INVESTIGATION OF BLOOD ANTIOXIDANT ENZYME LEVELS AND GLUTATHIONE PEROXIDASE, CATALASE, AND SUPEROXIDE DISMUTASE GENE POLYMORPHISM IN SHEEP WITH FLUOROSIS.

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ABSTRACT: Reactive oxygen species have been considered to play a significant part in the development of fluorosis, a critical public health issue in many parts of the world. OBJECTIVES: **The aims of this study, conducted on two groups of sheep, with and without fluorosis, was (i) to determine the effect of fluorosis on antioxidant enzymes, such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), catalase (CAT), and on malondialdehyde (MDA); and (ii) to investigate the relationship between the SOD, CAT, and GSH-Px genotypes and the antioxidant enzyme levels.** METHODS: **The two groups of sheep were: (i) 24 Akkaraman sheep with fluorosis living in Doğubeyazit province of Ağrı and (ii) 20 Akkaraman sheep with no signs of fluorosis living in Van. The MDA level and the antioxidant enzyme activities were identified spectrophometrically. The genotype characteristics were determined by the PCR-RFLP method using DNA extracted from blood.** RESULTS: **Compared to the control sheep without fluorosis, we found in the fluorotic sheep: (i) an increase in plasma MDA levels and GSH-Px-1 activity ($p<0.05$), and (ii) a decrease in SOD and CAT activities ($p<0.01$). There were significant differences found in the genotype and allele frequencies in both groups ($p<0.01$).** CONCLUSIONS: **Fluoride-induced oxidative stress affected the membrane structure resulting in elevated erythrocyte MDA levels and reduced enzyme activity of the enzymes SOD and CAT. According to our data, the gene polymorphisms show a tendency towards fluorosis for our local sheep population.**

Keywords: Catalase; Fluorosis; Glutathione peroxidase-1; Manganese superoxide dismutase; Polymorphism.

INTRODUCTION

The element fluorine is naturally present as the ion fluoride (F) in soil, rocks, and water throughout the world, with higher concentrations occurring in areas where there have been geologic activity, in the past or in recent years.[1,2] Chronic fluorosis is caused by long-term exposure to high levels of F and may be characterized by widespread pathology including changes in the liver, kidney, heart, muscle, gastrointestinal tract.[3,4] Because fluorine may be sequestered in the hard tissues of the teeth and skeletal system it may result in dental fluorosis and skeletal fluorosis.[5] Fluorosis may become a serious public health issue when drinking water contains more than 1–1.5 ppm of fluoride.[6-8] Chronic fluorosis is an endemic health problem in Turkey in Isparta and in Ağrı and its vicinity.[9,10]

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Oxidative stress is a normal phenomenon in the body. Reactive oxygen species (ROS) are produced as a normal product of plant cellular metabolism. Various environmental stresses lead to excessive production of ROS causing progressive oxidative damage. The main damage caused by ROS is lipid peroxidation of the polyunsaturated fatty acids of cellular membranes, from which malondialdehyde (MDA) is produced as a product. However, ROS also affect the activity of the main antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx).[11] Fluoride can inhibit or enhance various activities in blood cells. Taking into account the molecular basis of its mechanism of action, fluoride affects cellular enzymes, particularly antioxidants. Enzymes, in red blood cells in particular, are very sensitive to fluoride which causes various changes in the membrane function of erythrocytes in relation to structural and biochemical parameters. The most important antioxidant enzyme systems in erythrocytes are SOD, CAT, and GPx.[12] Many studies have investigated the connection between oxidative damage, the effects of antioxidants, and chronic fluorosis. However, the pathogenesis of endemic fluorosis remains poorly understood.[13] Fluorosis needs to be understood better at the biochemical and molecular levels.

This research aimed to investigate the variations in oxidative stress status by measuring the activities of SOD, CAT, GSH-Px-1 and the concentration of MDA in sheep with and without fluorosis. Furthermore, we evaluated whether or not the SOD, CAT, and GSH-Px-1 genotypes might be associated with the MDA, SOD, CAT, and GSH-Px levels in the blood plasma of sheep with fluorosis and controls without fluorosis in the Doğubeyazit Province of Ağrı.

