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Histochemical pattern of gastrocnemius muscle in fluoride toxicity syndrome

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ABSTRACT

Objective: To evaluate the effect of sodium fluoride (NaF) on cytochemical distribution of proteins, DNA, and RNA in the gastrocnemius muscle of rat in experimental fluorosis. **Methods:** Young Sprague Dawley albino rats were administered with NaF at 30, 45, and 75 mg/kg bw/day subcutaneously for 15 and 30 days, respectively. The control animals were given the vehicle (1cc deionized double distilled water/kg bw/day). **Results:** In the first phase of 15 days experimentation, the gastrocnemius muscle of rats intoxicated with NaF at 30, 45, and 75 mg /kg bw/ day showed decline in proteins including α amino acids as compared to control. In the second phase of 30 days experimentation, the muscle fibers of rat showed elevation in sarcolemmal and sarcoplasmic proteins in 30 mg NaF dose group, and angulated fibers exhibited increase in sarcolemmal proteins in 45 mg NaF group. The marginal regions of angular and rim fibers showed deeply stained rings of sarcoplasmic proteins whereas the split fibers were faintly stained in rats treated with NaF at 75 mg/kg bw/day for 30 days. In rats treated with NaF at 30 mg/kg bw/day for 15 days, the hypertrophied peripheral muscle fibers contained more DNA, however the atrophied fibers had more RNA. In 45 mg NaF group, RNA was located in sarcolemmal regions, while DNA content decreased as compared to control. In 75 mg NaF group, the muscle fibers had dark and light staining regions of DNA and RNA. In the 30 days of experimentation, the DNA level decreased whereas RNA content increased in the gastrocnemius muscle fibers of the rat treated with NaF at 30 mg/kg bw/day. In treatment group with NaF at 45 mg/kg bw/day, the RNA content slightly declined in comparison to control, in all treatments for 15 days as well as in treatment group with NaF at 30 mg/kg bw/day for 30 days whereas the amount of DNA slightly increased as compared to treatment group with NaF at 75 mg/kg bw/day for 15 days. The highest dose group revealed elevated amount of RNA whereas DNA content remained stable. **Conclusions:** The findings of present study demonstrate that certain concentrations of fluoride can induce muscle lesions and damage DNA, RNA, and protein in muscle cells and excessive intake and accumulation of fluoride is therefore a serious risk factor for muscular abnormalities in fluorosis.

1. Introduction

Fluoride is a biologically active ion and cumulative toxic agent with demonstratable effects on bone cells, bone osteoblasts and osteoclasts[1]. Detailed histological and histochemical studies of muscle biopsies in patients suffering from endemic skeletal fluorosis documented no evidence of direct toxic effect and the muscle changes were neurogenic, secondary to compressive radiculopathy[2]. In advanced stages, skeletal fluorosis causes crippling deformities and neurological complications. The neurological manifestations in skeletal fluorosis, radiculomyelopathy, arise primarily from mechanical compression of spinal cord and nerve roots from osteophytosis, sclerosed vertebral column and ossified ligaments[3]. Although fluoride increases bone mass or bone mineral density, the newly formed bone may have reduced strength, since fluoride increases widening of osteoid seams and creates cytological and matrix abnormalities. Increased brittleness and fragility due to increase in osteocytes resorption leads to an increase in the number of micro fracture in bone[4]. Toxic effects of fluoride in animals may be attributable to one or more of these metabolic and biochemical effects[5]. Many studies have suggested that fluoride can affect the collagen metabolism of cartilage[6-8].

Effects of fluoride on muscle pathology are evident but are less well characterized. Here an attempt has been made to study the fluoride induced cytochemical changes in proteins and nucleic acids in gastrocnemius muscle.

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2. Materials and methods

2.1.Experimental animals

Two to four months old male and female Sprague Dawley albino rats (100–300 g) were fed on pellet diet obtained from Hindustan Lever Ltd. Mumbai, India and water was supplied *ad libitum*. The rats were acclimatized for one week prior to the start of experiment. All rat experimentation was carried out in according with regulations and guidelines of the Ethical and Animal Use Committee of Punjabi University, Patiala, India.

