

## Research Article



## Sodium Benzoate (NaB) Induced Impairment of the Functions of Duodenal Visceral Smooth Muscle (VSM) *ex vivo* in Rat

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### ABSTRACT

Sodium benzoate (NaB) is a versatile food additive used to preserve packaged foods and beverages. Thus, people are unknowingly consuming it above the WHO recommended daily intake limit which draws our attention to experimenting with the effects of NaB on contractile functions of visceral smooth muscle (VSM) of the small intestine, the primary target organ exposed to NaB during digestion and absorption of food, in the albino rat model. We have observed a significant ( $p < 0.05$ ) increase in amplitude and decrease in the frequency of the contraction of the duodenum, the initial part of the small intestine, in dose and duration response manner in NaB exposed groups of rats compared to control contractions. This result indicates that NaB probably impairs the contraction of duodenal VSM by promoting the activity of myenteric cholinergic and/or the activity of adrenergic and/or nitrergic efferents. We have also found, a significant ( $p < 0.05$ ) decrease in the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) and an increase in the level of malondialdehyde (MDA) in duodenal VSM homogenate of groups of exposed rats compared to control. These results suggest that NaB probably impairs the contractile function of VSM by altering the functions of myenteric intrinsic efferents by producing oxidative stress in VSM and/or in intrinsic neurons. Further, we have found significant degenerative lesions and altered tissue architecture in the muscularis externa layer of the wall of the duodenum in NaB exposed hematoxylin and eosin stained duodenal tissue sections. These findings reveal that NaB might impair the contractile function of duodenal VSM by inducing the oxidative stress induced degenerations of the architecture of muscle at tissue and cellular levels. Hence, it is concluded that NaB impairs the contraction of duodenal VSM probably by inducing the cytoarchitectural degenerations of VSM structure and/or altering the functions of myenteric intrinsic efferent through oxidative stress.

**Keywords:** Sodium benzoate (NaB), Duodenal visceral smooth muscle (VSM), Antioxidant enzymes, Tissue architecture, Oxidative stress.

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### INTRODUCTION

Sodium Benzoate (NaB) is a highly water soluble sodium salt of benzoic acid<sup>1</sup>. Due to its potent antimicrobial and antifungal properties<sup>1</sup>, it is extensively used in food industries to preserve acidic food products and beverages, condiments, various cheeses, caviar, wine, and beer, and in so many personal care products<sup>2</sup>. It attains its antimicrobial activity by lowering the intracellular pH in microorganisms, thereby decreasing the activity of glycolysis regulatory enzyme phosphofructokinase to suppress the anaerobic fermentation of glucose to restrict cellular growth<sup>3</sup>. The Joint FAO/WHO Expert Committee on Food Additives (JECFA, 1973) evaluated this salt and accepted its uses as a food preservative limiting the daily intake of NaB to about 5mg/kg body weight<sup>4-6</sup>. But, because of its versatile applications, people are unknowingly being exposed to

NaB above its recommended dose by frequent consumption of the NaB tainted processed and packaged foods and beverages. Many pieces of experimental evidence suggest that overdosing on NaB could produce adverse effects on humans and other animals. It can damage vital organs like brain<sup>7</sup>, liver<sup>8,9</sup> and kidney<sup>9</sup>; causes reproductive toxicity in both genders by damaging ovaries<sup>10</sup> and testicular tissue<sup>11, 12</sup>; hinders fetal developments<sup>13</sup>; creates cognitive impairment and problems associated with learning and memory<sup>14</sup>. Several genotoxic effects on erythrocytes<sup>15</sup> and lymphocytes<sup>16, 17</sup> were also found in excess NaB consumption besides other hematological alterations<sup>18,19</sup>. Although, many studies had been conducted to understand the effects of NaB on different organs and different systems of various experimental models, but, no experimental evidence about the effects of NaB-induced alterations in the functions of small intestinal VSM has been reported till today.

