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Gallic Acid Provides Effective Protection Against Sodium Benzoate-induced Facilitation of the Contraction of Duodenal Visceral Smooth Muscle in Rat

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Abstract

People are often exposed to sodium benzoate (NaB), a versatile food preservative, above its WHO-recommended limit through NaB-tainted foods and beverages. This study was designed to examine the protective role of gallic acid (GA), a naturally occurring polyphenolic antioxidant, against NaB-mediated facilitation of the contractile function of small intestinal visceral smooth muscle (VSM) in albino rats of the Charles Foster strain following experimental, biochemical and histological studies. We observed a significant counteraction of the effect of NaB when NaB was administered with GA to obtain tracing of the contraction of the duodenum *ex vivo* in rats. Furthermore, GA significantly ($p<0.01$) counteracts the NaB-induced suppression of the activities of antioxidant enzymes- superoxide dismutase, catalase, glutathione peroxidase and glutathione reductase in dose dependent manner during co-exposure of GA with NaB. The level of malondialdehyde production was also significantly ($p<0.01$) reduced in the combined exposure of GA and NaB compared to the NaB-exposed group of rats. From light and scanning electron microscopic findings, it was observed that GA significantly counteracts the NaB-induced cyto-architectural alterations of duodenal tissue, probably induced by oxidative stress.

Major Findings: From the results it can be concluded that GA can provide effective protection against the NaB-induced facilitation of the contraction of duodenal VSM, presumably by oxidative stress.

Keywords: Duodenal Motility, Duodenal Visceral Smooth Muscle, Gallic Acid, Oxidative Stress, Sodium Benzoate

Abbreviations: ADI: Acceptable Daily Intake, BW: Body Weight, CAT: Catalase, dVSM: Duodenal Visceral Smooth Muscle, FAO/WHO: The Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO), FDA: Food and Drug Administration, FESEM: Field Emission Scanning Electron Microscopy, GA: Gallic Acid GPx: Glutathione Peroxidase, GR: Glutathione Reductase, H and E: Hematoxylin and Eosin MDA: Malondialdehyde, NaB: Sodium Benzoate, SOD: Superoxide Dismutase

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1. Introduction

Consumption of industrially processed and preserved foods has increased in recent decades with the advancement of civilisation. Therefore, food manufacturers use various preservatives to extend the shelf life of packaged foods and beverages to meet consumers' demand. Sodium benzoate (NaB) is one of the most commonly used food preservatives due to its stability, high water solubility, and strong antimicrobial property, especially at acidic condition[1,2].

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It is commonly used in a variety of products, including jam, jelly, pickles, sauces, condiments, caviar, carbonated beverages, soft drinks, beer, wine, fruit juices, cosmetics, and pharmaceutical products[3]. The FDA has considered that NaB is "Generally Regarded As Safe (GRAS)"[4] and the Joint FAO/WHO Expert Committee on Food Additives evaluates the Acceptable Daily Intake (ADI) of NaB to be 5mg/kg body weight[5]. However, excessive daily consumption of NaB-tainted foods and beverages may surpass the ADI limit. Various studies on humans and animals suggest that high levels of NaB consumption can have several harmful impacts on human health. It may damage tissues of vital organs like the kidney[6], liver[7,8], brain[9], and primary reproductive organs (testis and ovary)[10-12]. Additionally, NaB and its combinations with other preservatives may lead to genotoxicity, chromosomal aberration and DNA damage[13-15] and also cause developmental toxicity[16]. Furthermore, NaB may cause several gastrointestinal issues. It promotes gastric mucosal irritation by increasing the release of allergic mediators such as histamine, prostacyclin, prostaglandins, etc.,[17] and also leads to infiltration of immune cells into gastrointestinal mucosa, causing alteration of mucosal immunity[18]. Previously, our research conducted on a rat model also revealed that NaB exerts toxicities in duodenal tissue and impairs the contractile functions of duodenal visceral smooth muscle (dVSM)[19].

The small intestine is a vital part of the gastrointestinal tract, playing a crucial role in the digestion and absorption of ingested foods. These functions of the small intestine are facilitated by its contractile property, which results from the contraction of the VSM layer present in the wall structure of the small intestine. The intestinal lumen can get exposed to NaB through the consumption of NaB contaminated foods. Previously, we reported that high intake of NaB disrupts duodenal motility by inducing oxidative stress mediated cytoarchitectural damages of VSM and myenteric neurons found in its wall structure[19].

The antioxidants are good therapeutic agents against various diseases caused by oxidative stress[20]. GA or 3,4,5-trihydroxybenzoic acid is a well-researched naturally occurring antioxidant found in many plants as a secondary polyphenolic metabolite[21]. It is abundant in nuts, berries, pomegranates, grapes, tea leaves and vegetables[22,23]. Due to the special arrangement of hydroxyl groups in this planar molecule, GA exhibits strong antioxidant activity[24]. Researches have shown that GA can protect the vital organs like the brain[25], kidney, liver, etc. from oxidative damage[26-28] caused by toxic substances. Additionally, it has anti-inflammatory properties, which makes it useful in preventing inflammatory diseases, such as allergic inflammatory diseases (AIDs), rheumatoid arthritis, pelvic inflammation, hepatitis, pneumonia, gastroenteritis, colitis and many more[29]. Due to its antioxidant and anti-inflammatory roles, GA was considered to be used in this study to evaluate its protective efficacy against NaB-induced toxicities in the dVSM studied inthe rat model.

The probable protective role of GA against NaB-induced toxicity in dVSM has not been studied to date. Therefore, this study aimed to investigate the impact of GA on NaB-induced toxic effects on the contractile function of the dVSM, particularly involving oxidative stress.

2. Materials and Methods

2.1 Reagents and Chemicals

All the chemicals and reagents which were used in this study were of analytical grade. The sodium benzoate (C_6H_5COONa) (CAS Number: 532-32-1, purity ≥99.5%) was procured from Merck Life Science Private Limited (Mumbai, India), and the Gallic acid ($C_7H_6O_5.H_2O$) (CAS Number: 5995-86-8, purity ≥99.5%) was purchased from Loba Chemie Private Limited (Mumbai, India). All other reagents and chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), Sisco Research Laboratories Private Limited (Maharashtra, India) and Merck Life Science Private Limited (Mumbai, India).

