

Role of Selenium in Alteration of Erythrocyte Parameters in Bovine Fluorosis*

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ABSTRACT : Signs of dental discolouration, difficulty in mastication, bony exostosis and debility were observed in cattle from Qingtongxia Ningxia, China where fluoride concentration in drinking water, soil, fodder, serum, bone, teeth, haircoat and urine were significantly higher than the corresponding health site. The problem of fluorosis in beef cattle is attributable to water containing toxic levels of fluoride. The objective of this paper was therefore to evaluate the influence of fluoride on erythrocyte parameters in cattle under high fluoride and low selenium conditions, as well as the protective efficacy of selenium exposure in feedstuff for bovine endemic fluorosis. Sixteen 6 to 7 year-old high fluoride beef cattle were randomly allotted into two groups each with eight cows: high fluoride control group, and supplemented with 0.25 mg/kg selenium per day for 83 days respectively. In addition, eight 6 to 7 year-old normal control beef cattle were selected from a non-high fluoride site. Blood samples were collected on day 0, 30 and 83 for erythrocyte parameters analysis and scanning electronic microscopy. The results indicated that erythrocytes, hemoglobin, packed cell volume values and Na⁺-K⁺ ATPase activity from affected cattle on the high fluoride site were significantly reduced during the period as compared with the corresponding samples of normal control cattle, a great number of echinocytes were present in peripheral blood, and subsequent anaemia. However, affected cattle exposed to selenium revealed increasable erythrocyte parameters, the extent of elevation in these values being dependent on the duration of supplementation with selenium. These findings suggest that fluoride exposure can cause erythrocyte damage, whereas selenium supplementation can antagonize fluoride-induced generation of free radicals and cumulative effects of lipid peroxidation in erythrocytes. Selenium supplementation may help to alleviate the possible hazards associated with bovine endemic fluorosis. (*Asian-Aust. J. Anim. Sci.* 2006. Vol 19, No. 6 : 865-871)

Key Words : Cattle, Erythrocyte Parameters, Endemic Fluorosis, Selenium

INTRODUCTION

Fluorosis, characterized by chronic fluoride intoxication, is a worldwide health problem and is endemic in areas where the fluoride content of drinking water and fodder are high (Radostitis et al., 2000). Chronic ingestion of fluoride-rich fodder and water in endemic areas leads to development of fluorosis in animals. The disease is mainly characterized by clinical signs referable to the musculoskeletal system. They include discolouration, softening of teeth, difficulty in mastication, bony exostosis, lameness, painful gait and arthritis (Shupe et al., 1972; Patra et al., 2000). Besides, high fluoride concentration in the body also affects other systems leading to anaemia, debility, reduced production and incremental mortality (Radostitis et al., 2000). Fluorosis is endemic in many provinces in China with except of Shanghai (Wei et al., 2000). The condition continues to be difficult to prevent or correct. The major sources of fluoride ingestion are

drinking water, air pollution and additives in feedstuff. Dental fluorosis, skeletal fluorosis and non-skeletal fluorosis, from which the effect on hematology has been largely concentrated, arise from chronic fluorine intake. Spur-shaped erythrocytes were observed from rabbits with experimental or water-induced fluorosis through scanning electronic microscope (SEM) (Susheela et al., 1983). Yur et al. (2003) observed in sheep that fluoride-induced oxidative stress was reflected in elevated erythrocyte malonaldehyde levels that cause decreased enzyme activity of Na⁺-K⁺ ATPase and G6PD, which affect erythrocyte membrane structure. Bober et al. (2001) also found in humans that fluoride caused a decrease in Na⁺-H⁺ exchanger activity and an increase in intracellular H⁺ ion concentration. Mehdi et al. (1978) suggested from mouse studies that hemoglobin, packed cell volume and mean corpuscular hemoglobin concentration values could serve to detect preclinical effects of high fluoride intake with an added dose of as low as 125 mg/kg, or even less, for a period of four weeks or probably earlier. Han et al. (2002, 2004) reported from cattle studies that fluoride caused a decreased erythrocyte activity of superoxide dismutase, glutathione peroxidase and catalase and an increased content of free radicals and malonaldehyde during long-term intake of an overdose of fluoride. Actually, fluoride induces the generation of reactive oxygen species and affects lipid peroxidation accompanied by a decline in activities of some antioxidant enzymes (Inkielewicz and Krechniak, 2004). Selenium can antagonize fluoride-induced apoptosis, DNA damage, and lipid peroxidation in

