Research Article

Fluoride Facilitates Contraction of Duodenal Smooth Muscle in Rat by Inhibiting the Enzymatic Activity of Acetylcholinesterase and Promoting Oxidative Stress

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ABSTRACT

The aim of the present study was to examine the effects of fluoride on the contraction of visceral smooth muscle of duodenum, the initial segment of small intestine, in rat model. We have observed significant potentiation of the movement of the duodenum ex vivo in a dose and duration dependent manner in sodium fluoride exposed rats compared to control group of rats (7.5 mgNaF/KgBW/Day, 15 mgNaF/KgBW/Day, 30 mgNaF/KgBW/Day for 14 and 28 days durations). This result indicates that fluoride potentiates the duodenal movement probably by promoting the contraction of visceral smooth muscle found in the wall structure of duodenum. Further, a significant inhibition of the enzymatic activity of acetylcholinesterase (AChE) in duodenal smooth muscle homogenate in fluoride exposed groups of rats has been observed. This result suggests that fluoride induced potentiation of the contraction of duodenal smooth muscle might be due to inhibition of the enzymatic activity of AChE. To examine the involvement of smooth muscular oxidative stress in fluoride induced potentiation of smooth muscle contraction of duodenum, the effects of fluoride on oxidative stress variables have been studied. We have observed significant inhibition of the enzymatic activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferase (GST) and increase in the level of malondialdehyde (MDA) in smooth muscle homogenate of duodenum in exposed groups of rats. These findings indicate that fluoride might facilitate smooth muscle contraction by inducing oxidative stress. We have also found significant cytoarchitectural changes in stained duodenal wall structure in exposed groups of rats. This result suggests that fluoride may promote structural degeneration of muscle probably by inducing oxidative stress. In conclusion, fluoride potentiates the contraction of duodenal visceral smooth muscle of rat probably by inhibiting the enzymatic activity of AChE and inducing cellular oxidative stress.

Keywords: Fluoride, Duodenum, Acetylcholinesterase (AChE), Oxidative stress variables.

INTRODUCTION

Fluoride is involved in the process of physiological homeostasis of human body as one of the trace bio-elements. Although, the amount of fluoride in the human body in excess of the threshold amount (1.5ppm) may shift the set-point stability of some critical variables that lead to maladies or disorders in functions of many organ systems of humans[1-4]. Drinking water contamination with fluoride is one of the critical environmental health issues at present in India[5-10]. The gastrointestinal tract (GI) is the primary target organ for fluoride induced toxicity due to direct exposure of its tissues to fluoride during digestion and/or absorption of fluoride intoxicated food stuffs or drinking water[11]. The movement of small intestine in vivo helps in the digestion and absorption of digestive end products and the said movement is provided by the contractile function of visceral smooth muscle found in the wall structure of the small intestine[12-14]. It is expected that fluoride may alter the contractile function of small intestinal visceral smooth muscle due to chronic exposure of the smooth muscle cells to fluoride during digestion and absorption of fluoride contaminated food stuffs or drinking water in humans. The fluoride induced impairment of the contractile function of visceral smooth muscle could be considered to study the fluoride induced intoxication in small intestinal visceral smooth muscle. Therefore, the present study was undertaken to examine the probable toxic effects of fluoride on the contractile function of visceral smooth muscle found in the wall structure of the duodenum, the initial part of the small intestine, in the albino rat model.

The toxic health effects of fluoride on gastrointestinal (GI) tract function have been reported based on studies in animal models and affected humans indiscriminately [15-19]. Besides, several research studies have revealed that fluoride affects different organ systems in animals by producing oxidative stress-induced damages in tissues [20]. It has been reported that NaF increases reactive oxygen species (ROS) generation and malondialdehyde (MDA) level by decreasing the enzymatic activities of antioxidant enzymes in mouse liver [21,22]. But the probable toxic effects of fluoride on the motor function of duodenal visceral smooth muscle have not been reported till to date in rat model. Damage of duodenal smooth muscle layers due to chronic fluoride exposure has been observed by Shashi A,