2.2 Experimental Animals

For this study, adult male albino rats (*Rattus norvegicus*) of the Charles Foster strain, weighing between 120 and 130g and aged 12-16 weeks, were used. The procedures involving animals were conducted in accordance with the guidelines of the Institutional Animal Ethics Committee. The rats were kept in polypropylene cages in the animal house with adequate standard food and water. A 12-hour light and dark cycle and 25 ˚C-27 ˚C room temperature were maintained constantly. All animals were acclimatised in this controlled environment for 10 days before the commencement of the experiment. The Institutional Animal Ethics Committee (892/GO/Re/S/01/CPCSEA) approved this study on the Charles Foster rat (Approval no.: KU/22-11-23/02).

2.3 Study Design

The acclimatised rats were randomly divided into 8 experimental groups. The NaB and different doses of GA were applied to the study animals once daily by oral gavage method, and their applications were repeated for 15 and 30 consecutive days, as mentioned in Table 1. The doses of GA were applied after a 1hour interval of NaB administration. All these doses of NaB and GA as mentioned in Table 1 were selected from previously reported *in vivo* studies on NaB[30,19] and GA[31-34].

2.4 Animal Sacrifice and Tissue Collection

After the treatment period, the animals were sacrificed using the cervical dislocation procedure, and the duodenal segment of the small intestine was carefully removed and dissected. In this study, only the duodenum was chosen as the representative part of the small intestine. The duodenum is crucial because it is exposed to all orally consumed substances, absorbing them into the body either after digestion or directly without digestion. Experimental evidence indicates that the duodenum is the primary site for the exposure and absorption of orally ingested drugs[32,35]. Therefore, excess NaB may impact the activities of the duodenal smooth muscle, which is essential for the digestion and absorption of food. This high concentration of NaB, while being absorbed through the intestinal villi, could have adverse effects on health. Additionally, the motility pattern of the duodenum is more pronounced than that of other segments of the small intestine, which makes it easier to study the contractile activity *ex vivo*.

The duodenum part of the small intestine was collected from sacrificed rats and transversely incised into 2 cm segments. For contraction recording and biochemical analysis, different sets of duodenal segments were utilised. A 2 cm segment of the duodenum from each animal in each group was used for the contraction recording. For biochemical analysis, a tissue homogenate was prepared from distinct sets of duodenal segments collected from each rat.

2.5 Recording of the Contraction of Isolated Duodenum

The contraction of the isolated duodenal segment from experimental rats in each group was recorded *ex vivo* following our standard laboratory protocol[19,36]. The isolated duodenum was placed in the organ bath of Dale's apparatus, and motility was recorded using an isotonic transducer (IT-2245) connected with the data acquisition system of the RMS Polyrite-D machine (RMS, Chandigarh, India).

2.6 Biochemical Analysis

The duodenum was quickly excised from the sacrificed rat and rinsed with ice-cold saline. The tissue was then homogenised at a concentration of 2% (W/V) using a tissue homogeniser (RQ-127A, REMI, India) with 0.1M phosphate buffer (pH 8.0). Following homogenization, the tissue homogenate was centrifuged at 8000rpm for 10min by cooling centrifuge (C-24BL, REMI, India). After centrifugation, the supernatant was collected and stored at -20°C for further biochemical analysis[37].

The activities of antioxidant enzymes and the level of lipid peroxidation were analysed by using a spectrophotometer (Genesys, 10S UV-VIS, Thermo Fisher Scientific, India). The activity of enzyme superoxide dismutase (SOD) was determined as per the protocol proposed by Marklund and Marklund[38]. The activity of

Table 1. Experimental setup according to the doses of NaB and GA administered to the animals

Groups		Doses
Group-1	Control	0.5ml distilled water (no test compounds)
Group-2	GA-1	0.5ml of GA (5mg/kg BW/day)
Group-3	GA-2	0.5ml of GA (10mg/kg BW/day)
Group-4	GA-3	0.5ml of GA (20mg/kg BW/day)
Group-5	NaB	0.5ml of NaB (400mg/kg BW/day)
Group-6	NaB+GA-1	0.5ml of NaB (400mg/kg BW/day) + 0.5ml of GA(5mg/kg BW/day)
Group-7	NaB+GA-2	0.5ml of NaB (400mg/kg BW/day) + 0.5ml of GA(10mg/kg BW/day)
Group-8	NaB+GA-3	0.5ml of NaB (400mg/kg BW/day) + 0.5ml of GA(20mg/kg BW/day)

enzyme catalase (CAT) was measured by the method of Sinha[39]. Using the protocol described by Rotruck *et al.*, the activity of enzyme glutathione peroxidase (GPx) was assessed[40]. The activity of the enzyme glutathione reductase (GR) was examined by the method of Staal *et al*[41]. The level of lipid peroxidation was assessed by estimating the malondialdehyde (MDA) production following the method described by Buege and Aust[42].

2.7 Histo-toxicological Observations

2.7.1 Light Microscopic Observation of Duodenal Section

The duodenal segments of the sacrificed rats were processed according to the protocol described by Bancroft and Gamble for the observation of the morphology of duodenal tissues in experimental animals[43]. The tissue was fixed in 10% neutral buffered formalin solution and then gradually dehydrated in ascending grades of alcohol (70%, 90% and 100%). The dehydrated tissue was cleared by xylene and embedded in paraffin wax (56-58°C). Those paraffin-embedded tissues were sliced into 5µm thick sections using a rotary microtome (MT-1090 A, WeswoxOptik, India) and stained with hematoxylin and eosin (H and E) stain. An Olympus digital microscope (CH20i) was used to study the duodenal tissue architecture at 100x magnification, and the observed images were captured by an Olympus digital camera (E-620) equipped with the microscope. Morphological analysis of those photomicrographs was performed using image analysis software ImageJ (version 1.53k) to observe any cytoarchitectural changes compared to the control. The images were scaled, and the thickness of the dVSM layer was measured by ImageJ software in micrometres (µm).

Although histopathological evaluations were conducted without formal blinding, to reduce potential observer bias, assessments were guided by structured histopathological criteria and previously established approaches[44].

2.7.2 Scanning Electron Microscopic Observation of Duodenal Section

The duodenal samples were prepared following the standard protocol outlined by Murtey and Ramasamy for observing the ultrastructure of the duodenal tissue using a scanning electron microscope[45]. The duodenum was collected from sacrificed rats and rinsed in saline solution (0.9% W/V NaCl, 35°C). The luminal content was delicately washed out by warm saline. Then, the duodenum was cut into small tubular pieces and fixed in 2.5% V/V buffered glutaraldehyde fixative for 24 hours. The specimens were then washed in phosphate buffer (0.1M, pH 7.4) three times and placed in secondary fixative, osmium tetroxide, for 90 minutes. Thereafter, those were washed three times in distilled water and underwent dehydration by ascending grades of alcohol (50%, 70%, 90% and 100%). The samples were then kept in acetone for 1min and placed in a critical point dryer (Quorum) to dry using compressed CO_2. After that, the dried specimens were mounted on sample stabs attached with double-sided carbon adhesive and coated with a thin layer of gold using a gold sputter coater (Quorum). Finally, a topological examination of the muscle layer of the duodenal tissue samples was conducted using a Gemini 2- 450 Field Emission Scanning Electron Microscope, and images were captured at 3.5kX magnification.

