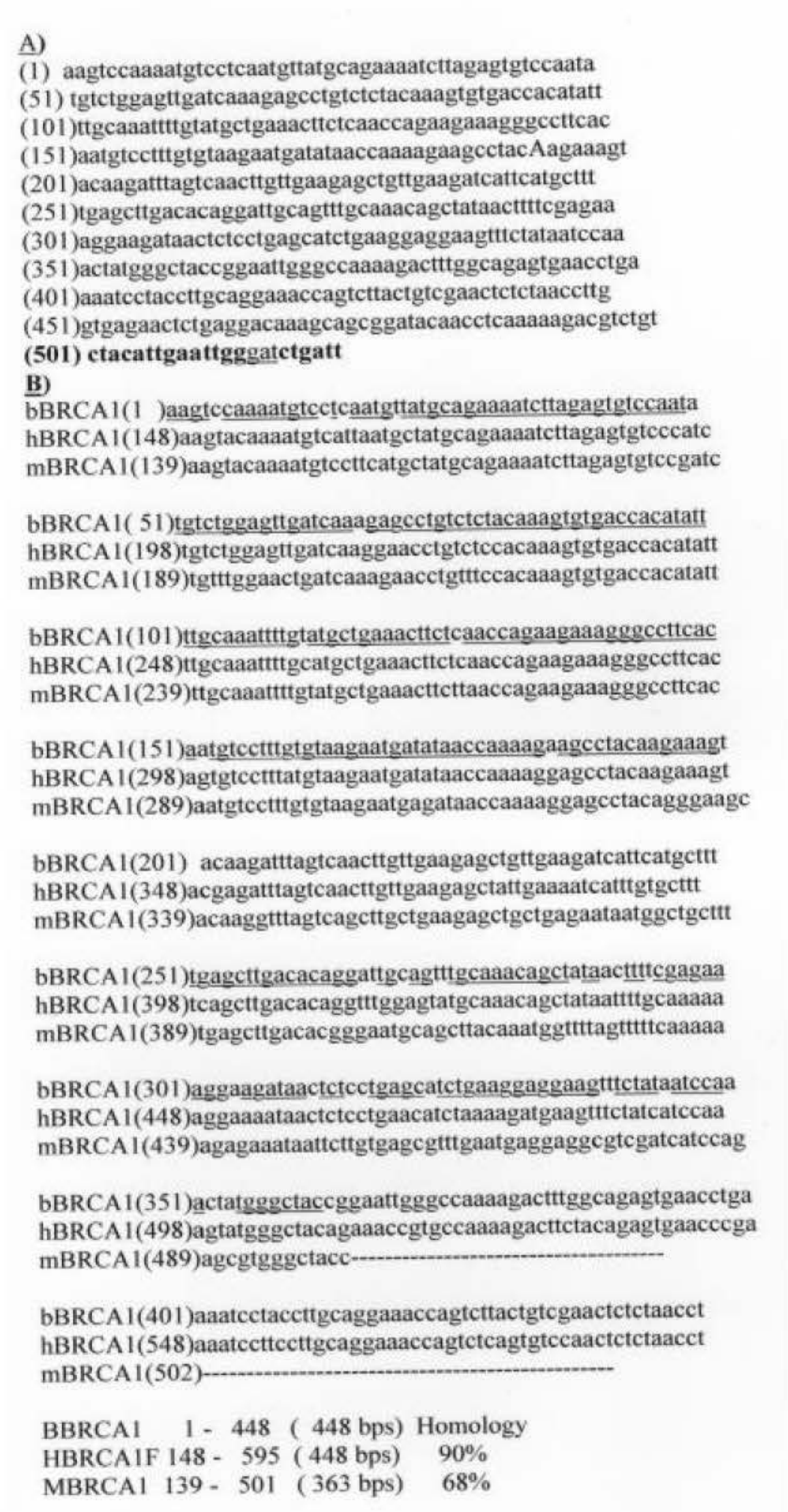
Cloning and sequencing of bovine BRCA1 : RT-PCR was performed with RNA from bovine mammary tissue, using primers derived from exons 3 and 24 of human BRCA1. Two PCR products (Fig. 1) were observed that would have derived from full length BRCA1 (i.e., about 5.5 kb, and 2.1 kb) as in human. Miki *et al.*, 1994 reported that a breast tissue-derived BRCA1 cDNA clone in which exons 9, 10, 11 were spliced out. These exons are 47, 76, and 3427 nucleotides in length, respectively. The small fragment was considered as exon 11 deletion splicing variant. This expectation was confirmed by northern analysis in which two transcripts were observed: one band around at 7.8-kb and the other one is at 4.4-kb (Fig. 2). Studies to determine whether splicing variants are functionally active are ongoing.

Bovine BRCA1 cDNA was highly conserved to the human. Five hundred base pairs of exon 11 (+3384/ + 3866, human BRCA1 cDNA position) and the C-terminus 1 kb were identical to the human. Seven hundred base pairs of the N-terminus, which contains two ring domains, showed 90 % homology to human BRCA1(Fig. 3). The sequence of 120 nt splicing junction, beginning at nucleotide 515, was as follows: GGgatc. (rest of 120 nt)YCTGC (lower case letters represent the 120 nt that are spliced spliced out). The Western blot analysis (Fig. 4) showed that size of bovine BRCA1 was quite similar to human's (i.e., 190-220 kDa).

Distribution of BRCA1 in bovine tissues : The distribution of bovine BRCA1 mRNA in spleen, kidney, liver and mammary tissues were analyzed by the RNase protection assay (Fig. 5). A probe spanning exons 2 to 8, as described in the methods section, was used for the RPA assay. Tissues were obtained from a cow in early lactation. The level of BRCA1 mRNA expression in all tissues was similar to that found in the mouse. The degree of bovine BRCA1 expression from highest to lowest was as follows: liver, spleen, mammary tissues and kidney.

Differential expression of BRCA1 in mammary tissue: BRCA1 mRNA expression was examined over developmental stages from biopsied mammary tissue by RNase protection assay (Fig. 6). The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded.