(2002) in young albino rabbits [23]. Another research finding suggests that due to chronic exposure of fluoride causes degeneration in lamina propria and muscular tissues, increase in number of goblet cells and breakage of villi in mice intestine[15]. It has also been reported that fluoride exposure alters gastrointestinal motility by modifying the activity of cholinergic signaling pathway in mice [24]. Fuji and Tamura, (1989) had shown that excess oral ingestion of sodium fluoride causes dilatation of blood vessels and inhibition of blood flow rate in the gastrointestinal mucosa leading to redness due to lack of blood circulation in the rat model [18]. Further, it has been seen that the exposure of fluoride also decreases the density of nNOS neurons at the myenteric plexus of rat's intestinal smooth muscle layers [25,26]. The oral administration of fluoride significantly enhances the process of lipid peroxidation and damages the small intestinal epithelial cell brush border membrane indicating the fluoride-induced alteration of the antioxidant system of intestinal smooth muscle cells in the rat model have also reported [20,27]. Furthermore, it has been reported that fluoride exposure induces the inflammation of the intestine to alter the gut micro biome which may produce intestinal abnormalities[28]. Clinically, the structural and functional abnormalities of the gastrointestinal tract due to chronic fluoride exposure through drinking water in the human subject have been reported by Das et. al., 1994 [16]. Fluoride intoxication also indirectly stimulates the occurrence of inflammatory bowel syndrome (IBD) [17]. So, from the above research findings, it is evident that fluoride may cause intoxication in smooth muscle of small intestine in human beings. So, the present study was designed to examine the effect of fluoride on the contractile function of duodenal visceral smooth muscle in fluoride exposed groups of rats. The knowledge obtained from the study could be extrapolated in human beings to let us know the probable effect of chronic exposure of fluoride on the contractile function of smooth muscle found in small intestine of human beings.

MATERIALS AND METHODS

Reagents and Chemicals

Chemicals used for this study were of analytical grade. Sodium fluoride (NaF) was purchased from E-Merck, India and sodium chloride (NaCl), potassium chloride (KCl), magnesium chloride ($MgCl_2$), sodium bicarbonate ($NaHCO_3$), sodium dihydrogen phosphate (NaH_2PO_4), dextrose, di-sodium hydrogen phosphate (Na_2HPO_4), sodium di-hydrogen phosphate (NaH_2PO_4), thiobarbituricacid (TBA), trichloroacetic acid (TCA), ethylene diamine tetra acetic acid (EDTA), bovine serum albumin (BSA), hematoxylin, eosin, copper sulfate ($CuSO_4$), sodium carbonate (Na_2CO_3), dipotassium hydrogen phosphate (K_2HPO_4), triton-X-100, pyrogallol, sodium azide (NaN_3), hydrochloric acid (HCl), nicotinamide-adenine dinucleotide phosphate ($NADPH.Na_4$, reduced), Ellman's reagent (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB), reduced and oxidized glutathione, acetyl thiocholine iodide, Folin Ciocalteu's phenol reagent, etc. were procured from Merck, India and Sisco Research Laboratory(SRL),India respectively.

Animal model

3-4 months old male albino rats of Charles Foster Strain (weight between 110-150gm) were used to carry out the study. The rats were fed standard laboratory feed and water was given ad libitum. Animals were maintained in the animal house of the Department of Physiology, University of Kalyani as per the recommendations of the Kalyani University Animal Ethics Committee.

Experimental design

After acclimatization of the experimental animals to the laboratory environment, the rats were randomly distributed into eight groups containing 7 rats (n=7) for each group. Animal grouping for the study is given below-

Exposure durations	Groups of rats (n=7)	
14 days	Control	Received 0.5ml distilled water
	Treated 1	Received 7.5mgNaF/KgBW/Day
	Treated 2	Received 15mgNaF/KgBW/Day
	Treated 3	Received 30mgNaF/KgBW/Day
28days	Control	Received 0.5ml distilled water
	Treated 1	Received 7.5mgNaF/KgBW/Day
	Treated 2	Received 15mgNaF/KgBW/Day
	Treated 3	Received 30mgNaF/KgBW/Day

Doses were selected according to the 5%, 10% and 20% of LD_{50} value of NaF (148.5mg/KgBW/day, EMerck, India followed by US-EPA 2007)[29,30]. The doses of NaF were given to the experimental animal by oral gavages.