Rats were injected with NaF at 30, 45, and 75 mg/kg bw subcutaneously and control group was given only deionized double distilled water for 15 and 30 days. The gastrocnemius muscles from various sites were excised, blotted free of blood and processed for morphological cytopathetic changes.

2.2.Cytochemical examination

Proteins including α –amino acids were stained by the method of Yasuma and Ichikawa[9]. The nucleic acids in the muscles of control and treated animals were assessed by using Methyl Green Pyronin Y technique[10].

3. Results

In control group, the Type–I and Type–II muscle fibers, perimysium and endomysium exhibited the normal distribution of proteins including α – amino groups. The endomysium revealed more concentration of proteins than perimysium. The marginal region of Type–II muscle fibers had more intensity of proteins than central and intermediate regions. The central region of Type–I fibers showed less quantity of proteins as compared to intermediate and peripheral regions (Figure 1).

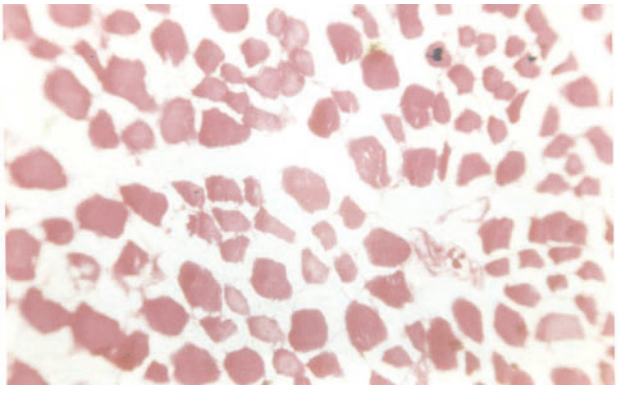

Figure 1. T.S. of gastrocnemius muscle of control rat (Ninhydrin Schiff 100×) showing the distribution of proteins in the muscle fibers and connective tissue sheath.

In the first phase of experiment, the animals were treated with NaF at 30, 45, and 75 mg/kg for 15 days, cytochemical alterations observed were as follows:

In the treatment group with NaF at 30 mg/kg bw, the Type II muscle fibers showed hypertrophy and invagination of sarcolemma, and extensive vacuolization. In this region, the intensity of protein decreased. Type I muscle fibers got fused and were deeply stained (Figure 2).

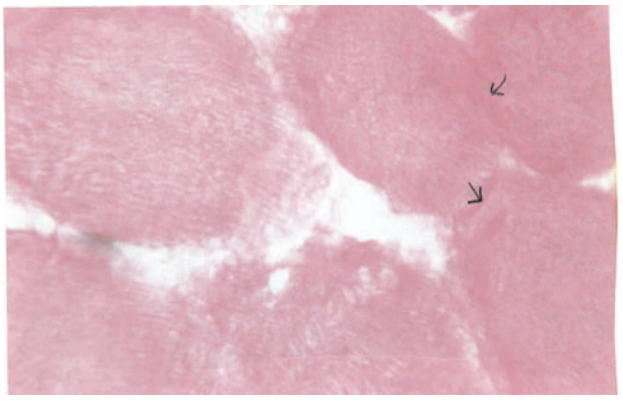

Figure 2. T.S of gastrocnemius muscle of 30 mg NaF treated rat for 15 days (Ninhydrin Schiff 400×) showing fusion of hypertrophied fibers and moderately stained sarcoplasm.

In the treatment group with NaF at 45 mg/kg bw, the hypertrophied Type II muscle fibers revealed focal necrotic areas with decrease in proteins concentration. In hypertrophied Type I fibers, the proteins were found concentrated in the sarcolemma. This region revealed moderate staining reaction with Ninhydrin Schiff reagent (Figure 3). Both Type I and Type II muscle fibers revealed atrophy and retraction from endomysial sheath in the treatment group with NaF at 75 mg/kg bw for 15 days. The sarcolemma showed invagination and disintegration along with low intensity of proteins. In the degenerative muscle fibers, the lightly stained patches from centre towards periphery became prominent. The endomysium exhibited moderate intensity of protein (Figure 4).