The small intestine is a vital part of our gastrointestinal system involved in the digestion and absorption of orally ingested foods. The wall of the small intestine has four layers from inside to outside which are the mucosa, submucosa, muscularis externa, and serosa. The muscle coat of the intestine has inner circular and outer longitudinal VSM fibers. An intrinsic nervous system called



the enteric nervous system (ENS) along with nerve supply from the autonomic nervous system (ANS) innervates the small intestine. The small intestine shows motility to digest and absorb food products, which is regulated by the VSM fibers and controlled by the intrinsic myenteric nerve plexus present in the muscularis externa of the wall of the intestine. Stimulation from the sympathetic adrenergic and nitrenergic myenteric efferents inhibits the contractile function of VSM of the small intestine whereas parasympathetic cholinergic myenteric efferents potentiate it.<sup>20-22</sup>

Every orally consumed chemical present in food products as an additive is exposed to our intestine and is absorbed in our body either after digestion or directly without digestion. So, there remains a possibility that excess consumption of NaB may affect the intestinal VSM activities due to its high concentration while absorbed through the intestinal villi and may generate several toxicological impacts.

As the probable toxic effects of NaB on the contractile functions of the small intestine have not been reported till date and considering the primary target organ for exposure to NaB during digestion and absorption of NaB tainted foods, the present study aimed to examine the probable toxic effects of NaB on the contractile function of VSM found in the wall structure of duodenum, the initial segment of the small intestine, due to NaB induced intoxication in VSM cells and myenteric efferent neurons.

## MATERIALS AND METHODS

### Reagents and chemicals

The reagents and chemicals which were used in this study were of analytical grade. The sodium benzoate ( $C_6H_5COONa$ ) (CAS No. 532-32-1, purity  $\geq 99.5\%$ ) was purchased from Merck Life Science Private Limited, Mumbai, India and the other reagents and chemicals were obtained from Sisco Research Laboratory Pvt. Ltd., Maharashtra, India; Merck Life Science Private Limited, Mumbai, India; and Sigma-Aldrich, St. Louis, MO, USA.

### Study animals

Adult male Swiss albino rats (*Rattus norvegicus*) aging around 90 to 120 days and weighing about 120-140 gm were chosen as the model animal for this study. They were maintained in the animal house following the guidelines of the Animal Ethical Committee of the University of Kalyani maintaining 12hr light and dark cycles at 25-27°C with a sufficient amount of standard food and water and acclimatized for 7 days in that controlled environment before initiation of the study.

### Study design

After acclimatization, the animals were randomly selected and divided into four experimental groups- Control, Exposed group 1, Exposed group 2, and Exposed group 3. Then they were exposed to NaB by oral gavage for 15 and

30 consecutive days according to the following doses mentioned in Table 1.

**Table 1:** The experimental design showing the animal grouping according to the doses of NaB used in this study.

Groups	Doses of NaB
Control	Received 0.5ml distilled water
Exposed group 1	Received 0.5 ml of 0.01 gm/kg BW/day (2.5% of LD <sub>50</sub> of NaB)
Exposed group 2	Received 0.5ml of 0.02 gm/kg BW/day (5% of LD <sub>50</sub> of NaB)
Exposed group 3	Received 0.5ml of 0.04 gm/kg BW/day (10% of LD <sub>50</sub> of NaB)

### Recording of the contraction of isolated duodenum

The contraction of the isolated duodenum of the rats from each experimental group was recorded following the standard laboratory protocol<sup>23</sup>. After 15-day and 30-day exposures to NaB, the control and NaB exposed rats were sacrificed by cervical dislocation and the duodenal segment of the small intestine was transversely incised from them. Then the lumen of those segments were cleared and placed longitudinally in the organ bath of Dale's apparatus filled with Tyrode's solution (containing 8.0 gm/l NaCl, 0.2 gm/l KCl, 0.2 gm/l CaCl<sub>2</sub>, 0.1 gm/l MgCl<sub>2</sub>, 1.0 gm/l NaHCO<sub>3</sub>, 0.05 gm/l NaH<sub>2</sub>PO<sub>4</sub> and 1.0 gm/l Glucose) of 7.4 pH. Continuous O<sub>2</sub> supply and 37±0.5°C temperature were maintained in the apparatus throughout the experimentation. The spontaneous rhythm of the duodenal contraction was recorded against a constant load by the isotonic transducer (IT-2245) connected with the RMS Polyrite D data acquisition system (RMS, India).