MATERIALS AND METHODS

Animal material: The animals studied were (i) a fluorosis group of 24 Akkaraman sheep, aged 2 yr or older, from Doğubeyazit Province of Ağrı and (ii) a control group of 20 Akkaraman age-matched sheep with no signs of fluorosis, from Van. The presence of fluorosis was determined by a clinical examination of the teeth and joints. The urine F levels of the animals were also determined.

Determination of fluoride: Urine samples were collected in polyethylene tubes and F levels were determined with an ion selective electrode (WTW PH / ION 738®).[14]

Biochemical analysis: Blood samples were collected from the jugular vein in all the animals. Lipid peroxidation in erythrocytes was assessed by detection of MDA using the method developed by Akkus (1995).[15] SOD and GPx activities were measured in hemolysates by using a commercially available kit (Randox Lab.). CAT activity was specified according to the method of Aebi (1974) and based on the rate constant (s^{-1}, k) of the H_2O_2 decomposition rate at 240 nm.[16] Haemoglobin (Hb) concentration was determined according to the oxyhemoglobin method.[17]

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) for genetic polymorphisms of Mn-SOD, CAT, GSH-Px-1: Genomic DNA

was extracted from blood by a commercially available kit (Qiagen, Hilden, Germany) using a method described in another study.[18] The PCR primers were designed as defined by Özbey et al. 2011:[19] (i) The Mn-SOD RFLP; F:5'-ACCAGCAGGCAGCTGGCGCCGG-3' and the R:5'-GCGTTGATGTGAGGTTCCAG-3' amplified a 107-bp PCR product; (ii) The GSH-Px-1 RFLP F:5'-GAAATCCCAGCCGCCTA-3' and the R:5'CACTCACCTTCGACTTCTCTTGCT-3' amplified a 260-bp PCR product. (iii) The CAT RFLP sense primer 5'-TAAGAGCTGAGAAAGCATAGCT-3' and antisense primer: 5'-AGAGCCTCGCCCCGCCGGACCG-3' amplified a 340-bp PCR product (IDT: Integrated DNA Technologies, Inc, Coralville, IA, USA).[19] Polymerase chain reaction (PCR) amplification of the genomic DNA was performed as described by Özbey et al. (2011), in a total volume of 25 μL, using approximately 100 ng DNA, 2.5 mmol/L $MgCl_2$, 200 mol/L dNTPs, 12.5 ng of each primer, and 0.5 units of Taq DNA polymerase (Promega, Madison, Wis) in the PCR buffer supplied by the manufacturer (10 mmol/L Tris-HCl, pH 9.0, and 50 mmol/L KCl).[19] The PCR cycling consisted of the following steps: a denaturation step at 95ºC for 5 min, 35 cycles at 95ºC for 1 min, 60ºC for 1 min, and 72ºC for 1 min, and a final extension step at 72ºC for 10 min using the Gene Amp PCR System 9700 thermocycler (PE Applied BioSystems). PCR products were analyzed by agarose electrophoresis in 2% agarose gel stained with ethidium bromide. Mn-SOD, CAT, and GSH-Px-1 genotypes were detected by using PCR-RFLP. The resulting 107-bp, 359-bp, and 340 bp PCR product was digested with the restriction endonuclease MroN I (New England BioLabs) at 37ºC for 16 hr, with Dde I (New England BioLabs) at 50ºC for 4 hr, and with Sma I (New England BioLabs) at 37ºC for 16 hr according to the manufacturer's recommendations. Restriction enzyme digestion results in a 107-bp product (allele 1 Val) or 89 and 18 bp products (allele 2 Ala), a 359-bp product (allele 1 C) or 299 and 60 bp products (allele 2 T), a 340-bp product (allele 1 C) or 185 and 155 bp products (allele 2 T) for Mn-SOD, GSH-Px-1, and CAT, respectively. The enzyme digested products were analysed on a 3% agarose gel with ethidium bromide and visualized on UV light using the image analysis system Kodak EDAS 120. The MnSOD, GSH-Px-1, and CAT genotypes were determined based on the presence or absence of the enzyme restriction sites, i.e., MroN I; VV, VA, AA, Dde I; TT, TC, CC, and Sma I; TT, TC, CC, respectively.