Recording of duodenal visceral smooth muscle contraction

Contraction of duodenal smooth muscle was recorded according to our standard laboratory protocol[31]. For the functional study of duodenal contraction, the rats were sacrificed by cervical dislocation after overnight fasting; the abdomen was immediately opened and the duodenal segments (3cm each) were removed by transverse incision and were used for the recording of duodenal contraction. Duodenal segment was then transferred to organ bath containing 40 ml Tyrode's solution (8.0 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $CaCl_2$, 0.1 g/l $MgCl_2$, 1.0 g/l $NaHCO_3$, 0.05 g/l NaH_2PO_4 and 1.0 g/l Glucose, pH 7.4),continuously bubbled with 95% O_2 and 5% CO_2 and temperature was maintained at 37˚C ± 0.5. After that, continuous recording of duodenal contraction was achieved with an isotonic transducer (IT-2245) coupled to RMS-Polyrite-D (RMS, Chandigarh, India). Before applying different drugs, the isotonic contraction was recorded by a constant 0.1 g load for 30 minutes to achieve spontaneous rhythmic contraction. Recording was taken at a sweep speed= 0.937 mm/sec, deflection=1000 mm, low filter = 0.3 Hz, high filter = DC and sensitivity=10 µV.

Tissue collection, fractionation and storage

After completion of the exposure period, rats from each group were sacrificed, the abdomen was opened via a midline incision and adjacent mesentery was gently removed from the duodenal segment. Afterwards, the duodenum of animals was removed and immersed in neutral buffer formalin fixative immediately for histology. For the biochemical study, duodenal tissues were snap-frozen by liquid nitrogen and after that stored at -20^{0}C until further biochemical assays.

Duodenal tissue homogenate preparation

For biochemical studies, duodenal tissues were blotted dry, weighed quickly and 2% w/v duodenal homogenate (Tissue homogenizer, RQ-127A, REMI, India) was prepared in standard 0.1 M phosphate buffer (pH 8.0) supplemented with 2mm EDTA and 0.5% Triton–X-100. Then the crude homogenate was subjected to differential centrifugation (Cooling centrifuge, C-24BL, REMI, India) and the supernatant was collected and stored at 20^{0}C until further biochemical assays for further observations.

Biochemical study

AChE activity of duodenal tissue homogenate was assayed by the method of Ellman et. al., 1961 followed by Srikumar et al., 2004[32,33]. The activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST) were measured following the method of Marklund and Marklund, 1974[34]; Sinha AK, 1972[35]; Rotruck et al., 1973[36]; Staal et al., 1969[37]; Habig et al., 1973[38] respectively with slight modifications. Lipid peroxidation was measured as malondialdehyde (MDA) production according to the modified method of Buege and Aust, 1978[39]. The total protein estimation of duodenal tissue homogenate was done by the standard method of Lowry et al.,1951[40].

Histological staining techniques for morphological study of duodenal smooth muscle

For the histological study of duodenal smooth muscle section, the normal hematoxylin and eosin staining was performed as per the method of Bancroft et al., 2002 with slight modification at our laboratory condition[41]. About 5-7μm thick paraffin embedded section of duodenal smooth muscle tissue fixed with neutral buffered formalin was used for this study. Tissue sections at first treated with xylene and graded percentage of ethanol in a sequential manner and rinsed with distilled water. Then the section was stained with hematoxylin solution for 1-2 minutes. To remove excess stain the tissue section was washed with distilled water. The tissue section was then placed into 2% Eosin Y solution for 2 minutes. After that section was dehydrated through graded ethanol, clear with xylene, mounted with DPX, and observed under microscope (model CH20i).

