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EXPERIMENTALSTUDYONTHERELATIONSHIPBETWEENFLUORIDE EXPOSURE ANDCARDIACOXIDATIVEBIOCHEMICALIMBALANCEAND ULTRASTRUCTURALDISRUPTIONSIN RATSUSINGSCANNINGELECTRON MICROSCOPY

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ABSTRACT

Fluoride contamination in drinking water due to natural and anthropogenic activities has been recognized as one of the major problems worldwide imposing a serious threat to human health. Excessive exposure to fluoride causes metabolic, functional and structural damages in many organs especially in the heart. This study aimed to investigate the ultrastructural and biochemical changes induced by sodium fluoride (NaF) in the cardiac tissue of rats, focusing on oxidative stress parameters and antioxidant enzyme activities. Rats were orally administered NaF at doses of 300 mg/kg b.w./day and 600 mg/kg b.w./day for 40 days and the control rats were given 1 mL of deionized water for the same period. After the experimentation, rats were sacrificed and cardiac tissue was processed for further examination. Scanning electron microscopic examination highlighted structural abnormalities in cardiac tissues of fluoride-treated rats, including mineral deposits, collagen fiber fragmentation, distorted myofibers, and calcium crystal formations. These ultrastructural changes were more pronounced with higher fluoride doses, suggesting dose-dependent tissue damage. Oxidative stress parameters such as malondialdehyde (MDA) levels showed significant increases in both fluoride-treated groups, indicative of lipid peroxidation. Reduced glutathione (GSH) levels decreased significantly, suggesting compromised antioxidant defense mechanisms. Activities of antioxidant enzymes catalase (CAT), mitochondrial superoxide dismutase (MnSOD), and glutathione peroxidase (GPx) were markedly reduced in fluoride-treated groups, further indicating oxidative stress-induced damage. These findings underscore the potential of fluoride to induce oxidative stress and ultrastructural changes in cardiac tissue, suggesting a mechanism whereby fluoride exposure may contribute to cardiovascular dysfunction.

KEYWORDS: Catalase, Fluoride, Glutathione peroxidase, Malondialdehyde, Mitochondrial Superoxide dismutase, Scanning electron microscopy

INTRODUCTION

Fluoride is one of the widespread environmental chemical pollutants in the earth's crust. Excessive fluoride exposure can cause fluorosis of drinking water.[1] Symptomatic fluorosis is more damaging to hard tissues i.e. dental and skeletal structures. The excessive intake of fluoride can disrupt multiple metabolic pathways, causing structural and functional damage to various organs including the heart.[2] The cardiovascular system is crucial in the transportation of blood, providing nutrients, oxygen, and removing waste products from tissues. Fluoride accumulates in the heart and reduces the capacity to eliminate free radicals, which leads to cell membrane damage.[3] However, the effects of fluoride on hard tissues are well established, but its long-term effects on cardiac tissue are still unknown.

Scanning Electron Microscopy (SEM) is a powerful imaging technique that allows the visualization of the surface morphology and ultrastructure of biological specimens at high magnification.[4] SEM uses a focused beam of electrons to scan the surface of a sample and then provides three-dimensional images that can reveal characteristics such as surface texture, morphology, and structural integrity.[5] SEM offers details on how fluoride exposure may alter cellular morphology, affect cellular adhesions, and alter overall cardiac tissue integrity.[6]

Oxidative stress is caused by the production of reactive oxygen species (ROS), and leads to modification of biomolecules and tissue damage. The defense mechanism of the body includes enzymatic antioxidants like catalase, superoxide dismutase and glutathione peroxidase, that counteract the effect of oxidative stress damage.[7] The production of excessive ROS leads to lipid peroxidation and malondialdehyde (MDA) is a key marker of lipid peroxidation. Reduced glutathione (GSH) is a non-enzymatic antioxidant that maintains the redox balance and catalase (CAT) and mitochondrial superoxide dismutase (MnSOD) are enzymes that neutralize hydrogen peroxide and superoxide radicals, respectively.[8] Fluoride induced oxidative stress impacts the cellular metabolism and the function of antioxidant enzymes leading to severe cellular damage.[9] Hence, the present investigation explores the fluoride-induced oxidative stress which affects the antioxidant defense mechanisms and cause ultrastructural alterations in cardiac tissue.