### Tissue collection, processing, and storage

After the planned exposure durations, all the exposed rats were sacrificed by cervical dislocation and the duodenal segment of the small intestine was collected from them, frozen in liquid nitrogen, and kept at -20°C for biochemical studies. For histological observations, the duodenum of sacrificed rats was cleared and fixed in 10% neutral buffered formalin and dehydrated with graded ethanol. Then the dehydrated tissues were embedded in paraffin (56-58°C) to prepare the permanent tissue sections for hematoxylin and eosin staining.<sup>24</sup>

### Determination of oxidative stress indices in duodenal tissue homogenate

For performing biochemical experiments in the control and NaB treated duodenal tissue of the rats, 2% w/v duodenal tissue homogenate was prepared by Tissue homogenizer (RQ-127A, REMI, India), using 0.1 M phosphate buffer (pH 8.0), 2mM EDTA and 0.5% Triton X-100 and then the supernatant was collected by centrifuging this homogenate by Cooling centrifuge (C-24BL, REMI, India). Following the standardized protocols, the studies on the activities of antioxidant enzymes and the level of lipid



peroxidation were performed by spectrophotometer (Genesys, 10S UV-VIS, Thermo Fisher Scientific, India). The activities of antioxidant enzymes viz. superoxide dismutase (SOD)<sup>25</sup>, catalase (CAT)<sup>26</sup>, glutathione peroxidase (GPx)<sup>27</sup>, and glutathione reductase (GR)<sup>28</sup> were analyzed and the level of malondialdehyde (MDA)<sup>29</sup>, a common oxidative stress biomarker produced by lipid peroxidation, was assessed.

### Histological observations

For the histological observation of the tissue architecture of the duodenum of the control and NaB exposed rats, 5-7  $\mu\text{m}$  thick sections of paraffin-embedded control and NaB exposed duodenum of the rats were obtained by rotary microtome (MT-1090 A, Weswox Optik, India). Then the tissue sections were stained by hematoxylin and eosin stain following the method of Bancroft and Gamble<sup>30</sup>. Then the muscle architecture of the duodenum was observed by Olympus light microscope (CH20i) at 100X magnification and the images of this observation were captured by Olympus digital camera (E-620) attached to that microscope. Then those photomicrographs were analyzed, using ImageJ (version 1.53k) software, to understand the NaB-induced cytoarchitectural changes.

### Statistical analysis

All the data of this study were expressed as mean $\pm$ SEM and the statistical analysis was executed by the Student's t-test. The graphical representations of the data were drawn by the statistical software GraphPad Prism (version 5.03, GraphPad Software, Inc.) considering the level of significance at  $p < 0.05$ .

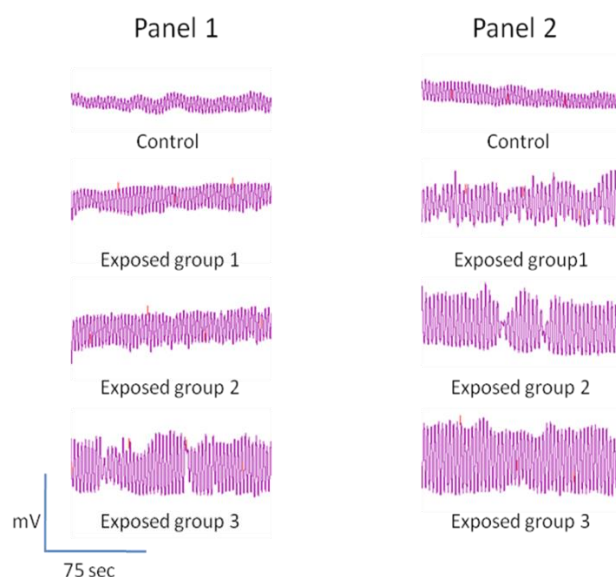
## RESULTS AND DISCUSSION

### Effects of NaB on the contraction of duodenal VSM *ex vivo* in rats

We have observed significant ( $p < 0.05$ ) potentiation of the amplitude and inhibition of the frequency of the movement of duodenum *ex vivo* in a dose and duration response manner in NaB exposed groups of rats in comparison with control groups of rats (Figure 1 and Figure 2). This finding indicates that NaB might potentiate the force of contraction and inhibit the frequency of contraction of VSM found in the muscularis externa layer of the wall structure of the duodenum which provides the movement of the duodenum.