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hepatocytes (Güven and Kaya, 2005). Laboratory and epidemiological investigations indicate that selenium can partially antagonize fluoride-induced lipid peroxidation. However, few reports have considered the erythrocyte ultrastructure changes in cattle with fluorosis, as well as the protection against oxygen radical damage involving the Se-dependent glutathione peroxidase, thioredoxin, reductases and possibly other selenoproteins containing Se in the form of selenocysteine. The present study was therefore undertaken to evaluate the influence of fluoride on erythrocyte parameters in cattle under high fluoride and low selenium conditions, as well as the protective efficacy of selenium exposure in feedstuff for bovine endemic fluorosis.

MATERIALS AND METHODS

Study site and sampling

The high fluoride study site is located in Guangwu county 20 km south of Qingtongxia city in Ningxia at longitude 106.2 E and latitude 38.0 N. However, the non-high fluoride site is located near Yongning city in Ningxia at longitude 106.5 E and latitude 38.4 N, and served as the normal control. Biological samples (blood, haircoat, teeth, bone and urine) were collected from cattle within the two sites. Representative fodder and soil samples were collected in polythene bags for analysis of fluoride content. Water samples from ponds were also collected for analysis of fluoride.

Animals

Sixteen 6 to 7 year-old Qin Chuan beef cattle weighing 300 to 370 kg, which were raised by farmers in Guangwu county of Qingtongxia city in Ningxia China, were selected from a natural high fluoride site, and clinically examined for fluorosis, namely dental lesions, bony exostosis on mandibles and lameness. They were randomly divided into two groups each with eight cows and fed with local high fluoride feedstuff. The groups were as follows: (1) high fluoride control group, (2) supplemented with 0.25 mg/kg selenium (sodium selenite) per day for 83 days, respectively. Each group was offered local high-fluoride water and feedstuff. In addition, eight 6 to 7 year-old non-high fluoride beef cattle selected from Yongning city in Ningxia, China, were offered water and feedstuff produced in the non-high fluoride site. The fluoride contents at both sites of bovine internal and external samples were measured, and whole blood was taken for baseline values including erythrocyte counts, hemoglobin, hematocrit and SEM. Thereafter, the selenium additive was given to the treatment group daily for 83 days, and the blood from each group was also tested for corresponding parameters on day 30 and day 83 respectively.

Collection of blood and urine samples

Blood samples were collected into 4 portions by jugular venepuncture. The first 5 ml portion from each animal was collected into a 10 ml vacuum blood collection tubes containing heparin and centrifugated immediately at 3,000 rpm for 10 minutes. The red blood cells were harvested and washed 3 times with equal volumes of isotonic saline solution. Red blood cells were lysed with 50 ml 10 mM Tris-HCl buffer and centrifugated at 12,000 rpm for 20 minutes, then washed 3 times with equal volumes of 50 ml 10 mM Tris-HCl buffer and the pellet suspended with 10 mM Tris-HCl buffer. The concentration was adjusted within 1 to 2 mg/ml and the suspension was stored at -20°C until assayed for Na⁺-K⁺ ATPase. The second 7 ml portion of blood from each animal was collected into a 10 ml vacuum blood collection tube,, stored at room temperature for several hours, and the serum was harvested after clotting of blood for fluoride analysis. The third 5 ml portion of blood from each animal was collected into 10 ml vacuum blood collection tubes containing EDTA, and stored at room temperature for measurement of erythrocyte parameters and selenium analysis. A fourth 2 ml portion from each animal was collected into 5 ml 2% glutaraldehyde tubes and stored at 4°C for SEM analysis. Urine samples, collected in plastic bottles, were transported to the laboratory for fluoride analysis. Likewise, both blood and urine samples were collected from eight healthy beef cattle maintained in a dairy farm at Yongning city.