Marquis *et al.*, 1995; Rajan *et al*, 1996 showed that BRCA1 is broadly expressed in tissues of the early embryo, and is expressed



A)
(1) aagtccaaaatgtcctcaatgttatgcagaaaatcttagagtgtccaata
(51) tgtctggagttgatcaaagagcctgtctctacaaagtgtgaccacatatt
(101)ttgcaaattttgtatgctgaaacttctcaaccagaagaaagggccttcac
(151)aatgtcctttgtgtaagaatgatataaccaaaagaagcctacAagaaagt
(201)acaagatttagtcaacttgttgaagagctgttgaagatcattcatgcttt
(251)tgagcttgacacaggattgcagtttgcaaacagctataacttttcgagaa
(301)aggaagataactctcctgagcatctgaaggaggaagtttctataatccaa
(351)actatgggctaccggaattgggccaaaagactttggcagagtgaacctga
(401)aaatcctaccttgcaggaaaccagtcttactgtcgaactctctaaccttg
(451)gtgagaactctgaggacaaagcagcggatacaacctcaaaaagacgtctgt
(501) ctacattgaattgggatctgatt

B)
bBRCA1(1)aagtccaaaatgtcctcaatgttatgcagaaaatcttagagtgtccaata
hBRCA1(148)aagtacaaaatgtcattaatgctatgcagaaaatcttagagtgtcccatc
mBRCA1(139)aagtacaaaatgtccttcatgctatgcagaaaatcttagagtgtccgatc

bBRCA1(51)tgtctggagttgatcaaagagcctgtctctacaaagtgtgaccacatatt
hBRCA1(198)tgtctggagttgatcaaggaacctgtctccacaaagtgtgaccacatatt
mBRCA1(189)tgtttggaactgatcaaagaacctgtttccacaaagtgtgaccacatatt

bBRCA1(101)ttgcaaattttgtatgctgaaacttctcaaccagaagaaagggccttcac
hBRCA1(248)ttgcaaattttgcatgctgaaacttctcaaccagaagaaagggccttcac
mBRCA1(239)ttgcaaattttgtatgctgaaacttcttaaccagaagaaagggccttcac

bBRCA1(151)aatgtcctttgtgtaagaatgatataaccaaaagaagcctacaagaaagt
hBRCA1(298)agtgtcctttatgtaagaatgatataaccaaaaggagcctacaagaaagt
mBRCA1(289)aatgtcctttgtgtaagaatgagataaccaaaaggagcctacagggaagc

bBRCA1(201) acaagatttagtcaacttgttgaagagctgttgaagatcattcatgcttt
hBRCA1(348)acgagatttagtcaacttgttgaagagctattgaaaatcatttgtgcttt
mBRCA1(339)acaaggtttagtcagcttgctgaagagctgctgagaataatggctgcttt

bBRCA1(251)tgagcttgacacaggattgcagtttgcaaacagctataacttttcgagaa
hBRCA1(398)tcagcttgacacaggtttggagtatgcaaacagctataattttgcaaaaa
mBRCA1(389)tgagcttgacacgggaatgcagcttacaaatggttttagttttcaaaaa

bBRCA1(301)aggaagataactctcctgagcatctgaaggaggaagtttctataatccaa
hBRCA1(448)aggaaaataactctcctgaacatctaaaagatgaagtttctatcatccaa
mBRCA1(439)agagaaataattcttgtgagcgtttgaatgaggaggcgtcgatcatccag

bBRCA1(351)actatgggctaccggaattgggccaaaagactttggcagagtgaacctga
hBRCA1(498)agtatgggctacagaaaccgtgccaaaagacttctacagagtgaacccga
mBRCA1(489)agcgtgggctacc----------

bBRCA1(401)aaatcctaccttgcaggaaaccagtcttactgtcgaactctctaacct
hBRCA1(548)aaatccttccttgcaggaaaccagtctcagtgtccaactctctaacct
mBRCA1(502)----------

BBRCA1	1 -	448	(448 bps)	Homology
HBRCA1F	148 -	595	(448 bps)	90%
MBRCA1	139 -	501	(363 bps)	68%

Fig.3: Ring finger domain (N-terminus) of Bovine BRCA1 cDNA Sequence. (A) The nucleotide sequence of bovine BRCA1 corresponds to human BRCA1 exon 2 to 8. The 120 bp splicing point is underlined. (B) Bovine BRCA1 sequence comparison with human and mice

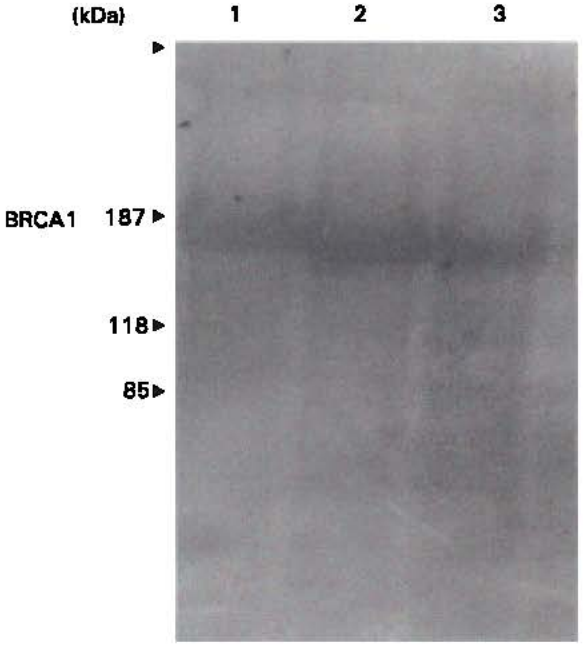


Fig.4: Western blot analysis of bovine BRCA.Each lane contains 120 μg of crude extracts prepared from lactating bovine mammary tissue. The extracts were electrophoresed on 5% SDS-PAGE and then transferred to PVDF membranes. Protein was detected using N-terminal BRCA1 monoclonal antibody (BRCA1-8F7).

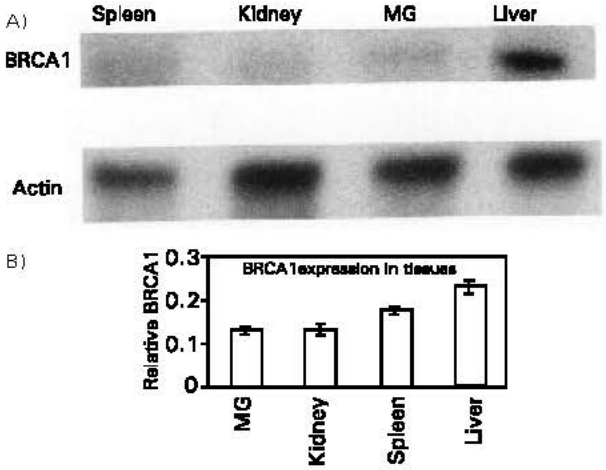


Fig.5: Tissue distribution of BRCA1 in a lactating cow. (A) Total RNA was isolated from a cow in early lactation and BRCA1 mRNA expression was analyzed by RNase protection assay, using a biotin labeled antisense BRCA1 probe. (B) Quantification of BRCA1 mRNA expression in (A). Relative BRCA1 mRNA expression to ß-actin mRNA expression is shown. Representative radiographs are shown in A. Means and standard errors are shown for three separate electrophoretic runs in B.

in an epithelial-specific fashion in the mammary gland of mice. The highest BRCA1 expression was found in the terminal end bud structures which contain rapidly dividing, relatively undifferentiated cell types that give rise to the differentiated mammary epithelial tree during ductal morphogenesis, during