The inherent rhythmic contraction of duodenal VSM is regulated by interstitial cells of Cajal (ICC), the intrinsic pacemaker cells, located in between circularly and longitudinally arranged smooth muscle layers in the muscularis externa layer of the wall of the duodenum<sup>20, 22</sup>. The contractile function of the duodenal VSM is controlled by three types of myenteric efferents- cholinergic efferents which are stimulatory; adrenergic, and nitrenergic efferents which are inhibitory in nature<sup>21</sup>. These myenteric efferents regulate the contractile function of duodenal VSM intrinsically by innervating the smooth muscle directly and also by innervating the ICC<sup>20, 22</sup>. In our study

NaB significantly potentiated the amplitude and inhibited the frequency of the contraction of the duodenum. From the results, we could hypothesize that NaB potentiates the force of contraction of duodenal VSM probably by promoting the activity of cholinergic myenteric efferents<sup>31</sup> and inhibits the frequency of contraction of duodenal VSM probably by promoting the adrenergic and/or nitrenergic efferents mediated inhibition of the frequency of the slow wave induced basal rhythmicity of the smooth muscle.



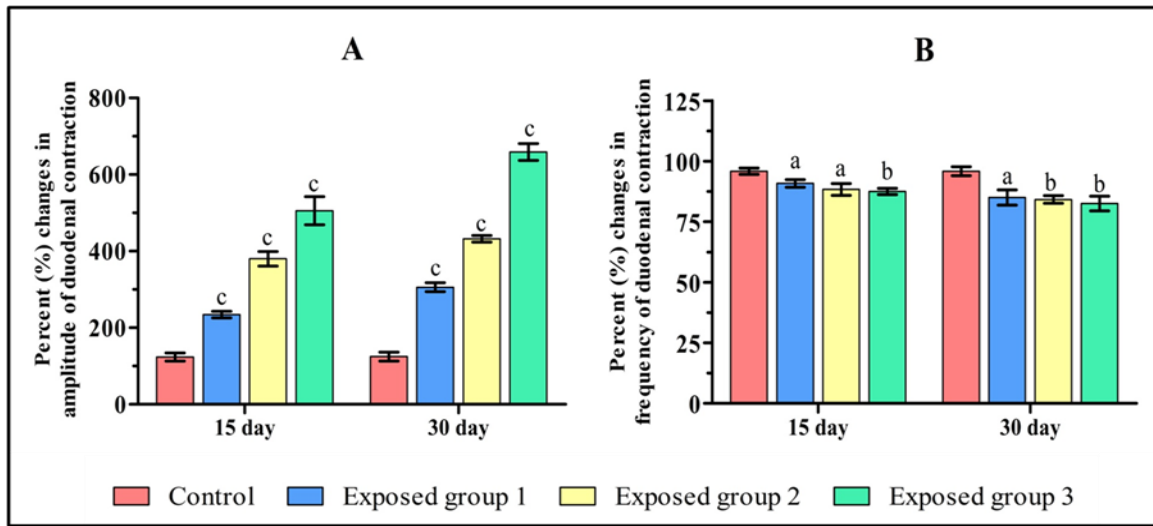
**Figure 1:** Representative records showing the effects of NaB on the movements of duodenum *ex vivo* of rats. Panel 1: Records of the movements of duodenum *ex vivo* in Control and 15 day NaB exposed groups of rats (for Control- 0 gm/kg BW/day, for Exposed group 1- 0.01 gm/kg BW/day, for Exposed group 2- 0.02 gm/kg BW/day, for Exposed group 3- 0.04 gm/kg BW/day); Panel 2: Records of the movements of duodenum *ex vivo* in Control and 30 day NaB exposed groups of rats (for Control- 0 gm/kg BW/day, for Exposed group 1- 0.01 gm/kg BW/day, for Exposed group 2- 0.02 gm/kg BW/day, for Exposed group 3- 0.04 gm/kg BW/day).