Analysis of fluoride, selenium and erythrocyte parameters

Fodder samples were processed by the ISI method (1983). The fluoride concentration in serum, urine and water samples was measured in mg/L by ion specific potentiometry using Total Ionic Strength Adjustment Buffer (TISAB)(pH5.5). The buffer was prepared by dissolving 37 g KCl, 68 g sodium acetate and 36 g EDTA in H₂O, adjusting to pH 5.5±0.2 with 1:1 HCl and diluting to 1 L. The method of Cernik et al. (1970) was followed with modifications using a portable fluoride ion specific electrode (Orion model 96-09) and ion specific electrode meter Orion. Model-290A. The detection range of the instrument was between 0.019 and 1,900 mg/L. Calibration of the instrument was made using five freshly prepared working standards. The accuracy and precision of the measurement were maintained by repeated analysis of the reference standard procured from Orion Research Incorporated Laboratory, USA. Selenium was measured by Shimadzu RF-540 (Japan) spectrofluorophotometer. Erythrocyte, hemoglobin and hematocrit were assayed by hematology analyzers (Sysmex F800, USA).

Na⁺-K⁺ ATPase activity in erythrocytes was measured as the release of inorganic phosphate from hydrolysis of ATP

Table 1. Fluoride and selenium contents in drinking water, soil and feedstuff (mg/kg)

Groups	Water		Soil		Fodder	
	Fluoride	Selenium	Fluoride	Selenium	Fluoride	Selenium
High F site	3.27±0.23** (n = 6)	0.0	8.0±0.87** (n = 6)	0.108±0.08* (n = 5)	33.9±1.46 (n = 6)	0.072±0.058 (n = 6)
Healthy site	0.58±0.21 (n = 5)	0.0	3.75±1.77 (n = 3)	0.194±0.02 (n = 2)	23.5±5.67 (n = 6)	0.087±0.073 (n = 5)

* Compared with healthy site $p < 0.05$, ** Compared with healthy site $p < 0.01$.

Table 2. The fluoride content of serum, bone, teeth, haircoat, urine and blood selenium in beef cattle (mg/kg)

Groups	Serum fluoride	Bone fluoride	Teeth fluoride	Haircoat fluoride	Urine fluoride	Blood selenium
High F cattle	0.62±0.12** (n = 8)	8,854.66±1,723.98 (n = 5)	9,393.46±48.99 (n = 5)	30.12±13.07 (n = 16)	21.59±4.23 (n = 16)	0.064±0.048** (n = 7)
Healthy beef cattle	0.24±0.01 (n = 8)	300-1,200 (n = 2)	240-625 (n = 2)	16-20	7.65±2.72 (n = 8)	0.114±0.056 (n = 6)

** Compared with healthy beef cattle $p < 0.01$.

(adenosine triphosphate) in the presence and absence of ouabain. Erythrocytes were incubated at 37°C for 60 min in 1 ml of a solution containing 3 mM ATP (pH 7.0), 50 mM NaCl, 20 mM KCl, 3 mM MgCl₂, and 100 mM tris-HCl buffer (pH 7.4). To inhibit Na⁺-K⁺ ATPase activity, 1 mM ouabain was added. The reaction was stopped by the addition of trichloroacetic acid. ATPase activity was expressed as nanomoles of phosphorus released per mg protein/h (Serpensu et al., 1978; Bildik et al., 2002).

Scanning electron microscopy (SEM)

The samples were fixed in 2% glutaraldehyde for 1 h. After rinsing with the buffer, the samples were post-fixed with 2% osmium tetroxide (OsO₄) for 50 min and rinsed with 60% ethanol. The rinsed samples were then dehydrated in a graded series of ethanol (60, 70, 80, 90, 95, 99 and 100%) for 10 min each and finally replaced with tert-butyl alcohol. The prepared samples were dried in a freeze dryer, mounted on specimen holders, sputter-coated with gold and examined on a SEM KYKY AMARY-1000B (USA) operating at up to 15 kV.