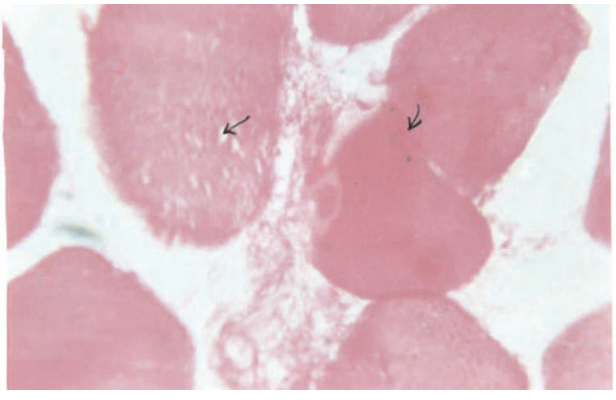

Figure 3. T.S of gastrocnemius muscle of 45 mg NaF treated rat for 15 days (Ninhydrin Schiff 400×) showing focal necrotic areas .

In the second phase of experiment, the animals were administered with NaF at 30, 45, and 75 mg/kg bw for 30 days. The cytochemical abnormalities noted were as follows:

In rats treated with NaF at 30 mg/kg bw /day for 30 days, the elevated level of proteins had been noted at the site of fusion of muscle fiber, endomysium and marginal fibers. The muscle fibers contained alternating dark and light region which was stained in deep and light, respectively. The staining pattern for proteins has been more intense in the sarcoplasm and sarcolemma as compared to the treatment group with NaF at 75 mg/kg bw for 15 days (Figure 5). In the treatment group with NaF at 45 mg/kg bw, there was

formation of angular fibers with a small subsarcolemmal rim of staining showed increase in intensity of proteins, while the sarcoplasm was very faintly stained. The sarcolemma of most of the groups of fibers showed fusion as well as invagination and revealed intense localization of protein (Figure 6). The number of rim fibers and angular fibers were more in the treatment group with NaF at 75 mg/kg bw. In these angular fibers, the marginal regions have prominent darkly stained rings of sarcoplasmic proteins. The disintegrated and split fibers were moderately stained. The sarcoplasmic masses were faintly stained, whereas necrotic areas were unstained (Figure 7).

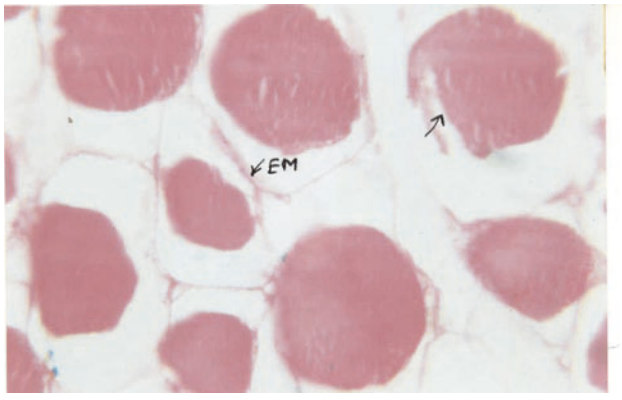

Figure 4. T.S of gastrocnemius muscle of 75 mg NaF treated rat for 15 days (Ninhydrin Schiff 400×) showing prominent disintegrated muscle fibers and invagination of sarcolemma.