### Effect of NaB on oxidative stress related variables in duodenal VSM of rats

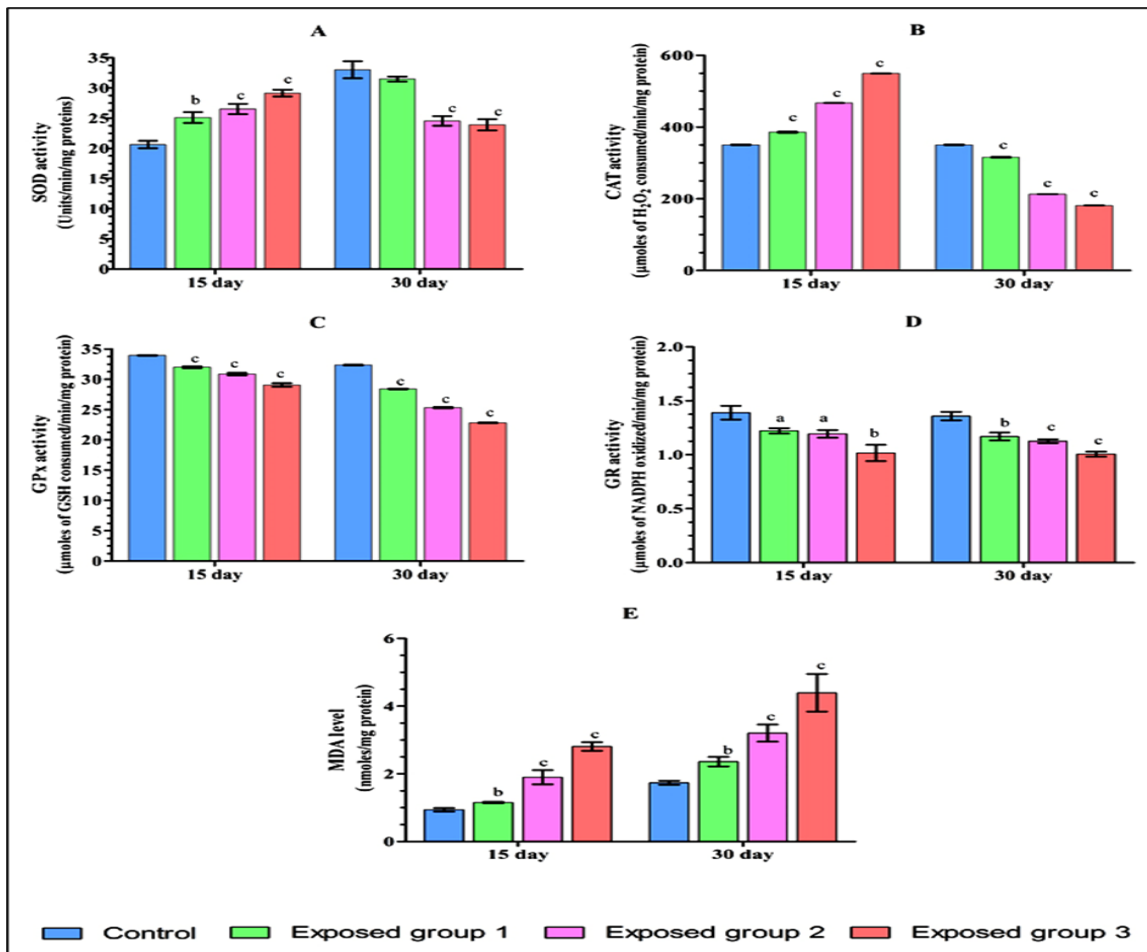
To find out the probable involvement of NaB-induced oxidative stress in duodenal VSM and intramural myenteric plexus in potentiating the force of contraction and inhibiting the frequency of contraction, the activities of the antioxidant enzymes (SOD, CAT, GPx, and GR) those scavenge the reactive oxygen species (ROS) generated in oxidative stress<sup>24, 32-35</sup>; and the level of MDA, a biomarker of lipid peroxidation<sup>36, 37</sup>, in duodenal tissue homogenate of NaB exposed and control groups of rats have been examined. In our study, we have observed a significant ( $p < 0.05$ ) decrease in the activities of SOD, CAT, GPx, and GR and an increase in the level of MDA in a dose-response manner in 30-day NaB exposed groups of rats (Figure 3). These results suggest that NaB induces oxidative stress in duodenal VSM probably by promoting the production of ROS within the duodenal VSM cells and enhancing the