Statistical analysis

The results were presented as the least squares mean value±standard deviation and one-way analysis of variance was performed using the General Linear Models Procedures of the SAS software (2000). Differences between means were tested using Duncan's multiple range tests. A significance level of 0.05 was used.

RESULTS

Clinical signs

At the beginning of the experiment on day 0 (in spring), the commonly observed signs of high-fluoride affected cattle were apparent including poor condition leading to emaciation, light-white teeth plagues with longitude fissures (mottling) and asymmetrical incisors. Bony exostosis on the

mandibles were noticed, and osteoproliferation fistula took a long time to recover. The affected cattle also presented with partially discolored rough haircoat and pronounced swelling of carpal and tarsal joints. At experimental day 50, the affected cattle showed debility and unthriftiness. At day 60, they still showed emaciation, weakness and systemic-covered old haircoat which persisted until the end of the experiment. However, selenium supplemented animals presented in better condition compared with the high fluoride control cattle, which lost old haircoat at day 30. At day 44, increased growth was observed in the group with good temperament, as well as bright fresh haircoat. At day 60, the selenium supplemented animals showed increased food intake, better temperament and body condition. At day 83 (in summer), better growth rate was observed in the supplemented group than in the high-fluoride affected animals. No emaciation, weakness and systemic-covered old haircoat were found in selenium supplemented animals before the end of the experiment, but light-white teeth plagues with longitude fissures (mottling) and asymmetrical incisors were unaffected.

Investigation of fluoride and selenium status between the internal and external environment

Average fluoride contents in the pond water, surface soil and fodder from the high fluoride site (see Table 1) were significantly higher than the corresponding healthy site. Although the fluoride content of the fodder was less than the tolerance limit of 40 mg/kg, in our field experiment we observed that the mean fluoride concentration of drinking water was 3.27 mg/L. Fluoride levels in water, consumed by the animals were much higher than the recommended permissible limit (1.0 mg/L). In view of the good agreement between laboratory and field measurement, it appears that the total intake of fluoride through contaminated water surely contributed to the development of fluorosis in the livestock. Furthermore the mean selenium level in these samples from the high fluoride site was similar to the

Table 3. Red blood cell counts ($\times 10^6/\mu\text{l}$), the haemoglobin contents (g/100 ml), the packed cell volume values (%), the changes of Na-K-ATPase activities of erythrocyte member in beef cattle ($\mu\text{mol Pi/mgprot/h}$)

Items	Groups	Day 0	Day 30	Day 83
Red blood cell counts	Normal control beef cattle	6.77 \pm 0.53	6.72 \pm 0.70	6.71 \pm 1.30
	High F control beef cattle	5.98 \pm 1.18**	5.84 \pm 0.99*	5.92 \pm 1.08*
	High F supplement Se beef cattle	6.12 \pm 1.06	6.18 \pm 1.28	6.69 \pm 1.35
Haemoglobin contents	Normal control beef cattle	11.10 \pm 1.12	11.03 \pm 1.31	10.96 \pm 0.93
	High F control beef cattle	8.58 \pm 2.03**	9.26 \pm 1.49*	8.60 \pm 1.71 **
	High F supplement Se beef cattle	9.98 \pm 1.22	10.55 \pm 1.56	10.41 \pm 1.38*
Packed cell volume values	Normal control beef cattle	32.25 \pm 3.56	32.13 \pm 2.23	32.14 \pm 3.98
	High F control beef cattle	26.88 \pm 3.31**	26.00 \pm 6.85 **	26.63 \pm 6.55 **
	High F supplement Se beef cattle	27.50 \pm 3.51*	28.38 \pm 2.62*	29.63 \pm 7.13
Na-K-ATPase activities of erythrocyte member	Normal control beef cattle	0.246 \pm 0.079	0.263 \pm 0.051	0.307 \pm 0.124
	High F control beef cattle	0.178 \pm 0.050	0.153 \pm 0.015*	0.214 \pm 0.073**
	High F supplement Se beef cattle	0.162 \pm 0.032*	0.183 \pm 0.083	0.292 \pm 0.101*

n = 8, * Compared with normal control group at the same time $p < 0.05$, ** Compared with normal control group at the same time $p < 0.01$.