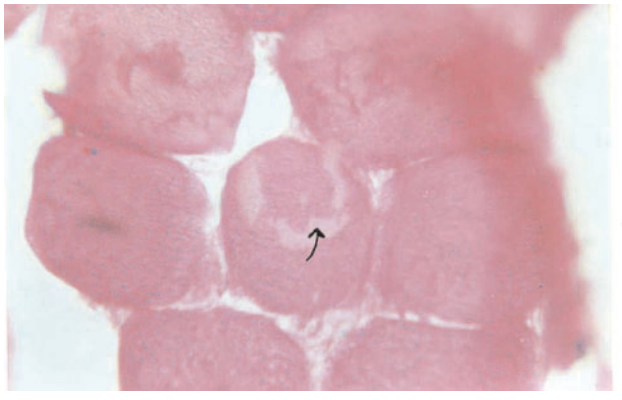

Figure 5. T.S of gastrocnemius muscle of 30 mg NaF treated rat for 30 days (Ninhydrin Schiff 400×) showing elevated level of protein at the site of fusion of fibers and patches of lightly stained sarcoplasm.

The cytochemical examination was made to study the effect of NaF on localization of DNA and RNA in the gastrcnemius muscle of rat at two different phases of experiment i.e 15th and 30th day.

In control rat, both DNA and RNA were present in sarcoplasm in appreciable amount. The endomysium faintly stained for nucleic acids. The DNA and RNA concentration was more towards sides of muscle fibers (Figure 8).

In the first phase of the treatment group with NaF at 30 mg/kg bw, the DNA concentration was more in the hypertrophied marginal muscles towards the periphery, whereas in atrophied muscle fibers, the quantity of RNA has been intensified (Figure 9). In the treatment group with NaF at 45 mg/kg bw, the DNA amount in hypertrophied and atrophied muscle fibers had been decreased greatly, but in endomysium DNA was present in the form of greenish patches. The granular RNA was very prominent in the majority of muscle fibers indicating increased synthesis of RNA. The sarcolemma showed strong intensity for RNA (Figure 10). The RNA was found concentrated in the centre of necrotic muscle fibers in the treatment group with NaF at 75 mg/kg bw. Some fibers were deeply stained for DNA towards the periphery (Figure 11).

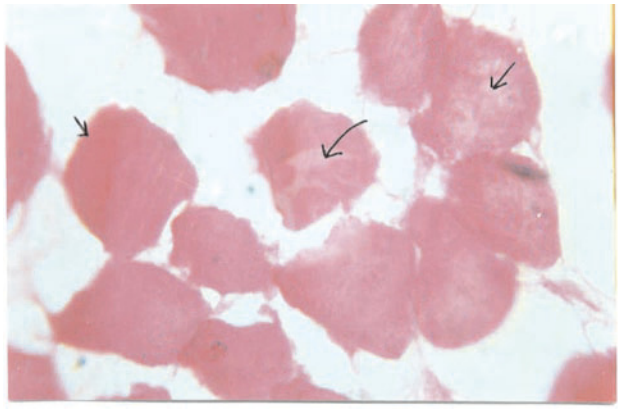

Figure 6. T.S of gastrocnemius muscle of 45 mg NaF treated rat for 30 days (Ninhydrin Schiff 400×) showing darkly stained rings of sarcoplasm in angulated muscle fibers.

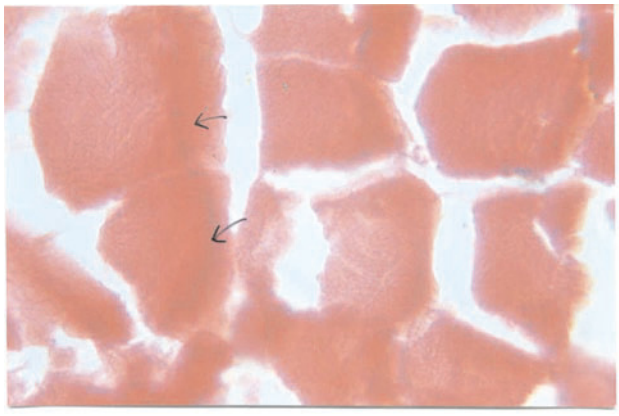

Figure 7. T.S of gastrocnemius muscle of 75 mg NaF treated rat for 30 days (Ninhydrin Schiff 400×) showing very intensely stained rings of sarcoplam in subsarcolemmal regions.