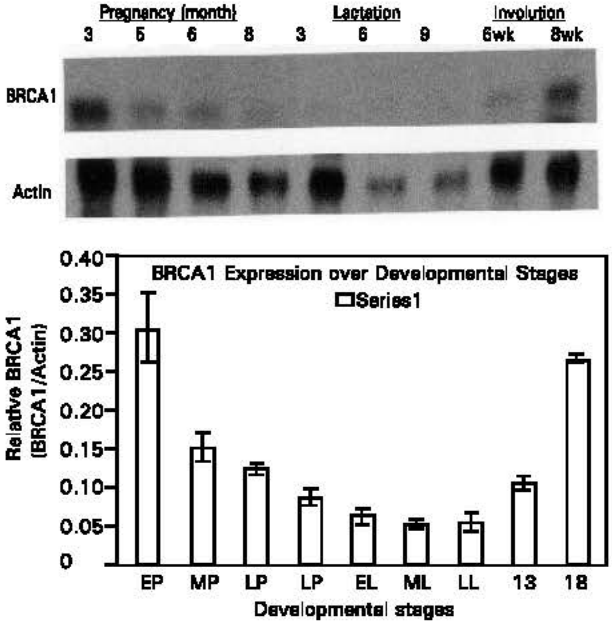


Fig.6: Bovine mammary gland BRCA1 expression over various developmental stages.(A) RNase protection assay (RPA) was used to quantify bovine BRCA1 mRNA expression. Top and mid-panel are RPA hybridized to the 550 nt, 500 nt bovine BRCA1 antisense riboprobe and 250 nt actin riboprobe, respectively. Each lane contains 15μg of total RNA extracted from indicated developmental stage of bovine mammary tissue. (B) Quantification of BRCA1 mRNA expression showed in (A). Relative BRCA1 mRNA expression to ß-actin mRNA expression is shown. EP (Early Pregnancy): 3 months; MP (Mid Pregnancy): 5 months; LP (Late Pregnancy): 6 months; LP (Late Pregnancy): 8 months; EL (Early Lactation): 3 months; ML (Mid Lactation): 6 months; LL (Late Lactation): 9 months; 6wk Involution, and 8wk Involution. Representative radiographs are shown in A. Means and standard errors are shown for three separate electrophoretic runs of three animals at each developmental stage in B.

puberty and in developing alveoli during pregnancy. Taken together, BRCA1 is considered to regulate cellular proliferation and differentiation.

Differentiation of the mammary epithelial cell is completed in mid-pregnancy in the bovine. Early pregnancy tissue (3 month) showed the highest BRCA1 expression, as expected. The expression of BRCA1 was maintained high until mid-pregnancy and then decreased until late lactation. So, if we consider these expression patterns, BRCA1 may serve as a regulator of cell proliferation in bovine mammary tissue. In other words, through all of pregnancy, BRCA1 may regulate the cell cycle and/or repair DNA. In addition, the interesting point is that BRCA1 expression increased over involution as in mice. The involution period includes a tissue remodeling process with wide ranging apoptosis taking place. Shao *et al.*,1996 showed that serum deprivation or calcium ionophore treatment of BRCA1 transfectants resulted in programmed cell death in NIH3T3 and MCF-7 cell lines. So far, there is no direct evidence that BRCA1 is directly involved in apoptosis during involution. Needless to say, further research is needed to answer it clearly. Marquis *et al.*, 1995 reported the mammary glands of parous mice that had undergone four weeks of postlactational regression express higher levels of BRCA1 mRNA than the mammary glands of age-matched virgin control mice.

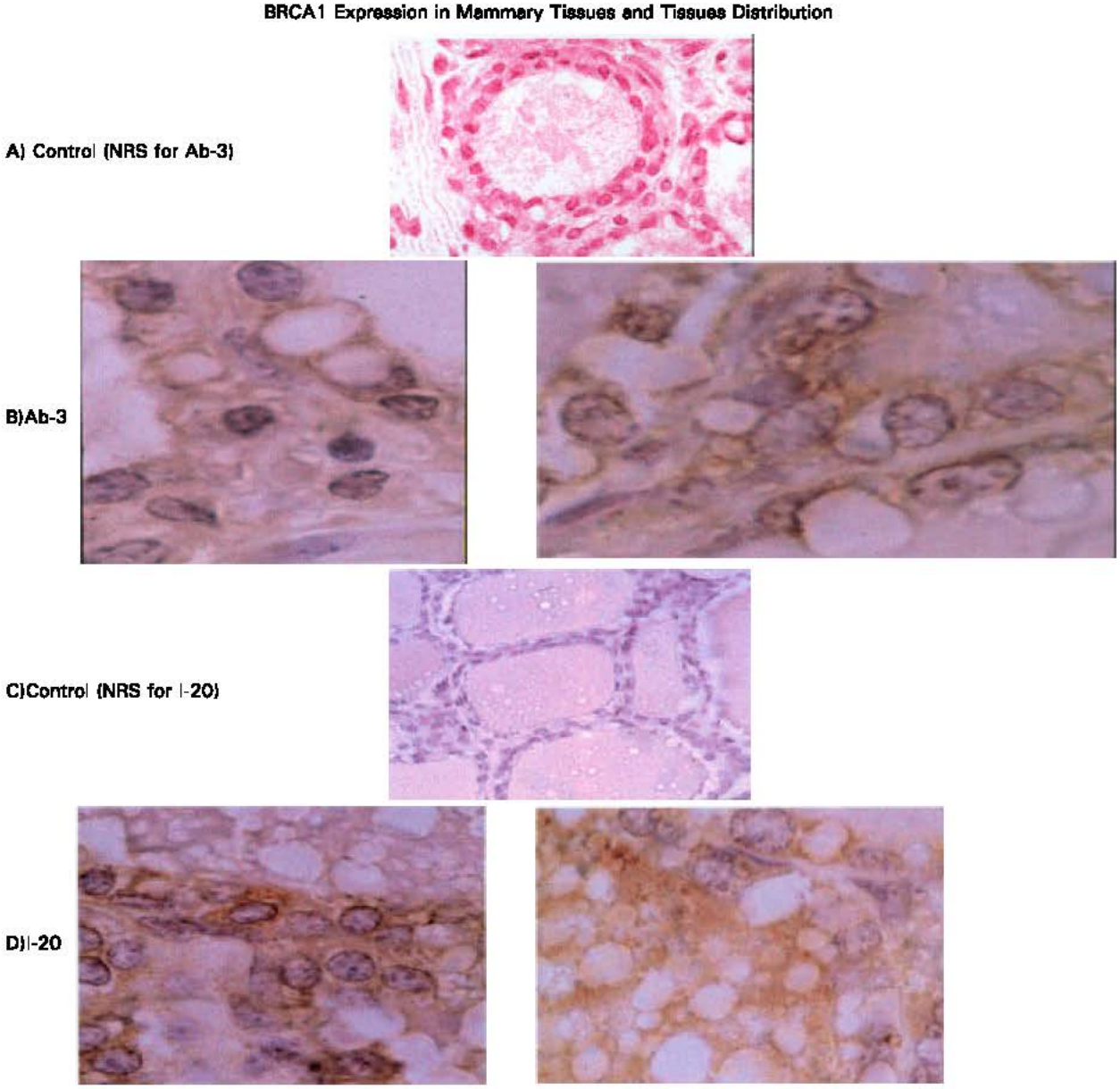


Fig. 7: Localization of BRCA1 in lactating bovine mammary tissue. All slides were processed using the Ab-3 (A, B) and I-20 (C, D) BRCA1 antibodies and immunohistochemistry procedures described in materials and methods. BRCA1 signals were photographed in tissue at 200X (A, C) and at 1,000X (B,D). Note that myoepitheial cells seen with elongated nuclei surrounding alveolar cells are not immunostained.