2.8 Statistical Analysis

The data obtained in this study were analysed by one-way analysis of variance (ANOVA), followed by Tukey's post hoc multiple comparison test and the results were represented as mean±SEM. Analysis and graphical representation of the data acquired in this study were conducted by computerised statistical software GraphPad Prism (version 5.03, GraphPad Software, Inc.).

3. Results

3.1 Effect of Oral Exposure of NaB and GA on Body Weight and General Toxicity Observations

3.1.1 Effect on Physical Parameters

During the study period of both 15-day and 30-day experimental durations, we did not observe any changes in fur, skin and eyes of the rats of different experimental groups.

3.1.2 Effect on Body Weight

The initial and final body weight of all groups of rats was measured. However, no significant change was found in the body weight of the rats after exposure to NaB and GA.

3.1.3 *Effect on Behavioural Changes*

In all experimental groups of rats, no behavioural changes, such as lethargy, salivation, alteration in postures and respiratory distress were noticed throughout the study.

3.1.4 *Effect on Food and Water Intake*

Throughout the experiments, food consumption and water intake patterns were the same in all study animals.

3.1.5 *Effect on Mortality*

In both control and treated groups, no animal died during either experimental duration.

These observations suggest that in this study, the doses of NaB and GA applied to the experimental animals had no effects on mortality and behavioural and physical parameters.

3.2 Effect of Oral Exposure of NaB on the Contraction of dVSM *ex vivo* of Rats

From the result of this study we have observed that oral exposure to 0.4gm/kg BW/day NaB for both 15 day and 30 day treatment durations significantly increases ($p<0.001$) the amplitude (Figure 1: A) and decreases ($p<0.001$) the frequency (Figure 1: B) of duodenal contraction compared to the basal motility of duodenum as observed in the control rats in a duration dependent manner. The representative recordings of the duodenal movements obtained from the control and NaB-treated rats *ex vivo* of both 15 and 30 day exposure durations are given in Figure 1.

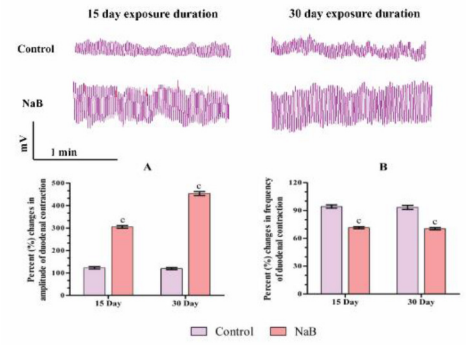


Figure 1. Representative records showing the *ex vivo* duodenal motility of the control and NaB-treated group of rats for 15-day and 30-day exposure durations. (Control: 0.5ml distilled water/day (no test compound), NaB: 400mg/kg BW/day NaB). **A:** represents the percent increase in amplitude of duodenal contraction for 15-day and 30-day exposure. **B:** represents the percent decreases in frequency of duodenal contraction for 15-day and 30-day exposure. The data are represented as mean±SEM of n=6. $^{c}p<0.001$ *vs.* Control; (c=***).

3.3 Effect of Oral Exposure to GA on the Contraction of dVSM *ex vivo* of Rats

To find out the effect of oral exposure to GA on duodenal contraction, the study animals were orally exposed to graded doses of GA (5, 10 and 20 mg/kg BW/day) for 15 days and 30 days, and the contraction of the isolated duodenum was recorded *ex vivo*. From the result of this study, no significant alterations in the force and frequency of duodenal contraction were found in GA exposed groups of rats of both 15-day and 30-day exposure durations as compared to the duodenal contraction of the control rats (Figure 2).

3.4 Effect of Post-administration of Graded Doses of GA Against NaB-induced Changes on the Duodenal Contraction *ex vivo* in Rats

To observe the protective efficacy, if any, of GA in NaB-induced facilitation of force and inhibition of frequency of contraction of dVSM, the NaB was co-administered with GA to the study animals for 15-day and 30-day exposure durations and the duodenal motility was recorded *ex vivo*. From the result of this study we have found significant opposition ($p<0.05$ *vs.* NaB; 49.84% and 67.56% protection by 20mg/kg/day dose of GA after 15-day and 30-day NaB exposure, respectively) in NaB-induced facilitation of the amplitude and decrease in frequency ($p<0.05$ *vs.* NaB; 27-29% protection by 20mg/kg/day dose of GA after 15-day and 30-day NaB exposure) of the contraction of dVSM in response to the application of NaB in association with GA recorded *ex vivo* (Figure 3 and Figure 4).

3.5 Effect of Oral Exposure to NaB on Inducing Oxidative Stress in dVSM of Rats

To find out the role of NaB-induced oxidative stress in NaB mediated alterations in duodenal contraction, the activities of the primary antioxidant enzymes were analyzed and the level of MDA, a biomarker of lipid peroxidation, was measured in the duodenal tissue homogenate of study