Statistical analysis

The data obtained from the study were represented as mean ± SEM. The force of contractions of duodenal smooth muscle was measured in terms of amplitude and frequency. For statistical data representation, the values of the treated preparations were expressed as percent change of the basal (or control) values of duodenal contraction recording. The data were analyzed by Student's t-test and ANOVA whichever is applicable by using GraphPad Prism (version 5.03) statistical software. $p \leq 0.05$ was considered as level of significance.

RESULTS

Effect of fluoride on duodenal contraction of NaF exposed rats

A significant increase in height of contraction of duodenum has been observed in a dose dependent manner in all duration exposures of sodium fluoride exposed groups of rats compared to the control groups of rats. But the frequency of the contraction has been increased significantly in case of T-3 for both durations (Figure 1 and Figure 2).

Effect of NaF on the activity of acetylcholinesterase (AChE) enzyme

In order to study the involvement of AChE in NaF induced potentiation of duodenal contraction, the enzymatic activity of acetylcholinesterse enzyme (AChE) in duodenal tissue homogenate of NaF treated rats has been observed biochemically. It was found that the AChE activity was decreased significantly at all doses of NaF treated rats compared to control group of rats in both 14 days and 28 days durations (Figure 3).

Effects of NaF on the activities of antioxidant enzymes in duodenal smooth muscle

Results obtained from the biochemical assay indicate a significant decrease in the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione S-transferase (GST) enzymes in NaF treated duodenal smooth muscle homogenate of both 14 and 28 days exposure groups of rats compared to control group (Table 1).

Effect of NaF on malondialdehyde (MDA) production in NaF exposed smooth muscle cells

NaF significantly increased MDA production (a known marker of lipid peroxidation) in all the NaF exposed groups of rats in a dose dependent manner for all exposure groups (14 days and 28 days) compared to control groups of rats (Figure 4).
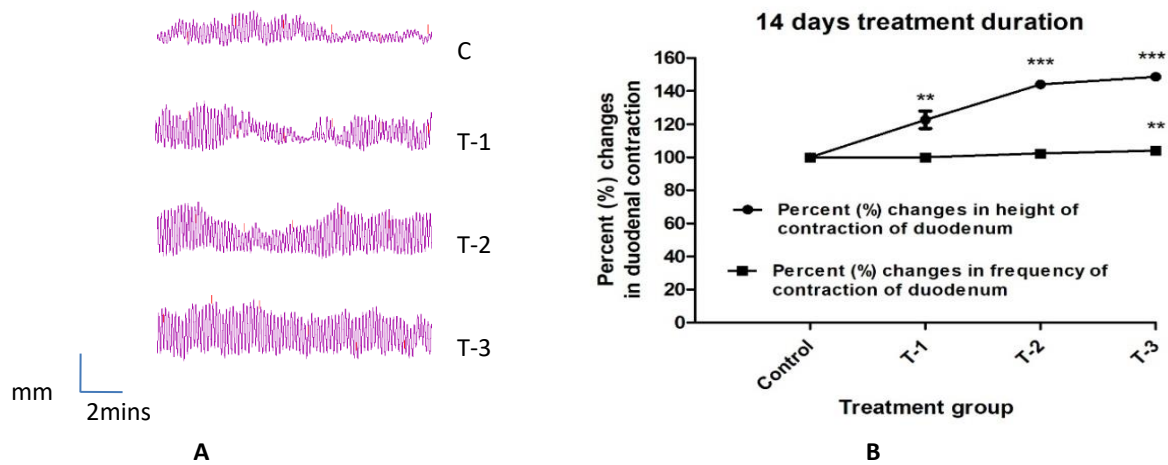
Figure 1: A. Representative records of the effect of graded doses of sodium fluoride (NaF) on the duodenal contraction. B: Line diagram showing the percent changes in height and frequency of duodenal contraction of control and treated groups. Values are expressed as Mean ± SEM (n=7), **p<0.01, ***p<0.001 vs. control. C-Control, T-1-Treated 1 (7.5 mgNaF/KgBW/day for 14 days), T-2-Treated 2 (15 mgNaF/KgBW/day for 14 days), T-3-Treated 3 (30 mgNaF/KgBW/day for 14 days).
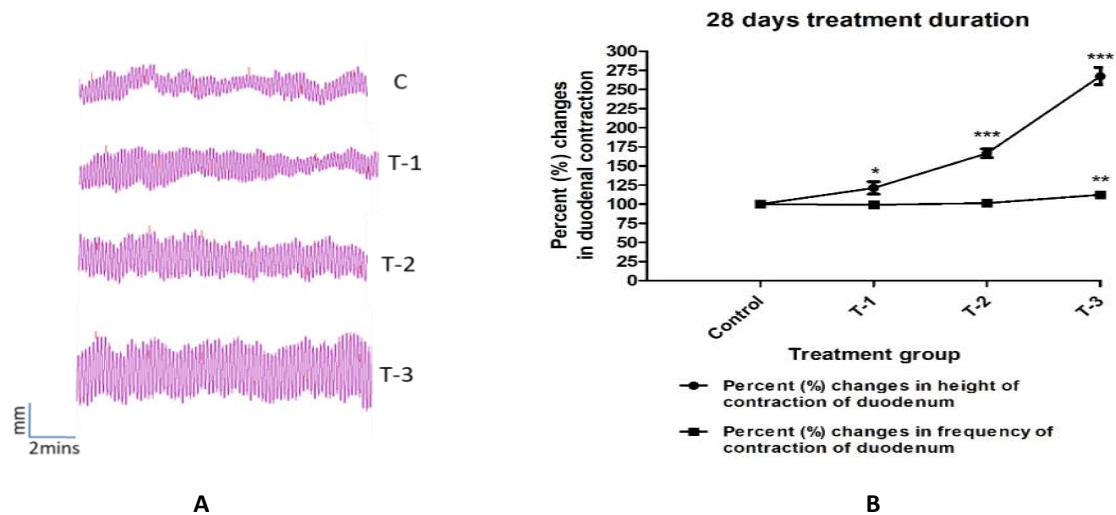