peroxidation of cellular membrane lipids of smooth muscle cells and intrinsic neurons<sup>24</sup>. These findings suggest that NaB might potentiate the force of contraction and inhibit

the frequency of contraction of duodenal VSM probably by inducing oxidative stress in smooth muscle cells and the intramural myenteric plexus neurons.



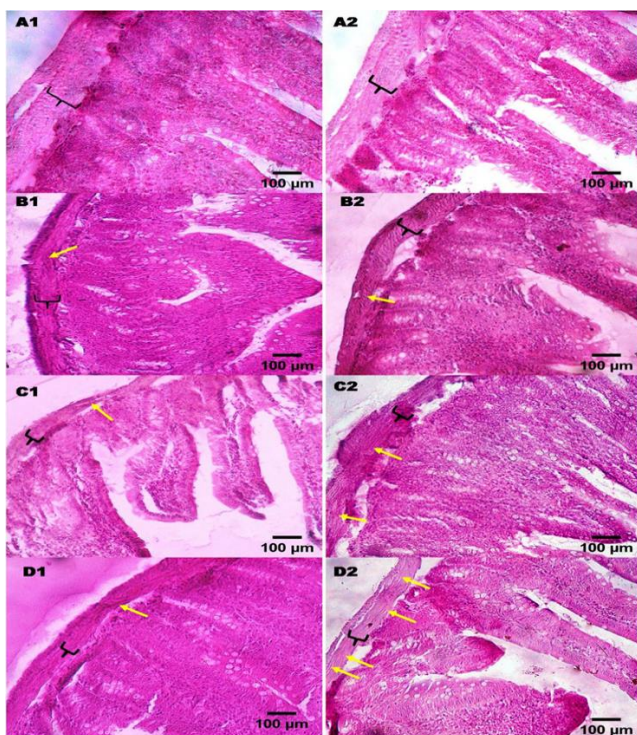
**Figure 2:** Column diagrams showing the percent changes in amplitude (A) and frequency (B) of duodenal movements of rats in response to the NaB exposure for 15-day and 30-day in exposed groups of rats compared to the control group of rats. The values are represented as mean±SEM (<sup>a</sup>p <0.05; <sup>b</sup>p <0.01; <sup>c</sup>p <0.001 vs. control) of n=5.



**Figure 3:** Column diagrams representing the effects of NaB on the activities of antioxidant enzymes and the level of MDA, a biomarker of lipid peroxidation in duodenal VSM of NaB exposed (15-day and 30-day exposure) and control groups of rats. A. Changes in the activity of superoxide dismutase (SOD), B. Changes in the activity of catalase (CAT), C. Changes in the activity of glutathione peroxidase (GPx), D. Changes in the activity of glutathione reductase (GR) and E. Changes in the level of malondialdehyde (MDA) in duodenal tissue homogenate of control and NaB exposed groups of rats. The values are represented as mean±SEM (<sup>a</sup>p <0.05; <sup>b</sup>p <0.01; <sup>c</sup>p <0.001 vs. control) of n=7.