Statistical analysis: Control groups' and fluorosis groups' frequency of genotypes and alleles were compared with each other applying the chi-square test. Comparisons for standard means of numerical variances were made using the Student-t test. Biochemical analysis were done using the Tukey test. p values less than 0.05 were accepted as being significant. Data are presented as mean±SD. All calculations were made with the SPSS (Statistical Packages of Social Sciences, SPSS for Windows Version 15.0 Inc, Chicago, IC, United States)

RESULTS

Levels of the MDA, SOD, CAT, and GSH-Px-1 in the blood plasma: Urinary fluoride levels in sheep with and without fluorosis are given in Table 1.

Table 1. Urinary fluoride levels in sheep with and without fluorosis (Data are expressed as mean±SD)

	Sheep with fluorosis	Sheep without fluorosis (control group)	T	p
	n=24	n=20		
Urinary F (ppm)	6.74±0.49*	1.50±0.30	–4.16	*p<0.01

Compared to control group: *p<0.01.

According to the data obtained, the mean urinary fluoride level of the sheep with fluorosis was found to be 4.5 times higher than in the control group, a difference that is highly significant ($p<0.01$).

The MDA, SOD, CAT, and GSH-Px plasma levels in the sheep with fluorosis and the control sheep were compared. The levels of MDA (an indicator of lipid peroxidation) were significantly higher in the sheep with fluorosis. The GSH-Px activity was an increased in the sheep with fluorosis compared to the control group ($p<0.01$) (Table 2). Moreover, we detected a statistically significant reduction in the CAT and SOD levels in the sheep with fluorosis from the region of endemic fluorosis compared to the control group without fluorosis ($p<0.05$).

Table 2. MDA levels and SOD, CAT, and GSH-Px activity in sheep with and without fluorosi (Data are expressed as mean±SD)

	Sheep with fluorosis	Sheep without fluorosis (control group)	p
MDA (nmol/ml)	4.11±0.85†	3.32±0.91	p<0.01
GSH-Px (U/gHb)	34.81±1.98†	14.88±0.45	p<0.01
CAT (k/gHb)	1.70±0.15*	10.99±0.87	p<0.05
SOD (U/gHb)	56.92±2.89*	69.85±3.39	p<0.05

Compared to control group: *p<0.05, †p<0.01.

Distribution of the MnSOD, CAT, and GSH-Px polymorphism in sheep with and without fluorosis: The genotype and allele distribution for the MnSOD, GSH-Px-1, and CAT gene promoter in the sheep with and without fluorosis are presented in

Table 3. The results of the genotype frequencies of MnSOD, GSH-Px-1, and CAT gene promoters, as summarized in Table 3, indicate that, compared with controls, the sheep with fluorosis had significantly lower frequencies of the Mn-SOD VV, GSH-Px-1 CC, and CAT TT genotypes and significantly higher frequencies of the Mn-SOD VA, GSH-Px-1, and CAT TC genotypes. A significant difference in the distribution of the genotype at each MnSOD, GSH-P-1, and CAT gene was observed ($p<0.01$).

Table 3. The frequencies of the allele and genotype of the Mn-SOD, GSH-Px-1 and CAT gene polymorphism in sheep with and without fluorosis

Gene	Group	Genotype frequency			Allele frequency	
		VV n (%)	VA n (%)	AA n (%)	Val	Ala
Mn-SOD	Sheep with fluorosis (n=24)	2 (8.33)*	22 (92.0)*	0 (0.0)	0.925†	0.458†
	Sheep without fluorosis (n=20)	17 (85)	3 (15)	0 (0.0)	0.541	0.075
		CC	TC	TT	C	T
GSH-Px-1	Sheep with fluorosis (n=24)	1 (2.17)*	23 (96)*	0 (0.0)	0.520†	0.479†
	Sheep without fluorosis (n=20)	16 (80.0)	4 (20.0)	0 (0.0)	0.9	0.1
		TT	TC	CC	C	T
CAT	Sheep with fluorosis (n=24)	2 (8.33)*	22 (92.0)*	0 (0.0)	0.458†	0.541
	Sheep without fluorosis (n=20)	18 (90.0)	2 (10.0)	0 (0.0)	0.05	0.95

*Comparing genotype frequencies between the sheep with and without fluorosis: $p<0.01$,
†Comparing allele frequencies between the sheep with and without fluorosis: $p<0.01$.