Murine mammary gland BRCA1 expression varies through all stages of mammary differentiation. It was reported that BRCA1 mRNA expression is very low during lactation in mice (Marquis *et al.*, 1995; Rajan *et al.*, 1996). Bovine BRCA1 mRNA expression is very similar to that of the mouse. BRCA1 protein expression, however, was quite high during lactation compared to other developmental stages. We feel that the elevated levels of BRCA1 protein seen during lacation are a result of regulatory mechanism(s) involved in translation, or RNA stability.

Identification of BRCA1 in mammary tissue, milk fat globule membrane and MCF-7 breast cancer cells

Mammary tissue: We used two antibodies in immunohistochemistry (IHC) to detect immunoreactive bovine BRCA1 in lactating mammary tissues. Both I-20, and Ab-3 antibodies localized BRCA1 in the nucleus, cytosol, and milk fat globule membrane (MFGM) (Fig. 7). The significance of BRCA1 localization in the MFGM shows that it is most likely a secreted protein. This finding supports the hypothesis for BRCA1 localization in the endoplasmic reticulum (ER)/golgi (Jensen *et al.*, 1996).

Our immuno-localization results showed the staining as very intense in epithelial cells, but not in myoepithelial cells, or fibroblasts (Fig. 7). This implies that the signal is cell specific.

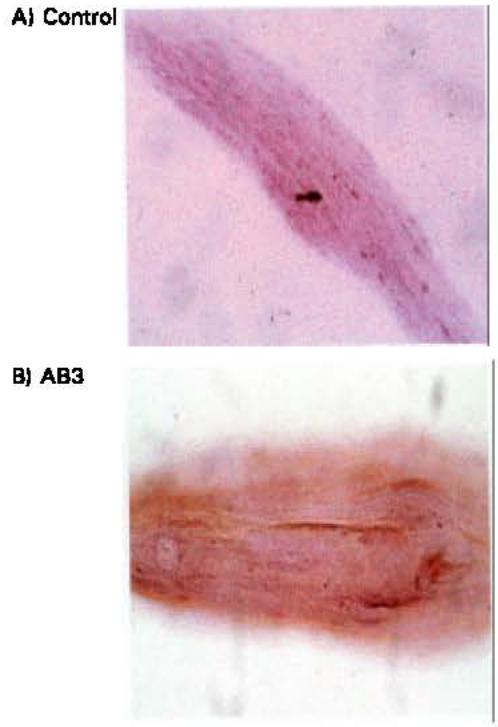


Fig. 8: Identification of BRCA1 in MFGM. All slides were processed using the Ab-3. (A, B) BRCA1 antibody an immunohistochemistry procedures were as described in materials and methods.

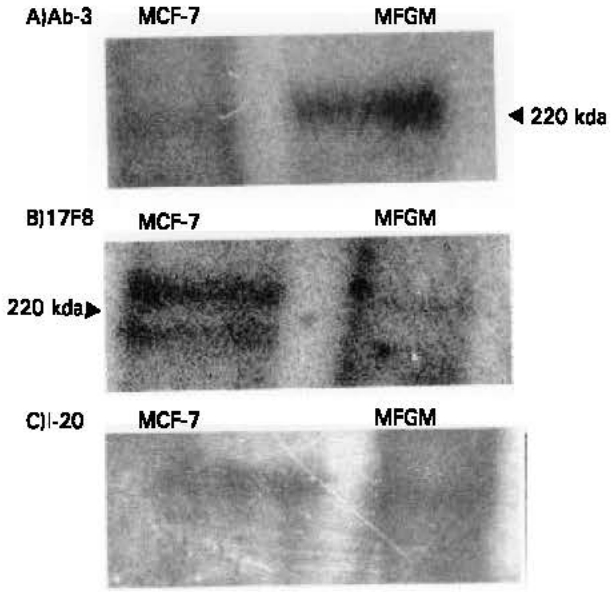


Fig. 9 Western analysis of BRCA1 from MCF-7 cell extracts and MFGM. 100 μg of isolated MCF-7 lysate and 200 μg of MFGM were loaded on 5 % SDS PAGE and then transferred on to a PVDF membrane. Protein was detected using Ab-3 (A), 17F8 (B), and I-20 (C) BRCA1 antibodies

Isolated MFGM: In addition, we observed intense BRCA1 staining in isolated MFGM (Fig. 8). The Ab-3 monoclonal antibody used in immuno-localization studies gives strong support for the secretion of BRCA1 in lactating tissues. It can be arguable that our isolated MFGM might be contaminated from secreted epithelial cells and proteins from dead cells. First, we can not rule out that there was contamination. However, we are sure through our experience that most of cells centrifuged at 3,000 x g for 50 min. This was confirmed under the microscope at the end of the procedure. We do not believe that cell contamination (membrane debris) contributed to our results, because a physiological buffer was used to isolate the MFGM which was isotonic [TBS (pH 7.2)]. We do not believe that isotonic TBS can precipitate free proteins at 100,000 x g for 90 min at 4 C. So, we ruled out the possibility of contamination during MFGM suspension and washing steps. From our data, we believe that bovine BRCA1 is a secreted tumor suppressor protein during lactation. Our next approach is to investigate the molecular mechanism(s) regulating BRCA1 synthesis and secretion.

Identification of BRCA1 in MCF-7 breast cancer cells: We isolated an MCF-7 total cell lysate isolates, as a positive control for our BRCA1 immuno-histochemistry studies since the protein is expressed in human MCF-7 breast cancer cells. We used three different antibodies for Western blot analyses. The three antibodies, I-20, Ab-3, and BRCA1 17F8, developed from exon 11, were used. Our Western blot results showed that all three antibodies, against BRCA1, were detected in the MCF-7 lysate (Fig. 9). This further suggested that BRCA1 was present in bovine tissues and the MFGM and helps confirm our cloning procedures. Even though our results are limited, with regard to unequivocaly proving that BRCA1 is a secreted protein, we speculate that a functional shift of epithelial cells to differentiated secretory alveolar cells allow BRCA1 secretion to occur in response to hormonal influences during lactation.

Bovine BRCA1 cDNA was highly conserved to the human. Five Bhundred base pairs of exon 11 (+3384/ + 3866, human BRCA1 cDNA position) and the C-terminus 1 kb were identical to the human. Seven hundred base pairs of the N-terminus, which contains two ring domains, showed 90 % homology to human BRCA1. The degree of bovine BRCA1 expression from highest to lowest was as follows: liver, spleen, mammary tissues and kidney.