In second phase of experiment for 30 days, in the treatment group with NaF at 30 mg/kg bw, the localization of RNA and DNA was more towards the marginal fibers. The sarcolemma showed most intense activity for RNA (Figure 12).

In the treatment group with NaF at 45 mg/kg bw for 30 days, the DNA amount slightly increased from 75 mg NaF and decreased in comparison to groups from control group to the treatment group with NaF at 30, 45, and 75 mg/kg bw for 15 days. The RNA content was also decreased from control group, the treatment group with NaF at 30, 45, and 75 mg/kg bw for 15 days and 30 mg NaF for 30 days. The DNA intensity was less whereas the RNA level was elevated in the muscle fibers. The sarcoplasm showed increased formation of RNA granules in all muscle fibers. The subsarcolemmal nuclei were deeply stained for DNA. The sarcolemma showed moderate staining pattern for nucleic acids (Figure 13). In the treatment group with NaF at 75

mg/kg bw, the RNA level increased intensively whereas the DNA content remained stable. The RNA amount elevated in the endomysium and stained deeply (Figure 14).

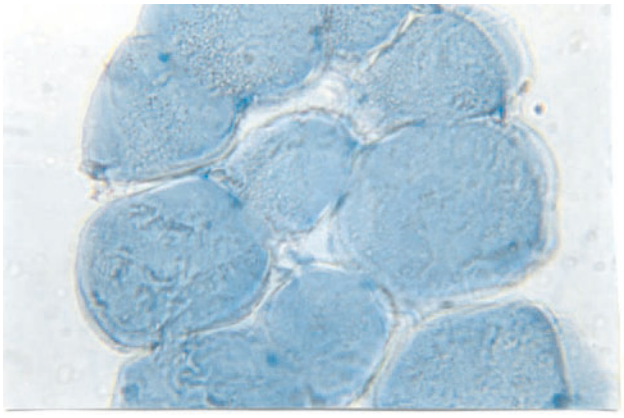

Figure 8. T.S of gastrocnemius muscle of control rat (Methyl Green Pyronin Y 400×) showing localization of DNA and RNA in sarcoplasm of muscle fibers.

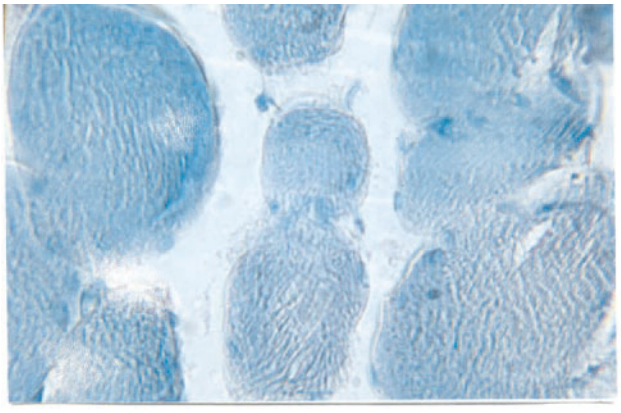

Figure 9. T.S of gastrocnemius muscle of 30 mg NaF treated rat for 15 days (Methyl Green Pyronin Y 400×) showing intense staining reaction for DNA in hypertrophied region and intensified RNA in atrophied region.

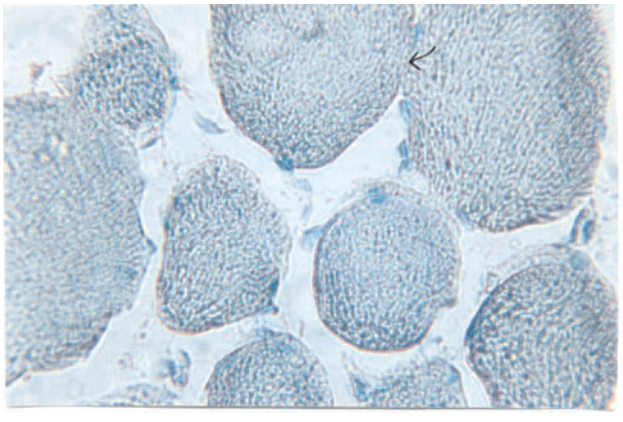

Figure 10. T.S of gastrocnemius muscle of 45 mg NaF treated rat for 15 days (Methyl Green Pyronin Y 400×) showing fusion of hypertrophied fibers. The DNA stainedlight and RNA stained dark.