Figure 2: A. Representative records of the effect of graded doses of sodium fluoride (NaF) on the duodenal contraction. B: Percent changes in height and frequency of duodenal contraction of control and treated groups. Values are expressed as Mean ± SEM (n=7), *p<0.05, **p<0.01, ***p<0.001 vs. control. C-Control, T-1-Treated 1 (7.5 mgNaF/KgBW/day for 28 days), T-2-Treated 2 (15 mgNaF/KgBW/day for 28 days), T-3-Treated 3 (30 mgNaF/KgBW/day for 28 days).
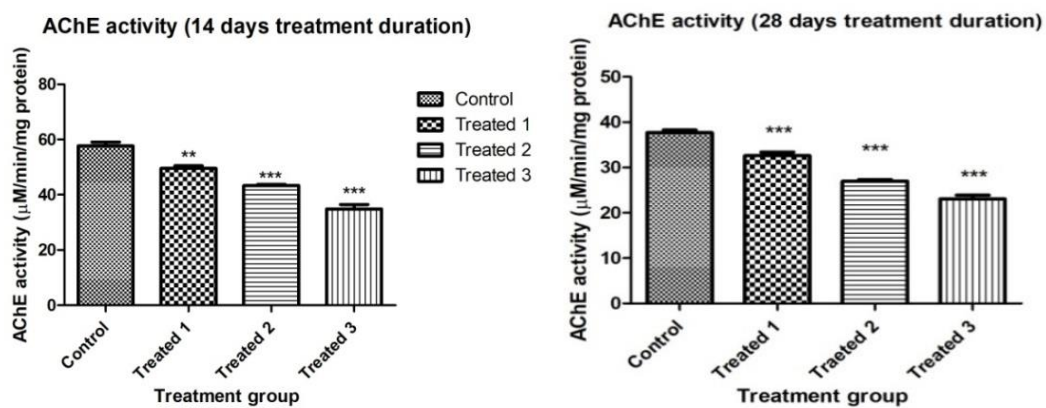

Figure 3: Bar chart showing the alterations of AChE activities in NaF treated rats for 14 days and 28 days treatment durations. Data represented as Mean ± SEM (n=5), **p<0.01 and *** p<0.001 vs Control. Treated 1- 7.5 mgNaF/kgBW/day; Treated 2-15 mgNaF/kgBW/day and Treated 3- 30mg NaF/kgBW/day for 14 days and 28 days exposure.