The mammary tissues of early pregnancy heifers (3 months) showed much higher mammary BRCA1 mRNA expression than other mammary developmental stages. BRCA1 expression declined during the remainder of pregnancy and remained low for lactating cows. However, its expression increased when involution proceeded. Immuno-histochemical studies showed that BRCA1 was localized in the nucleus and cytoplasm of mammary epithelial cells from lactating cows. BRCA1 was not found in myoepithelial cells. The protein was also localized in the milk fat globule membranes. Our data suggests that normal bovine mammary tissue contains immuno-reactive BRCA1 protein and that the BRCA1 gene is differentially expressed through various stages of mammary development and involution. The protein appears to be a secreted protein in normal mammary tissue, since immuno-reactive BRCA1 is present in the milk fat globule membrane. Even though our results are limited, with regard to unequivocaly proving that BRCA1 is a secreted protein, we speculate that a functional shift of epithelial cells to differentiated secretory alveolar cells allow BRCA1 secretion to occur in response to hormonal influences during lactation.

Further studies are necessary to determine the role of this protein in bovine mammary gland development and involution.

References

Chen, Y., A.A. Farmer, C.F Chen, D.C. Jones, P.L. Chen and W.H. Lee, 1996. BRCA1 is a 220-kDa nuclear phosphoprotein that is expressed and phosphorylated in a cell cycle-dependent manner [published erratum appears in Cancer Res. 1996 Sep. 1;56(17):4074] Cancer Res., 56:3168-72.

Futreal, P.A., Q. Liu, D. Shattuck-Eidens, C.J. Cochran, K. Harshman, S. Tavtigian, L.M. Bennett, A. Haugen-Strano, J. Swensen, Y. Miki, K. Eddington, M. McClure, C. Frye, J. W Weaver-Feldhaus, Z. Ding, P. Gholami, L. Söderkvist, A. Kamb, and R.W. Wiseman, 1994. BRCA1 mutations in primary breast and ovarian carcinomas. Science, 266:120-122.

Hall J.M., M.K. Lee, B. Newman, J. E. Morrow, L.A. Anderson, B. Huey and M. C. King, 1990. Linkage of early-onset familial breast cancer to chromosome 17q21. Science, 250:1684-1689.

Jensen R.A., M.E. Thompson, T.L. Jetton, C.I. Szabo, R. van der Meer, B.Helou, S.Tronick, M.C. King, D.L. Page and J.T. Holt, 1996. BRCA1 is secreted and exhibits properties of a granin. Nat. Genet., 12: 303-308.

Marquis S.T., J.V. Rajan, B. A. Wynshaw, J. Xu, G.Y. Yin, K.J Abel, B.L.Weber, and L.A. Chodosh, 1995.The developmental pattern of Brca1 expression implies a role in differentiation of the breast and other tissues Nat. Genet., 11: 17-26.

Miki, J. Swensen, D. Shattuck-Eidens, P.A. Futreal, K. Harshman, S. Tavtigian, Q. Liu, C. Cochran, L.M. Bennett, and W. Ding, 1994. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA-1. Science, 266: 66-71.

Paterson J.W., 1998. BRCA1: a review of structure and putative functions. Dis Markers., Feb., 13:261-274.

Rajan J.V., M. Wang, S.T. Marquis and L.A. Chodosh, 1996. BRCA2 is coordinately regulated with BRCA1 during proliferation and differentiation in mammary epithelial cells. Proc. Natl. Acad. Sci. U. S. A., 93:13078-83.

Romagnolo, D. Annab and T.E. Thompson, 1998. Estrogen upregulation of BRCA1 expression with no effect on localization. Mol. Carcinogenesis, 22: 102-109.

Ruffner, H.and I.M.Verma, 1997. BRCA1 is a cell cycle-regulated nuclear phosphoprotein. Proc. Natl. Acad. Sci. USA., 94: 7138-7143.

Sambrook, J., E.F. Fritsch, S. Maniatis and T. Hrsg, 1989. Molecular Cloning - A Laboratory Manual, 2 nd Edition. Cold Spring Habour Laboratory Press, New York.

Scully, R., S. Ganesan, M. Brown, J.A. DeCaprio, S.A. Cannistra, J. Feunteun, S. Schnitt and D.M. Livingston, 1996. Technical Comment: Location of BRCA1 in human breast and ovarian cancer cells. Science , 272:123-125.

Shao N.S., Y.L. Chai, E. Shyam, P. Reddy and V.N. Rao,1996. Induction of apoptosis by the tumor suppressor protein BRCA1. Oncogene, 13:1-7.

Spitsberg, V.L. and R.C. Gorewit, 1997. In Vitro phosphorylated bovine milk fat globule membrane proteins. Nutritional Biochemistry, 8: 181-189.

Pakistan journal of Nutrition 1(1) : 64-66, 2002

Nutrition in Pakistan: Estimating the Economic Demand for Calories

P. J. Dawson
Department of Agricultural Economics and Food Marketing,
The University, Newcastle upon Tyne, UK NE1 7RU
Email: P.J.Dawson@ncl.ac.uk

Abstract: In the last four decades, per capita calorie intake in Pakistan has grown from 1750-2450 (kilo)calories with an average annual growth rate of 0.90%. Nevertheless, 20% of Pakistan's population is still undernourished.This paper has examined the long-run relationship between daily per capita calorie intake and per capita income for Pakistan using cointegration analysis. Using annual data for 1961-1998, there is strong evidence taht such a relationship exists, and that a 1% increase in real per capita income raises the daily per capita calorie intake by 0.19 per cent. Further, causality tests indicate a unidirectional relationship from income to calorie intake; we find no evidence of causality in the opposite direction. This result substantiates Engel's law and provides no support for the hypothesis that income generation is constrained by calorie intake. There are two caveats to the results. First, data limitations restrict the number of observations to 38. Whilst it is not uncommon to find such small samples used in analyses of this type, some caution is necessary in interpreting the results as a consequence of the low power exhibited in some of the tests employed. Second, an aggregation problem arises from the use of national data since we are implicitly adding-up across non-linear relationships at the micro, household level; further, distributional changes in income have not been accounted for in the model. The implications of our results for development policies which seek to alleviate inadequate calorie intake in Pakistan are clear. First, the estimate of the calorie-income elasticity albeit low supports the conventional wisdom that income growth can alleviate inadequate calorie intake. However, nutritional status, measured in terms of nutrient deficiency, may not improve: as income increases, individuals may diversify their diets from a taste perspective as they substitute more expensive sources of calories for less expensive ones. Further substitution may occur by consuming complements to good nutrition, such as clean water, good sanitation or women's time in child care.

Key words: Calories, per capita calorie consumption, nutrition in Pakistan.