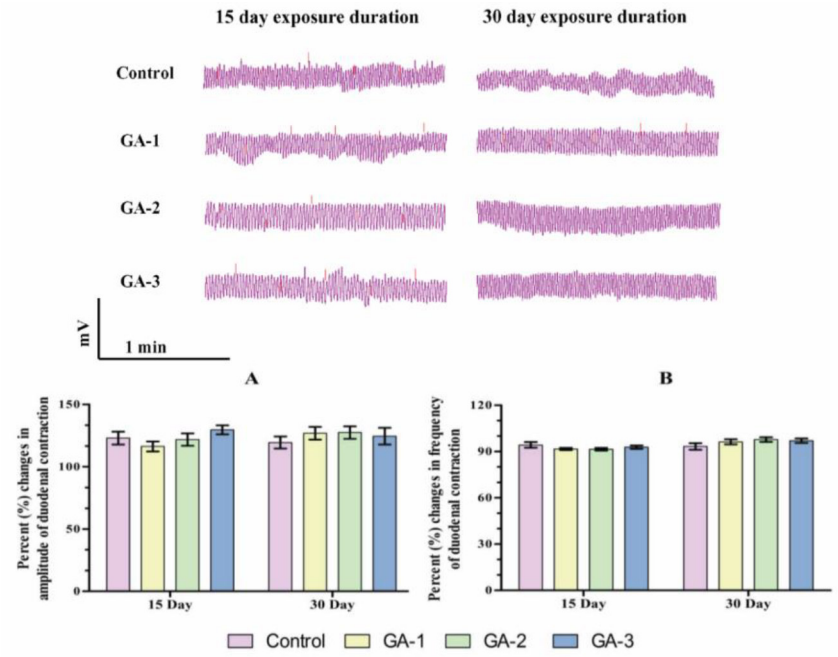


Figure 2. Representative recordings showing the *ex vivo* duodenal motility of the control and GA treated groups of rats for 15-day and 30-day exposure durations. (Control: 0.5ml distilled water/day (no test compound), GA-1: 5mg/kg BW/day GA, GA-2: 10mg/kg BW/day GA and GA-3: 20mg/kg BW/day GA). **A:** represents the percent (%) changes in amplitude of duodenal contraction for 15-day and 30-day exposure. **B:** represents the percent (%) changes in frequency of duodenal contraction for 15-day and 30-day exposure. The data are represented as mean±SEM of n=6.

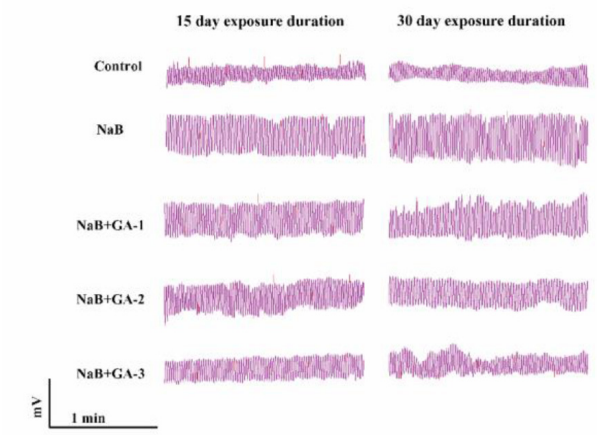


Figure 3. Representative recordings showing the *ex vivo* duodenal motility of the control and treated groups of rats for 15-day and 30-day exposure durations. (Control: 0.5ml distilled water/day (no test compound), NaB: 400mg/kg BW/day NaB, NaB+GA-1: 400mg/kg BW NaB + 5mg/kg BW/day GA, NaB+GA-2: 400mg/kg BW/day NaB + 10mg/kg BW/day GA and NaB+GA-3: 400mg/kg BW/day NaB + 20mg/kg BW/day GA).

animals. From the result obtained from these studies, as depicted in Table 2, we observed a significant increase ($p<0.001$ *vs.* Control) in the activity of SOD and CAT after 15-day NaB exposure. But, the enzyme activity of SOD and CAT was significantly decreased ($p<0.001$ *vs.* Control) after 30-day exposure duration. From the result, we also found dose dependently significant decrease in the ($p<0.001$ *vs.* Control) enzymatic activity of GPx and GR following NaB exposure for both 15-day and 30-day. A dose-dependent significant increase ($p<0.001$ *vs.* Control) in the level of MDA, a biomarker of oxidative stress induced lipid peroxidation, was observed after NaB administration for 15 days and 30 days (Table 2).

3.6 Effect of Oral Exposure to GA on Oxidative Stress Indices of dVSM of Rats

From this study, we found that in the duodenal tissue homogenate, the enzyme activities of SOD and CAT were not altered after application of GA as compared to the control (Figure 5). A significant increase ($p<0.001$ *vs.*

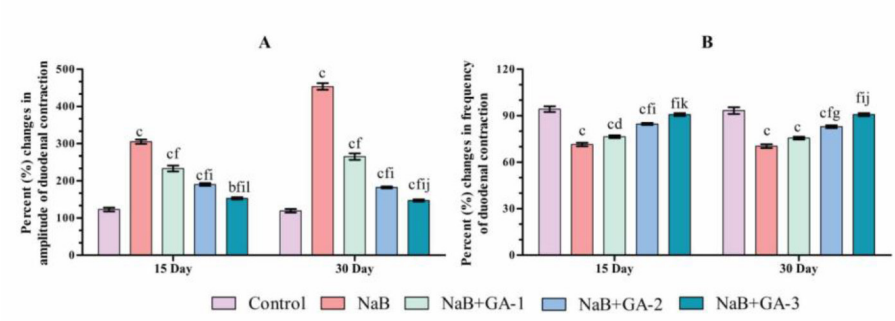


Figure 4. Graphical representations of the effects of GA on the duodenal motility of NaB exposed rats for 15-day and 30-day exposure durations. **A:** represents the percent (%) changes in amplitude of duodenal contraction for 15-day and 30-day exposure. **B:** represents the percent (%) changes in frequency of duodenal contraction for 15-day and 30-day exposure. The data are represented as mean±SEM of n=6. [b,c]p< 0.01, 0.001 *vs.* Control; [d,f]p<0.05, 0.001 *vs.* NaB; [g,i]p<0.05, 0.001 *vs.* NaB+GA-1; [j,k,l]p<0.05, 0.01, 0.001 *vs.* NaB+GA-2. (d, g, j=*; b, k=**; c, f, i, l=***)

Control) in the activity of GPx was observed compared to the control, only at the 20mg/kg BW/day dose of GA after a 15-day exposure duration. However, all graded doses of GA significantly increased (p<0.001 *vs.* Control) the GPx activity as compared with control after 30-day GA exposure (Figure 5). The activity of GR was also significantly increased (p<0.001 *vs.* Control) after both 15-day and 30-day GA exposure when compared to the control (Figure 5).

3.7 Effect of GA on NaB-induced Oxidative Stress in the Duodenal VSM of Rats

From the results as presented in Figure 6, we found that GA significantly counteracts the NaB-induced alteration of antioxidant enzyme activities when GA was administered along with NaB.