The identification of the 2 alleles at the polymorphic site was performed by incubating the PCR product with a restriction enzyme chosen to cut the 1 of the 2 alleles followed by electrophoresis on 3% agarose gels (Table 3). The results

obtained indicated that the Val/Ala, T, and C alleles for MnSOD, GSH-Px-1 and CAT, respectively, were more frequent in the sheep with fluorosis than in the controls ($p<0.01$). Accordingly, the carriers of the MnSOD Val and Ala, GSH-Px-1 T, and CAT C alleles were overrepresented in the fluorotic sheep compared to the controls ($p<0.01$) (Table 3). There were significant differences in the allele frequencies for each MnSOD, GSH-Px-1, and CAT gene between the sheep with fluorosis and the control sheep ($p<0.01$).

Relationships between the SOD, CAT, and GSH-Px-1 genotypes and the MDA, SOD, CAT, and GSH-Px-1 plasma levels: We investigated whether the SOD, CAT, and GSH-Px-1 genotypes might be associated with the MDA, SOD, CAT, and GSH-Px-1 levels in the blood plasma of the sheep with fluorosis and the control sheep. When compared with the controls, the sheep with fluorosis had a significantly higher frequency of the GSH-Px-1 TC genotype and the GSH-Px-1 activity was increased.

DISCUSSION

Exposure to high levels of fluoride, nutritional status, dietary habits, environmental factors, and body response to ingested or inhaled F are important in comprehending the etiology of fluorosis.[13] Water contamination and industrial pollution by F are considered to be among the most common environmental problems. F may adversely effect health if excessive amounts are ingested.[5]

There is a positive correlation between the amount of F present in the drinking water and amount of F excreted in the urine. Therefore, the measurement of F concentration in urine is used as a safe and accurate method for the diagnosis of fluorosis.[20] Şendil and Bayşu reported, in 1973, that urinary F levels of sheep in Eastern Anatolia, Turkey, where endemic fluorosis is common, ranged from 3.80 to 30.6 ppm.[21] The average urinary F level of sheep raised in that region was reported as 8.1 ppm by Ergun et al. in 1986.[9] The urinary F level of sheep with fluorosis in the Eskişehir-Beylikova/Kızılcaören region of Turkey was found to be 12.50±0.46 ppm.[10]

In our study, we found a significant increase ($p<0.01$) in the mean urinary F level in the sheep with fluorosis, 6.74±0.491 ppm, compared to that in the sheep without fluorosis, 1.50±0.30 ppm, which was consistent with the findings of other researchers (Table 1).[9, 22]

F intake in high doses increases the production hydrogen peroxide H_2O_2, the superoxide anion O_2^-, and the hydroxyl radical $^{\bullet}OH$. Chronic fluorosis due to high F levels occurring naturally is endemic in many regions in Turkey and in the world. Van city is one such region in Turkey. The Çaldıran and Muradiye regions in the Van Province of Turkey have a volcanic terrain and fluorosis occurs in humans and animals in these regions.[9,10,20-23] Ruminant animals such as sheep, goats, cattle, and other animals are more sensitive to fluorosis. In contrast, pigs and horses can partially hold their own against the disease and birds are fully resistant. Chronic degenerative disorders, including chronic fluorosis, have been associated with an enhanced production of reactive oxygen species, and lipid

peroxidation.[24] One of the most commonly used methods or deciding whether or not, and to what extent, F leads to lipid peroxidation has been to determine the MDA levels and the antioxidant enzyme activities in erythrocytes. The erythrocyte cell membrane is known to be highly sensitive to free radical oxidation due to its unsaturated fatty acid content.