Introduction

Substantial increases in per capita calorie consumption in Pakistan have taken place in the last 40 years, rising from 1753 (kilo)calories in 1961 to 2447 in 1998. (FAO, 2000). Notwithstanding this increase, 20% of the total population is still undernourished (United Nations, 2001). During the 1970s, such undernutrition was generally thought to reflect a lack of protein but by 1980, there was broad agreement on its cause: "Serious and extensive nutritional deficiencies occur in virtually all developing countries, though they are worst in low-income countries. They are usually caused by undernourishment - a shortage of food ... Malnutrition is largely a reflection of poverty: people do not have enough income for food. Given the slow income growth that is likely for the poorest people in the foreseeable future, large numbers will remain malnourished for decades to come. ... The most effective long-term policies are those that raise the incomes of the poor..." (World Development Report 1980, p.59).

Engel's law is derived from the relationship between the demand for calories and income: the proportion of income spent on food diminishes as income increases. This law is summarised in the income elasticity of calorie demand, ηM, which is the percentage change in calorie demand brought about by a 1% increase in income. It is expected that $0 < \eta M < 1$ and conventional wisdom (World Bank, 1986, pp.v and 10) is that calorie-income elasticities, while not equal to unity, are assumed to be substantially greater than zero. Much of the empirical literature on nutrition in LDCs has focused the calorie-income relationship but estimated calorie-income elasticities vary considerably. For example, Bouis and Haddad (1992) for the Philippines, and Ravallion (1990) for Indonesia provide estimates that are either close to, and/or insignificantly different from zero, whereas Behrman and Deolalilar (1987) for India and Strauss (1984) for Sierra Leone produce estimates of around 0.82 (Bouis, 1994, summarises this literature).

An alternative approach concerns the 'efficiency wages hypothesis' where income generation is affected by calorie intake. Again results are conflicting: for example, Strauss (1986) finds a significant relationship between farm productivity and calorie intake in Sierra Leone, while Deolaliker (1988) for India finds no evidence that nutrition determines wages (Bliss and Stern, 1978, survey this literature).

It is clear that causality in the calorie-income relationship can run in either (or both) direction(s). Recently, Dawson and Tiffin (1998) examine the long-run calorie-income relationship. Using annual data for India, results show that calorie intake is caused by income and the calorie-income elasticity is 0.34.

The focus here is to estimate the long-run relationship between per capita calorie intake and per capita income using aggregate annual data for Pakistan and to test for the direction of causality between calories and income. The remainder of the paper is organised as follows: Section 2 examines the empirical methodology, Section 3 discusses the data and results, and Section 4 summarises and concludes.

Materials and Methods

The method adopted to investigate the calorie-income relationship using time series data does not require the specification of causality prior to estimation. The vector autoregressive (VAR) model is the basis for this analysis and is expressed as:

$$\begin{bmatrix} C_t \\ M_t \end{bmatrix} = \begin{bmatrix} \mu_1 \\ \mu_2 \end{bmatrix} + \sum_{i=1}^{k} \begin{bmatrix} a_{k,11} & a_{k,21} \\ a_{k,21} & a_{k,22} \end{bmatrix} \begin{bmatrix} C_{t-i} \\ M_{t-i} \end{bmatrix} + \begin{bmatrix} \epsilon_{1t} \\ \epsilon_{2t} \end{bmatrix} \quad (1)$$

where C_t is calories, M_t is income, k is the lag length, μ and A_i are matrices of parameters to be estimated, and ϵ_t are error

Table 1: Unit root tests

Variable	ADF-Test Non-Trended Model	Trended Model	KPSS-Test Non-Trended	Trended Model Model
C_t	-2.26	-4.11	2.57	0.27
M_t	-1.66	-1.36	3.75	0.23
Critical value*	-2.93	-3.50	0.46	0.15

Note * 95% confidence level

Table 2: Trace Statistics

H_o (H_1)	Model 1	Model 2
$r=0 (r=1)$	50.85(15.41)	20.56(15.41)
$r\leq (r=2)$	11.40(3.76)	1.98(3.76)

Note:95 % confidence level in parentheses (Osterwald-Lenum, 1992)

Table 3: Normalised cointegrating vectors

Variable	β	α	β	α
C_t	-1.00	-0.57	-1.00	0.17
M_t	0.17	-0.13	0.19	0
		1.51		

Note: t-statistics in parentheses

terms.

As Harris (1995, pp.14-25) explains, many economic time series are non-stationary and that in general ordinary least squares regressions between non-stationary data are spurious. The presence of unit roots in a time series leads to non-stationarity. In such cases, each series must be first-differenced to make it stationary and it is referred to as integrated, or I(1). Therefore, the first step is to test for the presence of unit roots using the augmented Dickey-Fuller (ADF) test (Dickey and Fuller, 1981, and Said and Dickey, (1984): the null hypothesis is of a unit root while the alternative is stationarity. Often to substantiate conclusions from the ADF-tests, the KPSS-test (Kwiatkowski *et al.*, 1992) is used where the null hypothesis is of stationarity while the alternative is a unit root.

Where integrated series move together and their linear combination is stationary, the series are cointegrated and the problem of spurious regression is absent. Cointegration implies the existence of a meaningful long-run equilibrium (Granger, 1988); the Johansen (1988) procedure tests for cointegration and is based on the estimation of the VAR model in (1) transformed into its vector error correction model (VECM) form:

$$\Delta x_t = \mu + \sum_{i=1}^{k-1} \Gamma_i x_{t-i} + \pi x_{t-k} + \varepsilon_t \quad (2)$$

where $x_t = [C_t, M_t]'$, $\Delta x_t = x_t - x_{t-1}$, μ and Γ_i ($\Gamma_i = -[I - A_1 - \ldots - A_i]$ for $i = 1,\ldots,k-1$) are (2×1) and (2×2) matrices of parameters respectively, π ($\pi = -[I - A_1 - \ldots - A_k]$) is a (2×2) matrix of parameters and ε_t is a (2×1) vector of white noise errors. When the model is subject to unit roots, π is of reduced rank (r) and when $0<r<2$, π can be decomposed into $\pi = \alpha\beta'$. Where $r = 1$, (2) can be rewritten in full as:

$$\begin{bmatrix}\Delta C_t \\ \Delta M_t\end{bmatrix} = \begin{bmatrix}\mu_1 \\ \mu_2\end{bmatrix} + \sum_{i=1}^{k-1} \begin{bmatrix}\Gamma_{i,11} & \Gamma_{i,12} \\ \Gamma_{i,21} & \Gamma_{i,22}\end{bmatrix} \begin{bmatrix}\Delta C_{t-i} \\ \Delta M_{t-i}\end{bmatrix} + \begin{bmatrix}\alpha_1 \\ \alpha_2\end{bmatrix} \begin{bmatrix}\beta_1 & \beta_2\end{bmatrix} \begin{bmatrix}C_{t-k} \\ M_{t-k}\end{bmatrix} + \begin{bmatrix}\varepsilon_{1t} \\ \varepsilon_{2t}\end{bmatrix} \quad (3)$$

The Granger representation theorem (Engle and Granger, 1987) shows that $\beta' x_t$ is stationary implying that x_t is cointegrated with r distinct cointegrating vectors given by the columns of β. Johansen's (1988) procedure estimates (3); trace statistics are used to determine the rank of π which can then be decomposed to give the cointegrating vector, β.