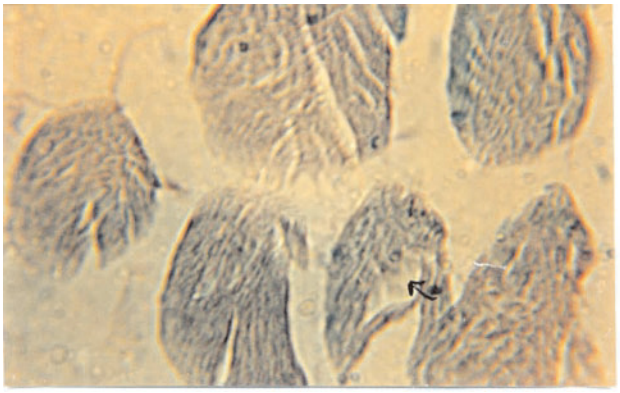

Figure 11. T.S of gastrocnemius muscle of 75 mg NaF treated rat for 15 days (Methyl Green Pyronin Y 400×) showing elevated level of RNA and necrotic areas.

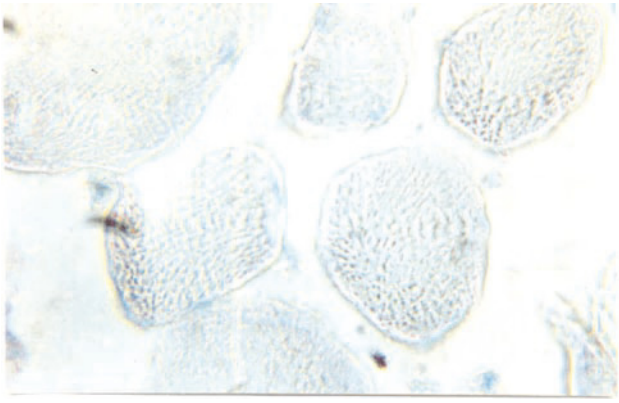

Figure 12. T.S of gastrocnemius muscle of 30 mg NaF treated rat for 30 days (Methyl Green Pyronin Y 400×)showing localization of DNA and RNA towards marginal fibers.

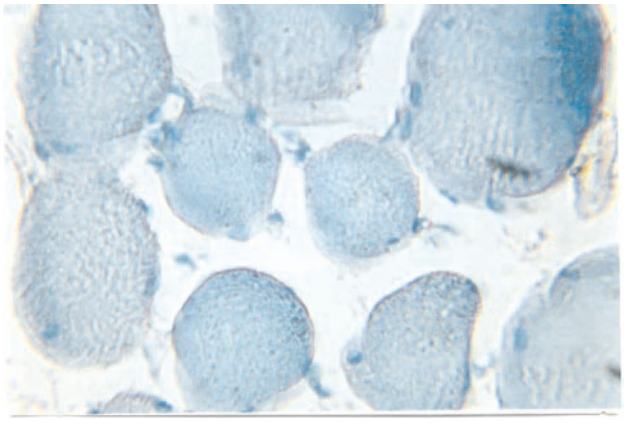

Figure 13. T.S of gastrocnemius muscle of 45 mg NaF treated rat for 30 days (Methyl Green Pyronin Y 400×) showing intense staining reaction for DNA and RNA in the subsarcolemmal region.

4. Discussion

In the present study, decline in gastrocnemius muscle protein of rats treated with NaF at 30, 45, and 75 mg/kg bw for 15 days has been observed.