Table 1: Showing the changes in the activities of antioxidants enzymes of duodenal smooth muscle homogenate of NaF treated rats.

Activities of antioxidant enzymes				
SOD activity (Units/mg protein)				
Treatment duration	**Control**	**Treated 1- 7.5mgNaF/kgBW/day**	**Treated 2- 15mgNaF/kgBW/day**	**Treated 3- NaF30mg/kgBW/day**
14 days	4.497±0.211	3.417±0.131**	2.444±0.188***	2.278±0.149***
28 days	5.702±0.178	4.370±0.098***	3.410±0.171***	2.126±0.284***
CAT activity (μmoles of H_2O_2 consumed/min/mg protein)				
14 days	57.920±1.620	45.390±1.062***	42.348±0.570***	40.220±1.748***
28 days	78.674±0.631	58.374±0.363***	51.724±0.366***	33.096±0.512***
GPx activity (μmoles of GSH consumed/min/mg protein)				
14 days	5.488±0.052	5.020±0.078**	4.040±0.040***	4.076±0.043***
28 days	11.052±0.143	9.760±0.300**	7.240±0.106***	6.702±0.077***
GR activity (μmoles of NADPH oxidized/min/mg protein)				
14 days	0.132±0.001	0.086±0.002***	0.070±0.001***	0.064±0.001***
28 days	0.094±0.002	0.087±0.002*	0.062±0.004***	0.054±0.004***
GST activity (nmoles of CDNB-GSH complex formed/min/mg protein)				
14 days	74.318±1.182	69.266±1.858	65.028±1.406***	29.304±2.239***
28 days	77.204±1.556	70.416±1.068**	65.908±2.366**	45.800±3.239***

Data showing as Mean ± SEM, level of significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. control (n=5)
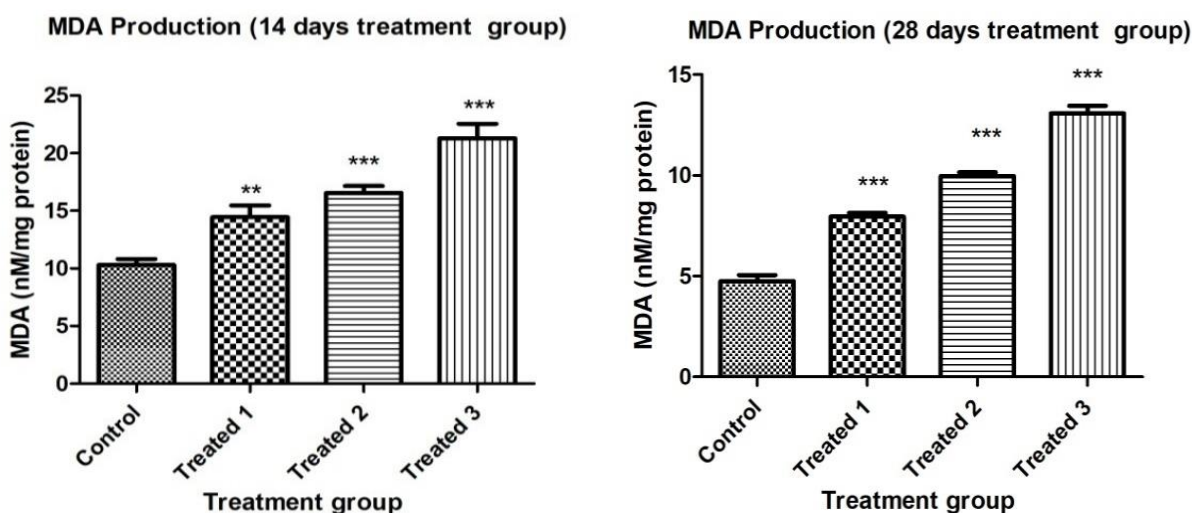

Figure 4: Bar chart showing the MDA production in NaF treated rats for 14 days and 28 days treatment duration. Data represented as Mean ± SEM (n=5), **$p < 0.01$ and *** $p < 0.001$ vs Control. Treated 1- 7.5 mgNaF/kgBW/day; Treated 2-15 mgNaF/kgBW/day and Treated 3-30 mgNaF/kgBW/day for 14 and 28 days exposure.