MATERIALS AND METHODS

Young Wistar albino rats weighing between 150-200 g were housed in polypropylene cages with stainless steel grill tops and given standard rat pellet diet (Hindustan Lever Limited, India) and water *ad libitum*. After a two-week acclimatization period, the rats were randomly divided into three groups of six animals each. Group II and Group III received daily oral gavage of 300 mg and 600 mg sodium fluoride (NaF) per kg body weight, respectively for 40 days, while the control group received equivalent volumes of double deionized water. Following the experimental period, the rats were fasted overnight, weighed, and excised. Cardiac tissues were rapidly taken out, rinsed with cold saline and processed further for ultrastructural and biochemical analysis.

Scanning electron microscopic examination

For SEM analysis, the cardiac tissue was rinsed with 0.1M phosphate buffer (pH 7.4) and fixed in 2.5% glutaraldehyde buffered with 0.1M phosphate buffer at pH 7.4 for 24 hours at 4°C, by the method of Karnovsky.[10] After washing with phosphate buffer (0.1M), the tissue was post-fixed in 1% osmium tetraoxide for 2 hours at 4°C. Dehydration was carried out using ascending concentrations of acetone, followed by critical point drying. Specimens were affixed to stubs, coated with gold using a sputter coater (Balzer Union SCD 020), and images were captured using a scanning electron microscope (JEOL JSM-6510) at All India Institute of Medical Sciences, New Delhi, India.

Biochemical Assays

Sample Preparation: The cardiac tissue was washed in 0.9% saline and homogenized quickly with 0.1 M phosphate buffer (pH 7.4) using a homogenizer to give a 10% homogenate. The homogenate was centrifuged at 10,000 rpm for 10 minutes and the supernatants were used for the estimation of various biochemical parameters.

Fluoride estimation

The level of fluoride in the cardiac tissue was measured by a potentiometric method by using the ion selective electrode.[11]

Oxidative Stress Markers

Malondialdehyde

Malondialdehyde (MDA) content in cardiac tissue of control and fluoridated rats was determined by the method of Ohkawa *et al*.[12]

Reduced glutathione

The level of reduced glutathione (GSH) in the cardiac tissue of control and fluorotic treated rats was determined by the method of Moron *et al.*[13]

Antioxidant Enzymes

Catalase

The activity of catalase (CAT) in the cardiac tissue of control and fluoride administered rats was assessed by the method of Aebi.[14]

Superoxide dismutase

The activity of mitochondrial superoxide dismutase (MnSOD) in the cardiac tissue of control and fluoridated rats was examined by the method of Das *et al.*[15]

Glutathione peroxidase

The activity of glutathione peroxidase (GPx) in the cardiac tissue of control and fluoride administered rats was determined by the method of Ellman.[16]

Statistical analysis

Statistical analysis was performed using SPSS 16.0 software (IBM). The data are presented as Mean ± standard deviation (SD). Differences between experimental groups were evaluated using one-way analysis of variance (ANOVA), followed by post hoc Bonferroni tests to compare specific group pairs. Statistical significance was considered at $P < 0.05$. Pearson's correlation analysis and simple linear regression tests were used to investigate associations between variables.

Ethical Approval

Experimental procedures were conducted following approval by the Institutional Animal Ethical Committee of Punjabi University, Patiala (Animal Maintenance and Registration No. 107/GO/ReBi/S/99/CPCSEA/2017-42).