Metabolic imbalance, oxidative stress, and lipid peroxidation are associated with the pathogenesis of chronic fluorosis. For this reason, many researchers have investigated the relationship between free radical reactions and fluorosis but the results of many studies are not consistent with the previous studies. In this respect, Shivarajashankara et al. reported that, in children aged 3 to 10 years with endemic fluorosis, erythrocyte MDA levels and GPx activity were increased.[25] Also, they demonstrated that rats exposed to F exhibited increased MDA and GPx activity in their red blood cells.[26] In another study, it was found that rats given F in drinking water at 40 mg F/L for 21 and 70 days had a significant increase in the level of MDA and the activities of GPx, CAT and a decrease in SOD activity. Çenesiz and Özcan reported similar results.[20] Yur et al. reported that erythrocyte MDA levels were significantly increased in fluorotic sheep.[24] Ha et al. found an increase in free radicals and the MDA level in 32 cows selected for grazing in pastures that contained a high level of F but the whole blood GPx, CAT, and SOD activities were lower in these cows than in the control group.[27] Guan et al. found a decrease in blood GSH-Px activity with no changes in SOD activity in F-intoxicated rats.[28] On the other hand, Reddy et al. did not find any changes in catalase, SOD, and GPx levels in the blood of rabbits with F intoxication of rabbits or in humans with fluorosis.[29]

In this study, we found that the erythrocyte MDA level and the GSH-Px activity were increased in fluorotic sheep ($p<0.01$) (Table 2). Other studies have reported a similar increase in erythrocyte GSH-Px activity.[20,25,26] We also detected, in comparison to the control group sheep, a statistically significant decrease in the CAT and SOD levels in the sheep with fluorosis from the endemic fluorosis area ($p<0.05$). These findings suggest that F induces oxidative stress in erythrocytes leading to elevated lipid peroxidation and decreased activity of the major antioxidant enzymes. In animals with fluorosis, it was observed that lipid metabolism was affected by exposure, for various periods of time, to high levels of F. The present study, performed on sheep with endemic fluorosis in Doğubeyazıt province of Ağrı, showed similar results. Factors affecting the severity of fluorosis in sheep include the severity of the exposure to F, the presence of organic and inorganic compounds, the presence of external factors which may reduce or enhance the effects of F, the type and dosage of F, the percentage of F in the compound, and the mode and duration of the F ingestion. It was established that the parameters of the animals diagnosed with fluorosis by examining the F level in the urine differed from healthy animals. In the light of these findings, we suggest that increased oxidative stress occurs in sheep with fluorosis.

In the molecular part of our study, for Mn-SOD, CAT, and GSH-Px-1 polymorphisms, we found significant differences in the genotype and allele

frequencies between the sheep with fluorosis and the control group ($p<0.01$). When compared with the controls, the sheep with fluorosis had a significantly higher frequency of the GSH-Px-1 TC genotype and the GSH-Px-1 activity was increased. Interestingly, we detected a statistically significant increase in the Mn-SOD VA and CAT TC genotypes and a decrease in CAT and SOD activity in the sheep with fluorosis from the endemic fluorosis region ($p<0.05$). The cause of this difference is related to environmental, medical, and racial differences as well as reflecting other social factor effects, the problems associated with having a small sample, and heterogeneity in the genetic background.

CONCLUSIONS

Consequently, oxidative stress and antioxidant enzymes play an important role in the pathogenesis of fluorosis and, according to our data, Mn-SOD, CAT, and GSH-Px-1 gene polymorphisms cause a predisposition to fluorosis for our regional sheep flocks. However, only limited analytical biochemical research data is available and we did not find any data in the literature on genetic studies. Therefore, the subject should be supported by a wider number of scientific studies. Further clarifying the relationship between oxidative stress and antioxidant enzymes and studying the molecular genetics with a larger sample size will enable meaningful scientific contributions to be made for understanding better the pathogenesis of fluorosis.

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