Effect of NaF on the histological structure of the smooth muscle layers in duodenum

Hematoxylin and eosin-stained duodenal tissue sections of control and NaF treated rats were observed under the light microscope at 100X magnification. After observing the hematoxylin and eosin-stained duodenal tissue section, it was seen that NaF remarkably altered the cytoarchitectural structure of the duodenal wall, including the inflammation of the muscularis layer and damage of the smooth muscle cell in muscularis layer in a dose dependent manner for all exposure durations (Figure 5).
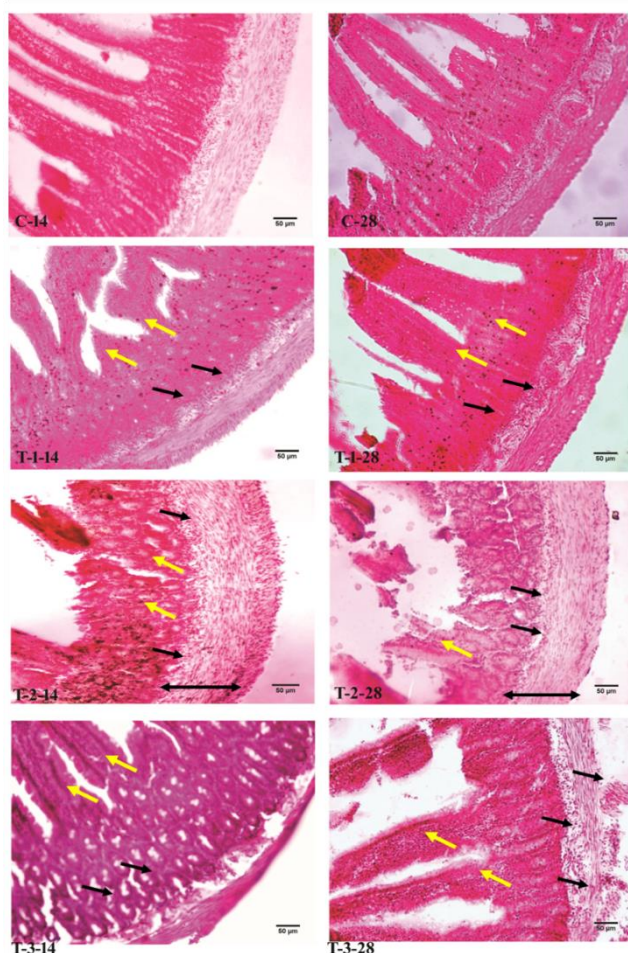
Figure 5: Photomicrographs of representative hematoxylin and eosin-stained duodenal tissue section of control and NaF exposed groups of rats. C-14 and C-28-duodenal smooth muscle tissue section of control groups of rats for 14 days and 28 days durations respectively; T-1-14 & and T-1-28-duodenal smooth muscle tissue section of NaF (7.5 mgNaF/kgBW/day) treated rats for 14 days and 28 days durations; T-2-14 and T-2-28- duodenal smooth muscle tissue section of NaF (15 mgNaF/kgBW/day) treated rats for 14 days and 28 days durations; T-3-14 and T-3-28- duodenal smooth muscle tissue section of NaF (30 mgNaF/kgBW/day) treated rats for 14 days and 28 days durations. Arrow heads indicate the changes of duodenal tissue layers structure due to NaF exposure. Black arrow heads indicate the inflammation of muscularis layer, damages of the smooth muscle cells, lesions and formation of necrotic debris in the smooth muscle layer. Yellow arrow heads indicate the thickening of the structure of villi. Both side arrow heads indicate the thickening of the muscle layers. Images were captured by digital SLR Olympus Camera (E-620) fitted with Olympus light microscope (CH20i) at 100X magnification. Bar indicates 50µM for histological analysis by ImageJ software.