Two possible models are admitted in (4). Model 1 is where there are no linear trends in the levels of the variables and the first-differenced series have a zero mean; here the intercept is restricted to the cointegration space. Model 2 is where there are linear trends in the levels of the variables and there is an intercept in the short-run model only. To test between these models, the Pantula principle (Harris, 1995, p.97) is used to test the joint hypothesis of both rank and the deterministic components.

Fig. 1: Per capita GDP and calorie intake in Pakistan (1961-1968

Equation (3) can be used to test the direction of causality (Granger, 1969) between calories and income. Following Hall and Milne's (1994) definition of (long-run) causality, C_t does not cause M_t if $\alpha_2 = 0$ in (3). Similarly, M_t does not cause C_t if $\alpha_1 = 0$ in (3). Bidirectional causality between M_t and C_t is also possible and is implied by $\alpha_1 \neq 0$ and $\alpha_2 \neq 0$.

Results and Discussion

The annual data relate to Pakistan for 1961-98 and are shown in Figure 1. Calorie intake is average per capita energy (calorie) intake per day, calculated on the basis of per capita dietary energy supply derived from national food balance sheets (source: FAO, 2000). It has trended erratically upwards and has varied between 1753 (kilo)calories in 1961 and 2447 in 1998 with an average annual growth rate of 0.90%. Real per capita GDP (in 1981 constant rupees) is real GDP/population (sources: World Bank, 2001). Real per capita GDP has trended upwards at a relatively constant average annual growth rate of 2.68%, varying between 1823 rupees in 1961 and 4970 rupees in 1996.

Augmented Dickey-Fuller (ADF) tests are used to test for unit roots in the series in logarithms. Lags are added so that the Breusch-Godfrey LM-statistic (Greene, 2000, p.541) rejects serial correlation up to fourth order. Table 1 presents the results of the ADF-tests performed with and without a linear trend. In the non-trended model, unit roots appear in both C_t and M_t; this conclusion is substantiated in the trended model for M_t but not for C_t. The KPSS-tests indicate that both C_t and M_t have unit roots irrespective of whether or not the model contains a trend. Therefore, we conclude on balance that C_t and M_t are I(1), that is, they are stationary after first-differencing. Therefore a cointegrating relationship between them is now sought.

The first step of the Johansen procedure is to select the order of the VAR. The LR-statistic, adjusted for small samples (Sims, 1980), is used to test the null hypothesis that the order of the VAR is k against the alternative that it is four where $k = 0,1,\ldots,4$, and results show that $k = 1$. The Johansen procedure and trace statistics are used to test between Models 1 and 2 and to test for the presence of a cointegrating

vector using the Pantula principle. From the results in Table 2, Model 2 is chosen with one cointegrating vector ($r = 1$).

Using the preferred Model 2, the cointegrating vector for the unrestricted model, normalised on C_t, is shown in Table 3 and implies that $C_t = 1.17GDP_t$. To test the direction of causality between C_t and M_t, the significance of the α-coefficients is tested. Testing $\alpha_1 = 0$, $\chi^2 = 2.01$ (p-value = 0.16) and the null is not rejected; testing $\alpha_2 = 0$, $\chi^2 = 13.70$ (p-value = 0.00) and the null is rejected. Both results are supported by the t-statistics shown in Table 3. Thus, the direction of causality is unidirectional from M_t to C_t, that is, changes in income lead to changes in calorie intake and there is no support for the hypothesis that income generation is determined by calorie intake. Also shown in Table 3 is the normalised cointegrating vector under the restriction that $\alpha_2 = 0$. The implied relationship is: $C_t = 0.19\ M_t$; the income elasticity of calorie demand is 0.19 which implies that a 1% increase in per capita income increases per capita calorie demand by 0.19%; Engel's law is valid in this case.

References

Behrman, J.R. and A.B. Deolalikar, 1987. Will Developing Country Nutrition Improve with Income? A Case Study for Rural India, J. Political Economy, 95: 492-507.

Bliss, C. and N. Stern, 1978. Productivity, Wages and Nutrition: Parts I and II: The Theory, J. Dev. Eco., 5: 331-398.

Bouis, H.E., 1994. The Effects of Income on Demand for Food in Poor Countries: Are our Databases giving Reliable Estimates? J. Dev. Eco., 44: 199-226.

Bouis, H.E. and L.J. Haddad, 1992. Are Estimates of Calorie-Income Elasticities Too High? J. Dev. Eco., 39: 333-64.

Dawson, P.J. and R. Tiffin, 1998. Estimating the Demand for Calories in India, American J. Agricl. Eco., 80: 474-481.

Deolaliker, A.B., 1988. Nutrition and Labor Productivity in Agriculture: Estimates for Rural India, Review of Economics and Statistics, 70: 406-13.

Dickey, D.A. and W.A. Fuller, 1981. Likelihood Ratio Statistics for Autoregressive Time Series with a Unit Root, Econometrica, 49: 1057-72.

Engle, R.F. and C.W.J. Granger, 1987. Cointegration and Error Correction: Representation, Estimation and Testing, Econometrica, 55: 251-76.

Food and Agriculture Organization, 2000. The State of Food and Agriculture. Rome: FAO.

Granger, C.W.J., 1988. Some Recent Developments in a Concept of Causality, J. Econometrics, 39: 199-211.

Greene, W.H., 2000. Econometric Analysis. London: Prentice-Hall, Inc.

Granger, C.W.J., 1969. Investigating Causal Relations by Econometric Methods and Cross-Spectral Methods, Econometrica, 37: 424-436.

Hall, S. G. and A. Milne, 1994. The Relevance of P-Star Analysis to UK Monetary Policy, Economic Journal, 104: 597-604.

Harris, R., 1995. Using Cointegration Analysis in Econometric Modelling, London: Prentice Hall, Harvester Wheatsheaf.

Johansen, S., 1988. Statistical Analysis of Cointegration Vectors, J. Economic Dynamics and Control, 12: 231-54.

Kwiatkowski, D., P.C.B. Phillips, P. Schmidt and Y. Shin, 1992. Testing the Null Hypothesis of Stationarity against the Alternative of a Unit Root, J. Econometrics, 54: 159-178.

Osterwald-Lenum, M., 1992. A Note with Quantiles of the Asymptotic Distribution of the Maximum Likelihood Cointegration Rank Test Statistics, Oxford Bulletin of Economics and Statistics, 54: 461-72.

Ravallion, M., 1990. Income Effects on Undernutrition, Economic Development and Cultural Change, 38: 489-515.

Said, S.E. and D.A. Dickey, 1984. Testing for Unit Roots in Autoregressive-Moving Average Models of Unknown Order, Biometrika, 71: 599-607.

Sims, C.A., 1980. Macroeconomics and Reality, Econometrica, 48: 1-49.