RESULTS

Scanning electron microscopy

In control rat, scanning electron microscopic examination showed the architecture of normal cardiac tissue. Cardiomyocytes exhibited a regular arrangement and were well-organized into tight muscle bundles. A dense network of extracellular matrix (ECM) components surrounded and interconnected the cardiac cells (Fig. 1). The epicardial layer revealed structural integrity along with abundant adipocytes (Fig. 2). The

expected ridged structure of the fibre bundles was evident (Fig. 3).

The rats treated with 300 mg NaF/kg bw/day revealed that the purkinje fiber's structure was distorted and the muscle trabeculae was fragmented at one end (Fig. 4). It revealed a dense collagen fiber network connecting two cardiac muscle cells. A distortion on the surface of a cardiac muscle cell was also noticeable (Fig. 5). The semi-spherical deposits containing minerals localized on the walls of the micro-cavity were observed within the organic matrix (Fig. 6). The results showed twisted cardiac myofibers and thick collagen fibers were also present (Fig. 7). The surface of cardiac cells had blebs, and tiny crystalline structures known as irregular prisms were discovered (Fig. 8).

The ultrastructural examination of rats treated with 600 mg NaF/kg bw/day for 40 days revealed dose effects and the changes were more severe than prior fluoridated group. A highly contracted myofibril was observed (Fig. 9). There was a fragmented collagen fiber (Fig. 10). Numerous calcium deposits as crystal prisms were observed. There was degenerated muscle bundles and profound dilation of the intercellular space with looseness of the extracellular matrix network (Fig. 11). There was distorted and collagen devoid muscle fiber appeared to be highly contracted (Fig. 12). At higher magnification, crystals of calcium deposits were also visible (Fig. 13).

Biochemical analysis

Oxidative stress markers

1. Malondialdehyde

The mean level of malondialdehyde (MDA) in cardiac tissue significantly increased in rats administered 300 mg NaF/kg bw/day and 600 mg NaF/kg bw/day ($F = 342.855$, $P < 0.0001$; Fig. 14), showing elevations of +119.766% and +284.211%, respectively.

Post-hoc Bonferroni multiple comparison tests indicated a significant ($P < 0.0001$) rise in the levels of MDA between and within groups (95% CI = -1.275 to -1.156) in cardiac tissue of fluoride-treated rats.

2. Reduced Glutathione

The mean level of glutathione peroxidase (GSH) in the cardiac tissue of test rats significantly ($F = 574.764$, $P < 0.0001$; Fig. 15) declined by -41.494% and -85.059% as compared to control after 40 days of fluoride exposure.

Post-hoc Bonferroni multiple comparison tests after ANOVA exhibited a significant ($P < 0.0001$) decline in the levels of GSH in cardiac tissue between and within groups (95% CI = 0.939 to 1.361) treated with fluoride for 40 days.

Antioxidant Enzymes

3. Catalase

The mean activity of catalase (CAT) in the cardiac tissue of test rats significantly decreased ($F = 345.034$, $P < 0.0001$; Fig. 16). There was a decline of -38.495% in 300 mg NaF and -65.269% in 600 mg NaF compared to control after 40 days of fluoride administration.

Post-hoc Bonferroni multiple comparison tests after ANOVA indicated a significant ($P < 0.0001$) decrease in activity of CAT in cardiac tissue between and within groups (95% CI = 0.666 to 0.704).

4. Mitochondrial Superoxide Dismutase

The mean activity of mitochondrial superoxide dismutase (MnSOD) in the cardiac tissue of test rats showed a significant decrease of -27.398% and -47.376% compared to control ($F = 128.924$, $P < 0.0001$; Fig. 17). Post-hoc Bonferroni multiple comparison tests after ANOVA indicated a significant ($P < 0.0001$) decline in the activity of MnSOD in cardiac tissue between and within groups (95% CI = 3.193 to 4.597).

5. Glutathione Peroxidase

The mean activity of glutathione peroxidase (GPx) in the cardiac tissue of fluoridated rats was significantly decreased ($F = 892.500$, $P < 0.0001$; Fig. 18), indicating declines of -44.496% and -68.223% in the 300 mg/kg NaF and 600 mg/kg NaF groups, respectively.