The SOD activity was reversed about 97% and CAT activity was reversed about 75.69% by 20mg/kg BW/day GA in NaB-exposed rats for 30 days (SOD activity: p<0.001 *vs.* NaB for 10 and 20 mg/kg BW/day doses of GA; CAT activity: p<0.001 *vs.* NaB for 5-20 mg/kg BW/day doses of GA) (Figure 6). The activity of GPx was highly significantly recovered (p<0.001 *vs.* NaB for each doses GA; 95% to 100% recovered by 20mg/kg BW/day GA after both 15-day and 30-day exposure durations) and the activity of GR was also significantly protected (p<0.001 *vs.* NaB for 10 and 20 mg/kg BW/day doses of GA; 69.34% and 54.83% protection by 20mg/kg BW/day dose of GA after 15 days and 30 days, respectively) by GA when GA was given after oral administration of NaB. A significant increase in the activity of GPx and GR was also found after GA exposure (Figure 6).

The NaB-induced increased level of MDA was dose-dependently and significantly restored towards normal in both durations when graded doses of GA were applied along with NaB (p<0.001 *vs.* NaB; 56.29% protection by 20mg/kg BW/day GA compared to NaB exposure) (Figure 6).

3.8 Effect of GA on NaB-induced Cytoarchitectural Alterations in the Duodenal Wall Structure of Rats

To find out the protective role of GA on NaB-induced cytoarchitectural degenerations of the duodenum, the structural organisation of histologically fixed post-mortem duodenal wall was examined following the H and E staining method under light microscope, and the ultrastructure of the dVSM layer of the study animals were observed under FESEM.

3.8.1 Light Microscopic Observations

From light microscopy, we observed that NaB remarkably altered the structural organisation of the duodenal wall and reduced the thickness of the muscularis externa of the duodenal wall in both 15-day and 30-day NaB exposure groups compared to the control groups of rats (Figure 7). However, GA effectively counteracted NaB-induced all degenerative changes in the duodenal wall structure and significantly thickened the muscularis externa layer of the duodenum in a dose and duration-dependent manner when GA was given after oral administration of NaB (Figure 8). But, no effects on structural alteration in the duodenum were found on GA-exposed groups of rats

Table 2. Showing the effect of oral exposure of NaB on the activities of antioxidant enzymes SOD, CAT, GPx and GR and the level of MDA in the duodenal tissue homogenate of rats

SOD activity (Units/mg protein)					
Effect of 15-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
19.941	0.430	30.160	0.404	300.015	<0.001[c]
Effect of 30-day exposure					
Control		NaB			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
21.177	0.889	8.327	0.240	194.785	<0.001[c]
CAT activity (µM of H_2O_2 consumed/min/mg protein)					
Effect of 15-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
272.404	4.490	420.991	10.428	171.645	<0.001[c]
Effect of 30-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
282.996	6.446	85.703	1.250	838.377	<0.001[c]
GPx activity (µM of GSH consumed/min/mg protein)					
Effect of 15-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
30.731	0.661	20.027	0.484	170.482	<0.001[c]
Effect of 30-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
31.303	0.783	16.628	0.597	234.758	<0.001[c]
GR activity (µM of NADPH oxidised/min/mg protein)					
Effect of 15-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
0.349	0.011	0.087	0.023	561.464	<0.001[c]
Effect of 30-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
0.383	0.010	0.063	0.002	891.524	<0.001[c]
MDA level (µM/mg protein)					
Effect of 15-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
1.639	0.047	4.833	0.111	610.951	<0.001[c]
Effect of 30-day exposure					
Control		**NaB**			
Mean	**SEM**	**Mean**	**SEM**	**F value**	**P value**
1.661	0.028	6.873	0.026	13715.365	<0.001[c]

Data expressed as n=6, df =10. [c] p<0.001 *vs.* Control; (c=***).

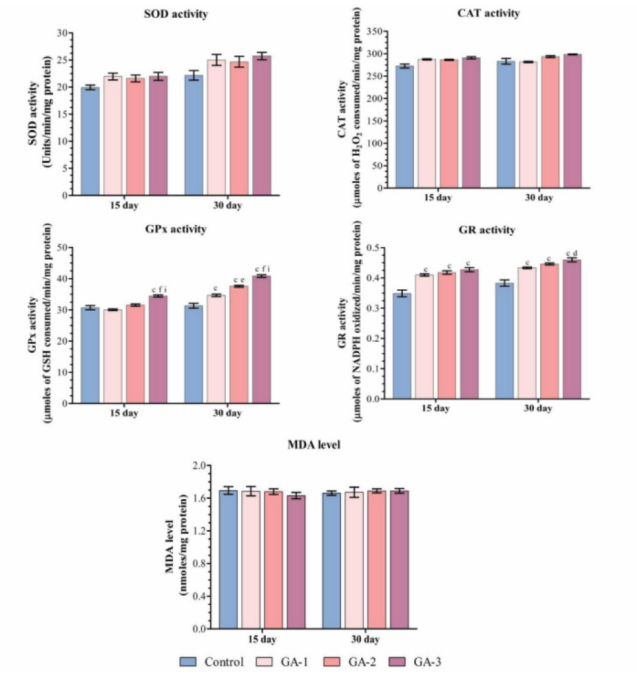


Figure 5. Graphical representations showing the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) and level of malondialdehyde (MDA) in duodenal tissue homogenate of control rats and GA exposed rats (graded doses) (Control: 0.5ml distilled water/day (no test compound), GA-1: 5mg/kg BW/day GA, GA-2: 10mg/kg BW/day GA and GA-3: 20mg/kg BW/day GA) for 15-day and 30-day exposure durations. Data are expressed as mean±SEM (n=6). $^{c}p<0.001$ *vs.* Control; $^{d,e,f}p<0.05$, 0.01, 0.001 *vs.* GA-1; $^{i}p<0.001$ *vs.* GA-2 (d=*; e=**; c, f, i=***).

compared to the control rats in both 15-day and 30-day exposure durations (Figure 7).

3.8.2 Scanning Electron Microscopic Observation

From the FESEM imaging of the duodenum, the intact muscle layers in the wall structure of the duodenum were spotted in control and GA-exposed rats. Whereas, in NaB-exposed rats of both 15-day and 30-day treatment durations, we observed prominent necrotic lesions and vacuolations in the muscularis externa layer of the duodenum and also revealed several necrotic lobular organisations in the circular and longitudinal VSM layers of the duodenum (Figure 9). However, no significant necrotic lesions were observed in the dVSM layer of the rats exposed to NaB in combination with GA, as compared to the NaB exposed groups of rats. It had been observed that the intactness of the muscularis externa layer of the duodenum was gradually restored in NaB+GA-exposed rats, especially at the 10mg/kg BW/day and 20mg/kg BW/day doses of GA (Figure 9).