DISCUSSION

In our experiment, significant potentiation of the contraction of duodenal visceral smooth muscle has been observed in a dose response manner for all exposure durations in NaF exposed groups of rats in comparison with control groups of rats (Figure 1 and Figure 2). This result indicates that fluoride induced potentiation of the contraction of smooth muscle might be due to augmentation of cholinergic intrinsic neurons and /or suppression of the activities of noradrenergic and nitrergic intrinsic neurons innervating the smooth muscle in the muscularis externa layers of the wall of the duodenum. In order to examine the involvement of cholinergic pathway in sodium fluoride induced potentiation of smooth muscle the enzymatic activity of AChE has been measured. In our result we have observed significant decrease in activity of AChE found in the smooth muscle membrane (Figure 3). Fluoride potentiates the smooth muscle contraction probably by facilitating cholinergic neuromuscular transmission. The facilitation of neuromuscular transmission might be due to prolongation of the action period of ACh released from cholinergic terminals at smooth muscle, because of the inhibition of AChE enzyme in presence of fluoride that terminates the action of ACh at neuromuscular junction by splitting acetylcholine into choline and acetate.

To correlate the fluoride induced potentiation of smooth muscle contraction and the probable oxidative stress in smooth muscle in presence of NaF, the activity of antioxidant enzymes and level of MDA have been determined in NaF exposed and control duodenal smooth muscle homogenate.

SOD and CAT are the antioxidant enzymes that provide defense against free-radicals damage in the cell. SOD destroys the superoxide-free radical by converting it into peroxide that can in turn be destroyed by CAT enzyme[42-44]. CAT decomposes the H_2O_2 into water and oxygen and neutralizes the oxidative damages[42-44]. Therefore, from the result, the decrease in activities of SOD and CAT in duodenal smooth muscle tissue homogenate might increase the level of H_2O_2 into the cell which may cause the potentiation of contraction of the duodenum (Table 1). Another antioxidant enzyme GPx helps in the detoxification process by converting the reduced glutathione (GSH) to its oxidized form (GSSG) and H_2O_2 to H_2O [42-44]. So, the decreased activity of GPx indicates that the excess production of H_2O_2 which leads to oxidative stress (Table 1). GR catalyzes the reduction of GSSG to GSH whereas GSH helps in the survival of cells from most aerobic organisms. So, the decreased activity of GR may inhibit this reaction which leads to cellular damage of duodenal smooth muscle cells (Table 1). From this study, it was observed that GST activity was also decreased after NaF exposure. GST catabolizes the conjugation of reduced glutathione (GSH) to a wide variety of endogenous and exogenous electrophilic compounds into the cell [43,44]. So, the decreased activity of GST may lead to cellular damage (Table 1). Reactive oxygen species (ROS) degrade the polyunsaturated lipids to produce MDA which is a biomarker of lipid peroxidation. MDA is a reactive aldehyde that can cause various toxic effects to the cells[45]. So, the increased production of MDA indicates oxidative stress in duodenal smooth muscle cells (Figure 4). To study the oxidative stress related morphological changes in duodenal smooth muscle layers; we have studied

histological structure of duodenal smooth muscle of NaF treated rats. We have found significant cytoarchitectural changes in the smooth muscle layers of duodenum of NaF treated rats (Figure 5). These degenerative changes of the smooth muscle of the duodenal wall may be due to changes in the antioxidant enzyme activity thus leading to cellular oxidative stress. Our research findings are supported by the results obtained from the previous research studies on liver tissue [21,46]. So, the structural degeneration of duodenal smooth muscle of NaF treated rats may be partially responsible for potentiation of duodenal contraction of NaF treated rats.

CONCLUSION

In conclusion, NaF potentiates the contraction of duodenal visceral smooth muscle. The NaF induced potentiation of duodenal smooth muscle contraction might be due to inhibition of the enzymatic activity of AChE and promotion of oxidative stress in smooth muscle cells.

Acknowledgement

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