Post-hoc Bonferroni multiple comparison tests after ANOVA revealed significant ($P < 0.0001$) decrease in the activity of GPx in cardiac tissue between and within groups (95% CI = 3.605 to 2.531).

Correlation Analysis

Pearson's bivariate correlation and simple linear regression analysis revealed a significant ($P < 0.0001$) positive relationship between levels of cardiac tissue fluoride and MDA ($R^2 = 0.902$, Pearson $r = 0.950$; Fig. 19) and negative relationship between levels of cardiac tissue fluoride and GSH in test rats ($R^2 = 0.951$, Pearson $r = -0.975$; Fig. 20).

There was a significant ($P < 0.0001$) negative relationship between level of cardiac tissue fluoride and activities of CAT ($R^2 = 0.964$, Pearson $r = -0.982$; Fig. 21), MnSOD ($R^2 = 0.947$, Pearson $r = -0.973$; Fig. 22) and GPx ($R^2 = 0.976$, Pearson $r = -0.988$; Fig. 23) in rats after 40 days of fluoride exposure.

DISCUSSION

The scanning electron microscopic examination of the control rat unveiled the structural integrity of myocardium in which cardiac myocytes were arranged into tight muscle bundles.[17]

The ultrastructural findings of present investigation revealed the alterations on the surface of cardiac tissue following sodium fluoride exposure. These alterations

are in line with the study of Wang *et al.*[18] who demonstrated the effect of sodium fluoride on myocardial cells, characterized by pits or blebs on the cell surface.

In the present study, dilation of the extracellular space within the necrotic myocardium was seen, including a partial degradation of the collagen matrix. These findings align with pathological processes such as myocardial infarction, ischemia, and isoproterenol-induced myocardial necrosis,[19,20,21] which exhibit structural abnormalities including substantial loss of the collagen framework.

According to Walentynowicz and Wrzolkowa,[22] the substances that break down the ECM are specialized proteinases and the ultrastructural changes observed in rat hearts exposed to high doses of Vitamin D3 were associated with double the activity of proteolytic enzymes in the homogenate of necrotic hearts as compared to control. Walentynowicz *et al.*[23] documented a reduction in the size of heart muscle bundles, which was associated with a decrease in the size of muscle fibers undergoing degeneration. This reduction was preceded by a decrease in the number of myofibrils. Additionally, the degenerative process of the muscle bundles coincided with morphological alterations in the extracellular matrix (ECM) components.

Crystalline structures were more prominent in the fluoride administered group. Saunders and Amoroso.[24] stated that presence of crystal deposition in the cardiac tissue may impede motion and possibly sever the sliding fibers with loss of function. Calcific deposition leads to tissue deterioration, distortion in extracellular matrix structure, and small voids as final stage of progressive mechanical tissue damage.[25,26]

Kanzaki *et al.*[27] outlined various morphological abnormalities observed in the three-dimensional structure of myocardium from a patient with hypertrophic cardiomyopathy. These abnormalities included disorganized, overlapping, and unusually branched cardiomyocytes within the hypertrophic cardiomyopathy heart sample.

During SEM investigation, there were degeneration and distortion of muscle fibers in the cardiac tissue of fluoridated rats. These findings concur with the study of Quadri *et al.*[6] who reported ultrastructural changes in the cardiac tissue of fluoride treated rats in comparison with control.