The fascicular atrophy, which is the characteristic feature in muscle undergoing neurogenic atrophy found in most of fibers could be attributed to the inhibition of the protein synthesis by fluoride[2,3].

Fluoride is known to cause altered protein levels by forming complexes with proteins or by inhibiting its synthesis or due to alteration in protein metabolism[11]. Earlier higher degradation of protein and amino acids in rabbits treated with NaF at 5–50 mg/kg bw for three and half months[12] and

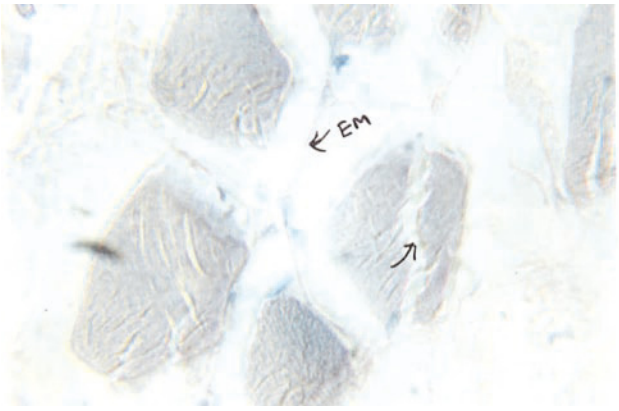

Figure 14. T.S of gastrocnemius muscle of 75 mg NaF treated rat for 30 days (Methyl Green Pyronin Y 400×) showing elevated amount of RNA in the endomysium and moderate color reaction for DNA in the sarcoplasm of subsarcolemmal region.

in mice treated with NaF at 10 mg and 200 mg/kg bw and aluminium fluoride for 30 days, has been reported[13]. It results in altered protein and oxidative metabolism and affects the contractive pattern of gastrocnemius muscles. Vani and Reddy[14] reported accumulation of fluoride and altered activities of some enzymes involved in free redical metabolism and membrane function in whole gastrocnemius muscle of female mice treated with NaF at 20 mg/kg bw for 14 days. The body weight and organo somatic index were significantly increased in gastrocnemius muscles. But in the dose group of rat treated with NaF at 30, 45 and 75 mg/kg bw for 30 days, the elevated level of proteins has been noted at the site of fusion of muscle fiber, endomysium and marginal fibers. Ekambaram and Paul[15] studied the effect of calcium on toxic effects of fluoride on adult female Wistar rats. They were treated with 500 ppm NaF in drinking water alone or in combination with $CaCO_3$ at 50 mg/kg/day for 60 days and found a decrease in concentration of protein and calcium in serum and skeletal muscle.

During present study, the hypertrophied peripheral muscle fibres contain more DNA, however, the atrophied fibres were having more RNA in the rats treated with NaF at 30 mg/kg bw, while decreased in DNA content and localization of RNA in sarcolemmal region was observed in the rats treated with NaF at 45 and 75 mg/kg bw. This may be taken as reason for decrease in protein concentration. Chinoy *et al*[16] demonstrated that the decrease in protein level occurred because of adverse or degenerative effect of fluoride, partly this effect perhaps also taken place of DNA. Some studies suggested that high concentration of NaF inhibited the synthesis of DNA and RNA in endocrine glands[17,18] and DNA strands breaks in rat osteoblast[1]. Bhatnagar *et al*[19] reported the concentration dependent total protein decrease in brain and muscle and increase in liver of female mice intoxicated with NaF at 60 and 120 ppm in drinking water for 30 days. He and Chen[20] gave Sprague–Dawley rats with NaF at 150 mg/L in the drinking water and after 4 weeks found that fluoride had induced oxidative stress and DNA damage, leading to apoptosis and cell cycle changes in rat oral mucosal cells and hepatocytes.

In conclusion, the findings of present study demonstrated that certain concentrations of fluoride can damage DNA, RNA, and protein in muscle cells and excessive intake and accumulation of fluoride is therefore a serious risk factor for muscular abnormalities in fluorosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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