Table 4. The percentage of echinocytes in bovine fluorosis through SEM (%)

Groups	Day 0	Day 83
High F control beef cattle	71.65 \pm 2.29 (n = 3)	75.48 \pm 8.77 (n = 6)
High F supplement Se beef cattle	73.65 \pm 2.29 (n = 3)	15.21 \pm 1.30** ^{aa} (n = 6)

** Compared with the same group at different time, $p < 0.01$; ^{aa} compared with the high F control group at same time, $p < 0.01$.

corresponding samples from the healthy site. Mean fluoride concentrations at the high fluoride site in serum, bone, teeth, haircoat, and urine samples (see Table 2) were significantly higher than the healthy site values for normal healthy beef cattle. However, selenium levels in cattle from the high fluoride site were statistically lower than the corresponding samples of health site ($p < 0.01$). Based on the clinical signs and fluoride content in water, soil, fodder, serum, bone, teeth, haircoat and urine, it was concluded that the problem of fluorosis in beef cattle is attributable to water containing toxic levels of fluoride.

Erythrocyte parameter alteration in bovine fluorosis

Erythrocyte counts, blood hemoglobin content and the packed cell volume are shown in Table 3; the values of the three parameters from high fluoride cattle on high fluoride site were significantly reduced during the experimental period compared with the corresponding samples from normal cattle. Together these values result in affected animals suffering anaemia. However, the affected animals supplemented with selenium revealed increased erythrocyte parameters and the extent of elevation in these values was, generally, dependent on the duration of dietary selenium supplementation. There was no deviation in these values from those in normal cattle after selenium supplementation for 83 days in high-fluoride cattle.

Na⁺-K⁺ ATPase activities recorded in Table 3 from high fluoride cattle on day 0, day 30 and day 83 were lower than

the corresponding values of Na⁺-K⁺ ATPase activity of normal cattle. However, Na⁺-K⁺ ATPase activities of selenium supplemented high-fluoride animals on day 0, day 30 and day 83 were significantly higher than the corresponding values of high fluoride control beef cattle, while still lower than the corresponding Na⁺-K⁺ ATPase activities of normal cattle. The results indicated that selenium exposure causes elevation of Na⁺-K⁺ ATPase activities and also results in improved erythrocyte parameters.

Morphological observation of erythrocytes in bovine fluorosis through SEM

Erythrocytes from the normal control group were bright smooth biconcave discs with a thick brim and a thin centre, and few had a deformed shape (Figure 1a). At day 0, deformed erythrocytes from high fluoride control cattle had short, bulky and spur-shaped processes, which had blunt tops under moderate state and sharp tops under heavy state numbering 3 to 5 or 7 to 8, and resulted in polygonal or scantha-shaped erythrocytes, these kind of erythrocytes had the shape of an amoeba with pseudopodia like folds projecting in different directions. Such erythrocytes were termed echinocytes (Figure 1b and 1c). All of the erythrocytes with a spur shape were observed in high fluoride control cattle through SEM. At day 83, the majority of erythrocytes in selenium supplemented cattle trended toward normal shape, and spur-shaped erythrocytes were decreased (Figure 1d). Statistical results of morphological observation at day 0 and 83 are presented at Table 4.

DISCUSSION

The clinical signs revealed a significant poor condition, hypoplasia, debility and lower selenium within two sites. Dental lesions such as mottling, brownish discolouration and deformity of the teeth leading to painful mastication