Fluoride is known to cause oxidative stress and hinder the activity of antioxidant enzymes in soft tissues.[28,29,30] In the present investigation, the cardiac tissue of rats administered with 300 and 600 mg NaF/kg b.w./day for 40 days, displayed significant ($P < 0.0001$) reduction in the activity of antioxidant enzymes such as catalase, mitochondrial superoxide dismutase and glutathione peroxidase, along with elevation in the level of malondialdehyde and decrease in the level of reduced glutathione. The findings of the current study are in line with the study of Yildrim *et al.*[31] who reported similar results in the cardiac tissue of rats administered with 15 ppm of sodium fluoride. Several other studies also witnessed increased lipid peroxidation and decreased activity of antioxidant enzymes.[8,32,33]

Lipid peroxidation is the important indicator of oxidative damage, and malondialdehyde (MDA) is used as a marker of lipid peroxidation.[34] The elevation of cardiac lipid peroxidation after fluoride administration may play a major role in the impairment of myocardial function. The present study also revealed that chronic fluoride exposure appears to exacerbate the damage to the heart muscle due to persistent oxidative stress, primarily due to suppression of fatty acid oxidation and increased MDA levels.[35]

Reduced glutathione (GSH), a tripeptide containing a sulfhydryl group, directly reduces free radicals and is a substrate for GPx and glutathione S-transferase (GST) which are essential for the removal of hydrogen peroxide, lipid hydroperoxides and electrophilic compounds and thus has a crucial role in cellular protection against oxidative stress. The current study revealed significant ($P < 0.0001$) decrease in the level of GSH in cardiac tissue of fluorotic rats as compared to control. This decrease in GSH concentration could be due to the decrease in nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) or due to the use of GSH in the elimination of peroxides.[36]

Glutathione peroxidase (GPx) is involved in the reduction of peroxides and in the process it uses GSH and converts it to glutathione disulfide (GSSG) which is then reduced back to GSH by glutathione-disulfide reductase (GSR).[37] The present investigation showed significant decrease ($P < 0.0001$) in the activity of GPx in fluoride-administered rats and this decline could be attributed to the excessive production of superoxide radical anions. To neutralize the free radicals, GPx uses GSH in the process and the decrease in GSH content due to fluoride toxicity leads to a decrease in GPx activity as well.[38]

The current study exhibited significant decrease ($P < 0.0001$) in the activity of mitochondrial superoxide dismutase (MnSOD) and catalase (CAT) in fluoridated rats. SOD and CAT are the two important antioxidant enzymes that protect the heart by reducing the superoxide radicals (O^{2-}) and hydrogen peroxide radicals into harmless water and oxygen.[39]

It is widely known that free oxygen radicals, which are known to cause lipid peroxidation, are effectively scavenged by antioxidant enzymes such as CAT, GPx and SOD. However, when the concentration of hydrogen peroxide (H_2O_2) is high, the enzyme superoxide

dismutase which is involved in the conversion of superoxide radicals to H_2O_2 is affected. Furthermore, high concentration of oxygen (O^2) in the environment also affects the activity of catalase and glutathione peroxidase.[40] Zhou *et al.*[41] found that the high levels of fluoride reduce the activities of SOD, GPx, and the total antioxidant capacity, which in turn increases reactive oxygen species (ROS) and reactive nitrogen species (RNS) levels.

The accumulation of fluoride leads to oxidative stress and alters the cellular antioxidant defense system. Research carried out on animals and human beings living in endemic fluorosis regions have reported higher levels of ROS and reduced activity of antioxidant enzymes in blood and tissues.[33,42] Fluoride at even low concentration stimulates the production of ROS such as superoxide anion, peroxide, hydroperoxides and hydrogen peroxide which are involved in the pathogenesis of various ailments found in inhabitants of endemic fluorotic areas.[43] Sharma *et al.*[44] postulated that the decrease in antioxidant system activities, which was characterized by the reduced CAT, SOD and GPx, might be due to an overwhelmed response to the oxidative stress caused by fluoride. As a result, there was a decrease in the ability to eliminate free radicals generated after fluoride administration.

CONCLUSION

The present study reveals that increased production of reactive oxygen species due to fluoride-induced oxidative stress disrupts the antioxidant defense system of heart, leading to lipid peroxidation and severe alterations in the ultrastructure of cardiac tissue, as evidenced by scanning electron microscopy. Enhancing the antioxidant defense mechanisms could be helpful in mitigating fluoride-induced cardiac damage, preserving both structural integrity and cardiac function.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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