4. Discussion

In the present study, toxicodynamic investigation has been performed on the VSM found in the duodenal wall of the small intestine. Duodenum is the primary site of orally ingested drug exposure and absorption[32,35]. The VSM found in the duodenal wall and also in the wall of the jejunum and ileum of the small intestine helps in the digestion and absorption of the ingested foodstuffs by providing motility[46].

We have already reported in our published paper that NaB hampers contraction of the dVSM by inducing oxidative stress in the smooth muscle cells[19]. In the present study, we report here that GA, a secondary plant metabolite[47] having anti-microbial, anti-fungal, anti-inflammatory, anti-allergic, anti-cancer, anti-hypertensive, anti-diabetic, neuroprotective and potent antioxidant effects in the biological system[48,49], protects the contractile properties of the dVSM from the toxic effects of NaB.

Figure 1 shows that oral exposure to NaB for consecutive 15 and 30 days significantly increases the force and decreases the frequency of the duodenal contraction recorded *ex vivo* in NaB-exposed rats in a duration-dependent manner as compared to the duodenal contraction of the control rats. From the results, we could suggest that NaB might exert toxicity on dVSM by inducing oxidative stress.

We have also observed significant induction of the oxidative stress in the dVSM as NaB decreases the activities of the antioxidant enzymes such as SOD, CAT, GPx and GR and increases the level of membrane lipid peroxidation biomarker, MDA in the duodenal tissue homogenate of NaB-exposed rats as compared to control rats (Table 2). We have also reported in our earlier paper that NaB probably alters structural characteristics of the dVSM, as shown by reduction of the thickness of the muscularis externa layer of duodenum, along with necrotic lesions and lobular organisations and vacuolations in the muscularis externa layer of duodenum by oral exposure to NaB in a dose and duration-dependent manner[19]. In this context, from the results, it may be suggested that the structural alterations of dVSM in response to the exposure of NaB might be due to the induction of oxidative stress.

In this present study, we have found a significant reversal of the contractile characteristics of dVSM

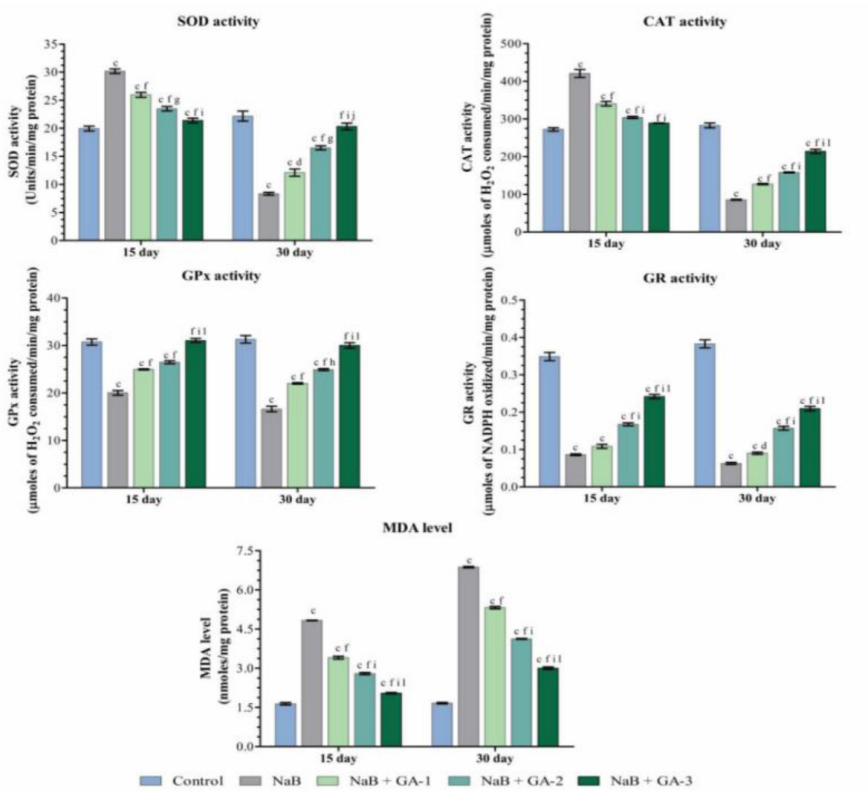


Figure 6. Graphical representations showing the activities of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GR) and level of malondialdehyde (MDA) in duodenal tissue homogenate of control rats; NaB exposed rats and NaB along with graded doses of GA exposed rats (Control: 0.5ml distilled water/day (no test compound), NaB: 400mg/kg BW/day NaB, NaB+GA-1: 400mg/kg BW/day NaB + 5mg/kg BW/day GA, NaB+GA-2: 400mg/kg BW/day NaB + 10mg/kg BW/day GA and NaB+GA-3: 400mg/kg BW/day NaB + 20mg/kg BW/day GA) for 15-day and 30-day exposure durations. Data expressed as mean±SEM of n=6. $^{c}p<0.001$ *vs.* Control; $^{d,f}p<0.05$, 0.001 *vs.* NaB; $^{g,h,i}p<0.05$, 0.01, 0.001 *vs.* NaB+GA-1; $^{j,l}p<0.05$, 0.001 *vs.* NaB+GA-2 (d, g, j=*; h=**; c, f, i, l=***).

induced by NaB by GA. We have found that oral exposure to GA for 15 and 30 days has no significant effect on the contractile function of the duodenum recorded *ex vivo* as compared to the contraction of the duodenum of control rats (Figure 2). However, GA significantly counteracted the potentiation of amplitude and inhibition of frequency of duodenal contraction induced by NaB when graded doses of GA were post-administered after NaB for 15 and 30 day exposure durations (Figures 3 and 4). These results indicate that GA could mitigate the effects of NaB on dVSM, probably by protecting from the NaB-induced oxidative stress in dVSM cells.