Strauss, J., 1984. Joint Determination of Food Consumption and Production in Rural Sierra Leone: Estimates of a Household-Firm Model, J. Dev. Eco.nomics, 14: 77-103.

Strauss, J., 1986. Does Better Nutrition Raise Farm Productivity? J. Political Economy: 94: 297-320.

United Nations, 2001. Human Development Report. http://www.undp.org/hdr2001/

World Bank, 2001. World Tables 1998, World Bank: Washington DC.

World Bank, 1980. World Development Report. Washington DC .

World Bank, 1986. Poverty and Hunger-Issues and Options for Food Security in Developing Countries.

Pakistan Journal of Nutrition 1(1) : 67-68, 2002

Journal Selections; Let's Support our Students' Futures

Susan L. Meacham[1*], Lesley J. Johnson[2] and Eva D. Stowers[3]
[1*]Department of Nutrition Sciences, [2]Department of Food and Beverage Mgt.
[3]Lied Library, University of Nevada Las Vegas,
4505 SO. Maryland Parkway Box 453026
Las Vegas, NV 89154-3026, USA
smeacham@ccmail.nevada.edu

Dietetic educators concur that use of professional journals in the undergraduate curriculum promotes student reading skills (McCabe *et al.*,1995), exposes students to current research, enhances computer skills (Evers,1996; Parks,1994; Raidl *et al.*,1995; Carew *et al.*,1997; Merkel *et al.*, 1998; Kipp ,1996) and prepares dietetic students for the real world environment. Those of us in educational institutions are continually asked to review our university library holdings; prioritizing on the basis of department selections, cost, rate of inflation, use by faculty and students and availability through interlibrary loans and other document retrieval procedures. No doubt, those in industry and clinical and private practice are also watching their budgets and are asked to review expenses for professional publications.

As 69% of American Dietetic Association (ADA) members regularly read the Journal of the American Dietetic Association (JADA), a logical place to begin journal prioritizing is to review the journal's "New in Review" department (Anonymous, 2000). "New in Review" appears monthly in JADA and lists article abstracts and titles of interest to members from other professional journals. In an attempt to develop a priority list for our university library's journal holdings for students studying nutrition at the University of Nevada Las Vegas, we tabulated the journals cited by JADA in "New in Review" over the past 5 years. Over 4,500 article abstracts and titles were cited from 53 different professional journals from January 1996 to December 2000. The journals were categorized into topic areas: (Table 1): clinical (31), dietetic association publications (4), education (2), foods (6), health promotion (4), and management (6).

Table 1: Topic areas represented (%) by professional journal article abstracts and titles from 1996-2000 in the Journal of the American Dietetic Association "New in Review"

Topic Area	1996	1997	1998	1999	2000	% Total
Clinical	79%	78%	81%	86%	86%	82%
Assoc. Publ.	1	1	1	0	2	1
Education	3	4	3	3	4	3
Foods	7	6	7	5	3	6
Health	6	5	4	4	3	4
Management	4	6	4	2	2	4

The top ten periodicals over the 5 year period, ranked according to the most article abstracts and titles referenced, all represented clinical practice.

American Journal of Clinical Nutrition	(25%)
European Journal of Clinical Nutrition	(6%)
American Journal of Epidemology	(6%)
Journal of Nutrition	(5%)
Diabetes Care	(4%)
Journal of the American College of Nutrition	(3%)
International Journal of Obesity Research and Metabolic Disorders	(3%)
Journal of the American Medical Association	(3%)
Archives of International Medicine	(3%)
Medicine and Science in Sports and Exercise	(3%)

The distribution of referenced articles and the selected journals was fairly consistent throughout the five-year period with clinical articles representing 78-86% of all articles cited. The journals representing other topic areas were typically:

Dietetic Association Publications-Journals of the Canadian, Australian, and New Zealand Dietetic Associations

Education-Journal of Nutrition Education and Diabetes Educator

Foods-Food Technology, Journal of Food Protection, Journal of Food Composition and Analysis, Journal of Food Science

Health Promotion-American Journal of Public Health, American Journal of Health Promotion, Journal of Women's Health and Public Health Reports

Management-Food Management, Harvard Business Review, Health Care Manager, Hospital and Health Networks, School Food Service Research Review and Training Development.

According to the most recent survey of the ADA membership the primary positions held by registered dietitians in 1997 were as follows: clinical nutrition, 47.8%, food and nutrition management, 17.6%, community nutrition, 14.9%, consultation and business 11.7%, and education and research, 8.0%. In order to adequately serve the profession our educational programs' library holdings should parallel the employment trends for registered dietitians (Bryk, 1997). This indicates the need for dietetic educators to broaden the scope of professional readings offered to dietetic students to coincide with current employment.

Expanding our reading beyond the clinical area can also better prepare dietetic students for the increasing demand for multi-skilled health care providers. As we promote the dietitian's active participation in nontraditional roles on health care teams, continued exposure to the readings of other health professionals should also be available.

This brief analysis may raise more questions than it answers. For institutions beginning new educational programs, where do we begin when we ask our libraries to support our dietetics programs? Do the journals holdings reflect the needs of the membership? Offer support for entry-level dietitians? Do they broaden our scope of practice allowing for diverse employment opportunities? Are there other mechanisms by which we can offer publications for educational programs and professional development for registered dietitians? Journal selections should coincide with current and future job opportunities. Periodic review, therefore, is needed to maintain relevant journals for professional development and dietetic student education.

References

Bryk, J.A. and T.K. Soto,1999. Report on the 1997 membership database of the American Dietetic Association J. Am. Diet. Assoc., 99:102-107.

Carew, L.B., V.M. Chamberlain and F.A. Alster, 1997. Evaluation of a computer-assisted instructional component in a college level nutrition course. J. Nutr. Ed., 29:327-334.

Evers, W.D., 1996. Communications and technology in the profession. J. Am. Diet. Assoc., 96:756-757.

Kipp, D.E., 1996. Reflections on teaching graduate students how to use and publish information on the world wide web. J. Nutr. Educ., 28:7-13.

McCabe, B.J., S.D. Koury, J.W. Tysinger, M.T. Hynak-Hankinson and S. Foley, 1995. Reading skills of dietetic interns and readability of dietetics literature. J. Am. Diet. Assoc., 95:874-878.

Member Needs Assessment/Satisfaction Study Review Group, 2000. Report on the American Dietetic Association's Member Needs Assessment/Satisfaction Study. J. Am. Diet. Assoc., 100:112-116.

Merkel, J.M. and K.L. Dittus,1998. Cybernutrition on line: Educating nutrition students and the public in cyberspace. J. Nutr. Ed., 30:66A.

Parks, S., 1994. Challenging the future: Impact of information technology on dietetics practice, education and research. J. Am. Diet. Assoc., 94:202-204.

Raidl, M.A., O.B. Wood, J.D. Lehman and W.D. Evers, 1995. Computer-assisted instruction improves clinical reasoning skills of dietetic students. J. Am. Diet. Assoc., 95:868-873.