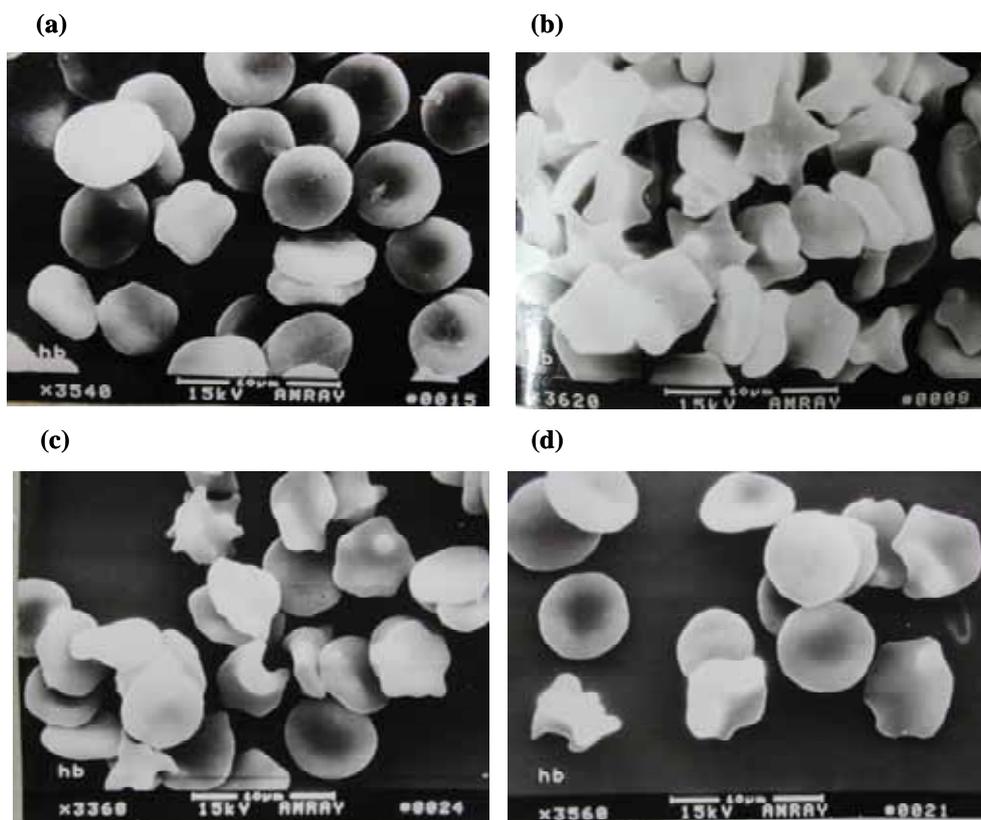


Figure 1. (a) Erythrocytes of normal control cattle, which were bright smooth biconcave discs with thick brim and thin center, observed through SEM with few deformed shape. (b) Erythrocytes of high fluoride control cattle viewed by SEM on day 0, deformed erythrocytes had short, bulky and spur-shaped processes, which had blunt tops under moderate state and sharp tops under heavy state numbering 3 to 5 or 7 to 8, and resulted in polygonal or scantha-shaped erythrocytes; these kind of erythrocytes had the shape of an amoeba with pseudopodia like folds projecting in different directions. Such erythrocytes were termed echinocytes. (c) Erythrocytes of high fluoride control cattle viewed by SEM on day 83. (d) Erythrocytes of high fluoride cattle supplemented with 0.25 mg/kg selenium per day for 83 days, the majority of erythrocytes in selenium exposed cattle trended toward normal shape, and there were no obvious spur-shaped cells.

were invariably recorded in all the fluorotic cattle, that is why the affected animals maintained a rough hair coat and debility. Swelling carpal and tarsal joints were very prevalent, although few bony exostosis on the mandibles were noticed in cattle. It was therefore confirmed that dental lesions were the main external sign of fluoride intoxication in cattle. These results confirmed previous reports that the diagnosis was based on the clinical signs and analysis of the fluoride levels in bone, urine and serum in cattle. High concentrations of fluoride in bone and urine were suggested as evidence of fluoride toxicity (Jubb et al., 1993). The diagnosis of chronic fluoride toxicosis is consistent with the high concentrations of fluoride found in the dental lesions, bone, fodder and water samples of the animals. A level of approximately 2 mg/L fluoride in drinking water or 40 mg/kg in forage has been shown to cause chronic dental fluorosis in livestock (McDowell, 1992; Loganathan et al., 2001; Cronin et al., 2002), and as bone fluorine concentrations approach 6,000 mg/kg, clinical signs of osteofluorosis appear. The consumption of fodder and water

was responsible for the development of chronic fluorosis in the cattle studied here. Actually, cattle are more susceptible to chronic fluorosis probably due to their higher intake of water (Sahoo et al., 1998).