Furthermore, we observed a significant dose-dependent increase in the enzyme activity of SOD, CAT, GPx and GR and decreased level of MDA (Figure 6) in the duodenal tissue of rats exposed to NaB in combination with graded doses of GA as compared to the NaB-exposed rats for both 15-day and 30-day exposure durations. Scientific researches indicate that GA enhances the transcription of enzymatic and non-enzymatic antioxidants (SOD, CAT, GPx, GSH, etc.) to combat oxidative stress by scavenging the free radicals[50,26,51]. GA can modulate the Nrf2 signalling pathway[52]. Activation of Nrf2 may upregulate transcription of antioxidant enzymes[53]. Thus, GA exposure may induce the tissue to produce more antioxidant enzymes to increase their overall activity against reactive species. In this study, we observed a dose-dependent significant increase in the activity of GPx and GR in GA exposed rats (Figure 5). Study suggests that GA dose dependently promotes a reduced (GSH)/oxidised glutathione (GSSG) ratio[54], which indicates the increased activity of GR. It may also indirectly increase the GPx activity, as GSH is the substrate used for GPx. So, the result we obtained from this study implies that GA

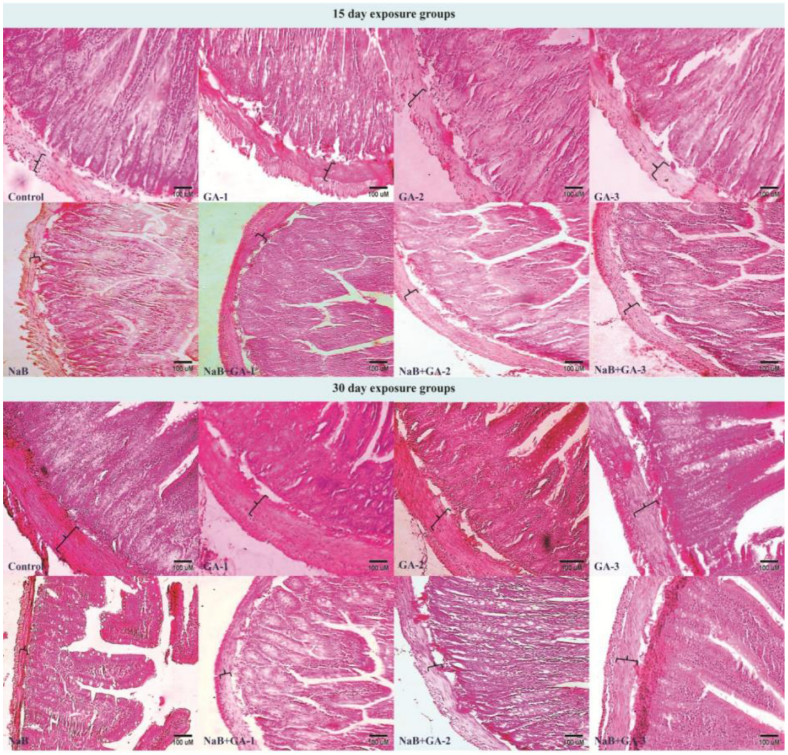


Figure 7. Representative photomicrographs showing the Hematoxylin and Eosin-stained permanent tissue sections of the duodenum in Control (0.5ml distilled water/day (no test compound)), GA (GA-1: 5mg/kg BW/day GA, GA-2: 10mg/kg BW/day GA and GA-3: 20mg/kg BW/day GA), NaB (400mg/kg BW/day NaB) and NaB+GA (NaB+GA-1: 400mg/kg BW/day NaB + 5mg/kg BW/day GA, NaB+GA-2: 400mg/kg BW/day NaB + 10mg/kg BW/day GA and NaB+GA-3: 400mg/kg BW/day NaB + 20mg/kg BW/day GA) exposed groups of rats for 15-day and 30-day exposure durations. The brackets indicate the decreasing thickness of the muscularis externa layer of the duodenum. Photographs were taken with an Olympus digital camera (E-620) attached with Olympus light microscope (CH20i); magnification: 100X. The bars indicate 100µm length.

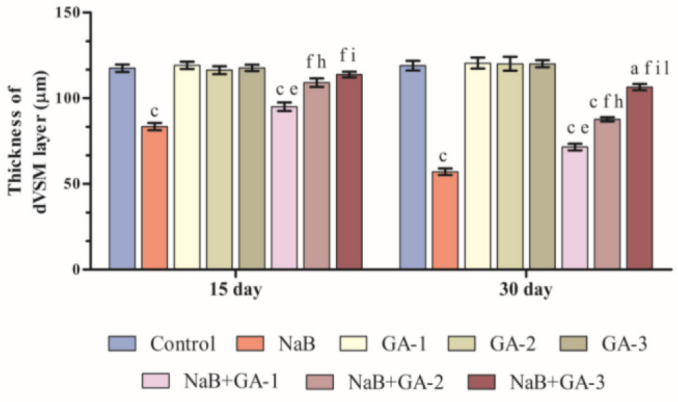


Figure 8. Graphical representation showing the alteration of the thickness of dVSM layer in Hematoxylin and Eosin-stained permanent tissue sections of the duodenum in Control (0.5ml distilled water/day (no test compound)), GA (GA-1: 5mg/kg BW/day GA, GA-2: 10mg/kg BW/day GA and GA-3: 20mg/kg BW/day GA), NaB (400mg/kg BW/day NaB) and NaB+GA (NaB+GA-1: 400mg/kg BW NaB + 5mg/kg BW/day GA, NaB+GA-2: 400mg/kg BW/day NaB + 10mg/kg BW/day GA and NaB+GA-3: 400mg/kg BW/day NaB + 20mg/kg BW/day GA) exposed groups of rats for 15-day and 30-day exposure durations. Data expressed as mean±SEM of n=6. $^{c}p<0.001$ *vs.* Control; $^{e,f}p<0.01$, 0.001 *vs.* NaB; $^{h,i}p<0.01$, 0.001 *vs.* NaB+GA-1; $^{l}p<0.001$ *vs.* NaB+GA-2 (e, h=**; c, f, i, l=***).