### Effect of NaB on smooth muscle architecture in the muscularis externa layer of the duodenum:

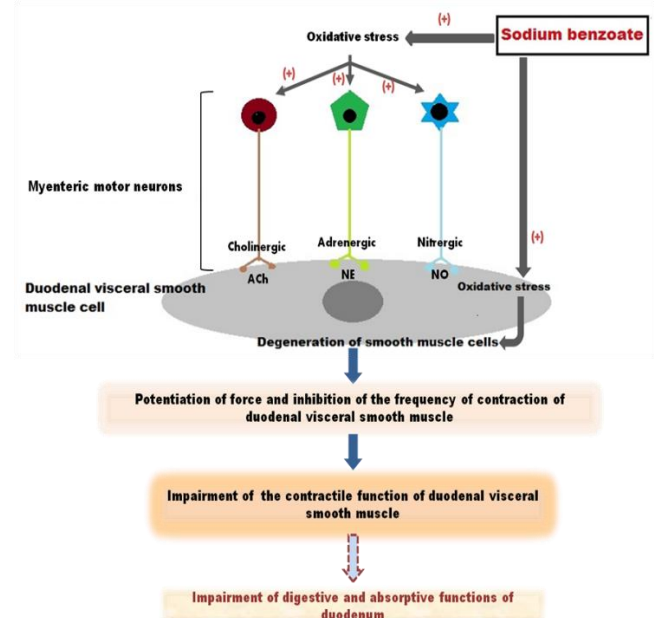
The effects of NaB on the architectural organization of circular and longitudinal smooth muscle in the muscularis externa layer of the duodenal wall have been examined to find out the probable involvement of the architectural degenerative changes of duodenal VSM in sodium benzoate induced impaired smooth muscle contraction. We have found significant marks of the lesion and necrotic lobular organizations in circular and longitudinal smooth muscles in the muscularis externa layer of the duodenal wall structure; besides, the thickness of the muscularis layer is found to be decreased probably due to degenerations of the muscle cells and the intramural myenteric neurons in a dose-response manner in hematoxylin and eosin stained permanent tissue sections of the duodenum in NaB exposed and control groups of rats (Figure 4).



**Figure 4:** Representative photomicrographs showing the hematoxylin and eosin-stained permanent tissue sections of the duodenum in control and NaB-exposed groups of rats (for 15-day and 30-day exposure durations). A1 represents the section of control and B1, C1 and D1 represent the duodenal sections of NaB exposed rats with 0.01, 0.02, and 0.04 gm/kg BW/day for 15 days. A2 represents the section of control and B2, C2 and D2 represent the duodenal sections of NaB exposed rats with 0.01, 0.02, and 0.04 gm/kg BW/day for 30 days. The arrowheads indicate lesions and necrotic lobular organizations and the brackets indicate the decreasing thickness of the muscularis externa layer of the duodenum. Photographs were taken with Olympus digital camera (E-620) attached with Olympus light microscope (CH20i); magnification: 100X. The bars indicate 100µm length analyzed by ImageJ (version 1.53k) software.

These findings indicate that NaB might promote oxidative stress mediated cytoarchitectural degenerations of the smooth muscle cells and intramural myenteric plexus<sup>31</sup>. The NaB-induced structural degenerations might be responsible for the inhibition of the frequency of smooth muscle contraction in NaB-exposed groups of rats.

Thus, from the results of this study, it is proposed that NaB impairs the contractile functions of duodenal VSM by augmenting the oxidative stress induced cytoarchitectural degeneration of VSM and myenteric motor neurons of ENS present in the wall of the duodenum. The findings on the probable mechanisms of NaB-induced functional impairment of duodenal VSM are summarized here (Figure 5).



**Figure 5:** Schematic representation showing the probable mechanism of action of NaB in impairing the contractile function of duodenal VSM in the rat. ACh: Acetylcholine, NE: Norepinephrine, NO: Nitric oxide.

### CONCLUSION

It can be concluded that NaB potentiates the force of contraction and inhibits the frequency of contraction of duodenal VSM which may impair the digestive and absorptive functions of the duodenum. The NaB induced said impairment of the contractile function of the duodenal VSM might be due to the promotion of the activities of the stimulatory cholinergic efferents and/or inhibitory adrenergic or nitrenergic efferents of the intramural myenteric plexus. The NaB-induced promotion of the activities of myenteric efferents might be mediated through induction in biochemical mechanisms to produce oxidative stress in smooth muscle cells and myenteric efferents and the pathological mechanisms involved in degenerations of the smooth muscle cells, intrinsic neurons of intramural myenteric plexus and mesenchymal ICC pacemaker cells. The results obtained from this study could be extrapolated in humans and the knowledge obtained could be utilized to mitigate NaB-induced intoxicative effects on the duodenal

motility required for digestion and absorption of the ingested food stuffs.

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