This study clearly indicated that values of the three parameters including haemoglobin, red blood cell and packed cell volume were significantly lowered during the experimental period in high-fluoride control cattle as compared with the normal control and selenium supplemented cattle. However, appearance of the symptoms in selenium exposed cattle did change the trend in the above values. In accordance with our findings, Susheela et al. (1983) and Mehdi et al. (1978) reported on the influence of erythrocyte parameters and showed similar decremental changes in rabbit and mouse. Being a cumulative poison, fluoride induces many metabolic disturbances in different organs, blood being the primary target (Han et al., 2004), with bone and teeth being the second deposition site (McDowell, 1992). Therefore, anaemia, emaciation, loss of production, and infertility observed could be attributable to

the polysystematic effect of chronic fluoride toxicity (Han et al., 2004).

As the erythrocyte membrane is an important structural entity which contains the chemical factors responsible for blood group substances, considerable work on membrane structure and function has been carried out. It is now known that when fluoride is ingested, it will also accumulate on erythrocyte membrane, besides other cells, tissues and organs. The erythrocyte membrane becomes pliable and is thrown into folds. The erythrocytes attain the shape of an amoeba with pseudopodia-like folds projecting in different directions. Such erythrocytes are termed as echinocytes. The echinocytes will be found in large numbers, depending upon the extent of fluoride poisoning and duration of exposure to fluoride. The echinocytes undergo phagocytosis and are likely to be eliminated from the circulation. This would lead to low haemoglobin levels in animals chronically ill due to fluoride toxicity.

Generation of free radicals, lipid peroxidation, and altered antioxidant defense systems are considered to play an important role in the toxic effects of fluoride (Shivarajashankara et al., 2002). The increase in lipid peroxidation causes damage to cell membranes, and the products of lipid peroxidation can easily penetrate cell membranes by simple diffusion and directly attack DNA, leading to apoptosis (Wang et al., 2004). Glutathione peroxidase, thioredoxin, reductases, which are the main antioxidant, are Se-dependent, so Se supplementation improves enzyme activity and elimination of fluoride-induced reactive oxygens (Han et al., 2004). A number of studies indicate that fluoride induces the generation of reactive oxygen species and affects lipid peroxidation accompanied by a decline in activities of some antioxidant enzymes. A decreased glutathione peroxidase activity and an increased production of thiobarbituric acid reactive substances were reported in rats exposed to 12 mg F⁻/L in drinking water over 15 days. Guan et al. (2000) reported a decrease in glutathione peroxidase activity and GSH level in erythrocytes and an increase in lipid peroxidation in serum of rats given 10 or 30 mg F⁻/L in drinking water for 8 months. Shivarajashankara et al. (2002) found higher levels of GSH and MDA as well as lower activity of superoxide dismutase in plasma of rats receiving 100 mg F⁻/L in drinking water during a 4 month period. The concentration of GSH and MDA and the activity of glutathione S-transferase were also elevated in brain and liver (Inkielewicz and Krechniak, 2004). ATPase is one kind of enzyme in erythrocyte membrane. Once overdose of fluorine deposition occurs in erythrocytes, the membrane cholesterol increases, and fluidity of membrane lipid is decreased, transportation of the Na⁺ and Ca²⁺ pump is blocked and, furthermore, endocytic ion concentration is changed. Endomembrane-dispersed heme and actin

connects into the network through the action of disulfide cross-bridges, and discocytes are changed into spur-shaped cells. Selenium activates Na-K-ATPase activity, and therefore can protect S-H bonds of membrane protein, avoid formation of disulfide cross-bridges and reduce connection into the network by heme and actin, thereby protecting erythrocytes from disco-shape. In conclusion, suitable recommendations for selenium supplementation may alleviate the possible hazards associated with bovine endemic fluorosis.

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