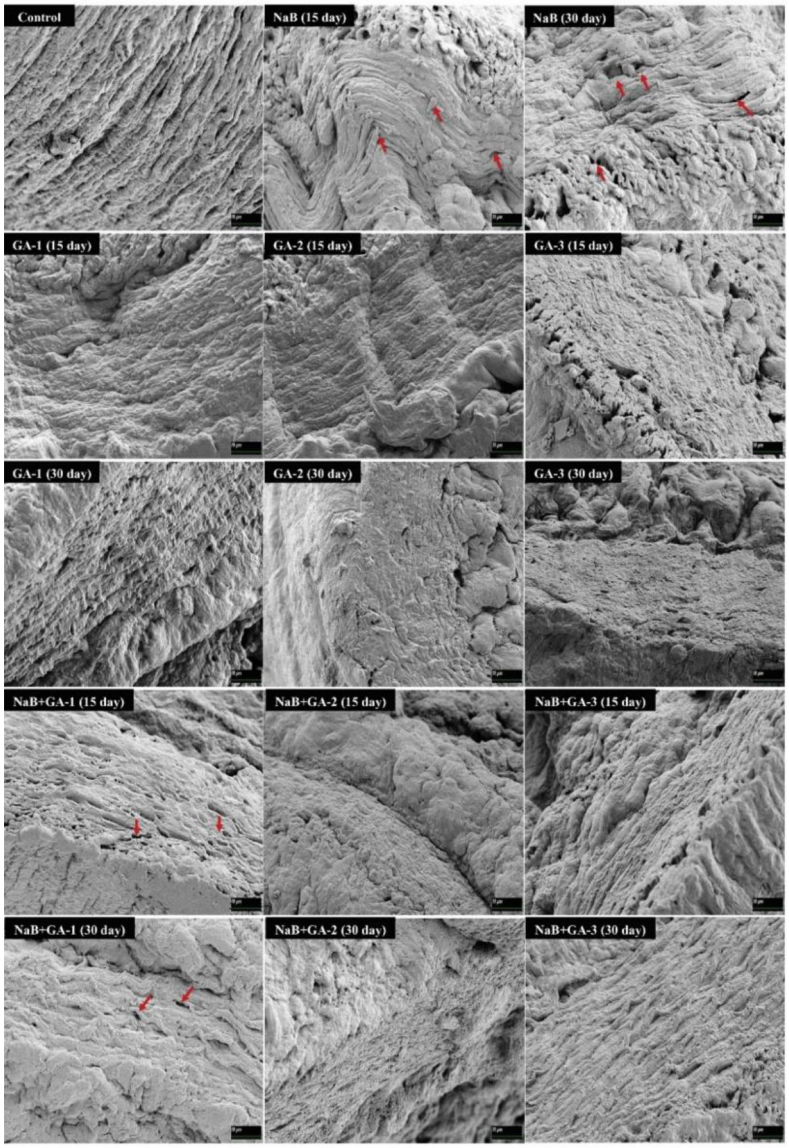


Figure 9. Representative photomicrographs showing the ultrastructure of the muscularis externa layer of the duodenal wall of the Control (0.5ml distilled water/day (no test compound)), GA (GA-1: 5mg/kg BW/day GA, GA-2: 10mg/kg BW/day GA and GA-3: 20mg/kg BW/day GA), NaB (400mg/kg BW/day NaB) and NaB+GA (NaB+GA-1: 400mg/kg BW/day NaB + 5mg/kg BW/day GA, NaB+GA-2: 400mg/kg BW/day NaB + 10mg/kg BW/day GA and NaB+GA-3: 400mg/kg BW/day NaB + 20mg/kg BW/day GA) exposed rats for 15-day and 30-day study durations. The study was performed by GeminiSEM 450-8216010130 Field Emission Scanning Electron Microscope, and the photographs were taken at 3.50kX magnification (EHT: 3.00kV, I Probe: 66 pA). The bars indicate 10µm length, and the arrows indicate the necrotic lesions.

effectively counteracts the NaB-induced oxidative stress by promoting the activities of antioxidant enzymes, which further reduces the free radicals-mediated peroxidation of membrane phospholipids and decreases the production of MDA in the duodenal tissue of NaB-exposed rats. Thus, these results suggest that GA counteracts the NaB-mediated oxidative stress in dVSM and restores the normal contractile pattern of the duodenum, presumably by preventing the oxidative stress-related damages to the duodenal tissue due to oral NaB exposures.

Further, the photomicrographs obtained from the light and electron microscopic imaging also show that the NaB-mediated all cytoarchitectural alterations of the duodenal muscle layer and reduced thickness of the

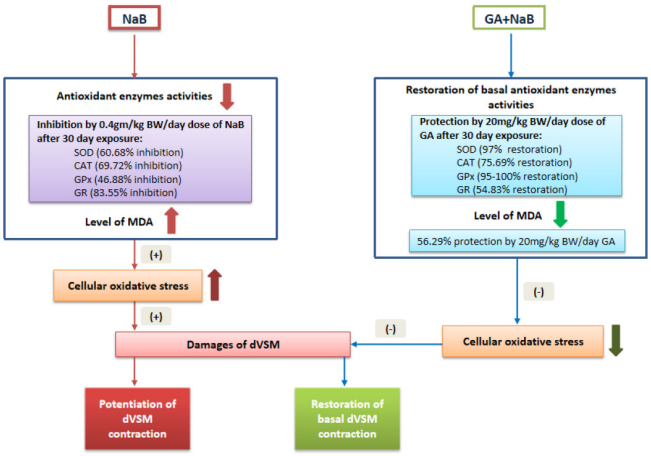


Figure 10. Schematic diagram showing the probable mechanism of action of GA in protecting the NaB-mediated facilitation of contraction of dVSM. NaB - Sodium benzoate, GA - Gallic acid, SOD - Superoxide dismutase, CAT - Catalase, GPx - Glutathione peroxidase, GR - Glutathione reductase, MDA - Malondialdehyde, dVSM - Duodenal visceral smooth muscle. (+) indicates stimulation and (-) indicates inhibition, ↑ indicates increase and ↓ indicates decrease.

muscularis externa of the duodenal wall were reversed by GA when GA was applied after NaB (Figure 7, Figure 8 and Figure 9). These findings indicate that GA, a potent antioxidant, provides protection against NaB-mediated oxidative stress and may prevent structural disruption of dVSM layers mediated by NaB. The research findings on the probable mechanisms of the protective action of GA against the NaB-induced functional impairment of dVSM are outlined in Figure 10.

Thus, the histotoxicological observations further corroborate our earlier hypothesis that GA protects from NaB-induced facilitation of contraction of dVSM by presumably inhibiting NaB-mediated oxidative stress-induced cytoarchitectural alterations of duodenal tissue.

5. Conclusion

From the findings of this study, we can conclude that GA can effectively mitigate the NaB-mediated facilitation of dVSM contraction, which might protect the normal functionality of the duodenum in NaB-exposure. GA restores the basal contractile property of the duodenum, presumably by preventing the NaB-mediated oxidative stress-induced damage of duodenal tissue by recovering the basal activities of antioxidant enzymes and preventing the membrane lipid peroxidation in duodenal smooth muscle cells in NaB exposure. Hence, the results of this study could be extrapolated to humans, and GA could be utilised as a therapeutic agent to mitigate NaB-induced toxicities in the small intestine.

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Author Contributions

CD designed the study, performed experiments, acquired and analysed data and prepared the manuscript; KS contributed to the conceptualisation, preparation and revisions of the manuscript; MD contributed to the study design and original draft preparation; MGR, DR and SB contributed to original draft preparation; GP contributed to conceptualisation and study design, editing and revising the manuscript; GP supervised the study; All authors read and approved the final manuscript.

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