Potentiation of the contraction of duodenal visceral smooth muscle in rat through oxidative stress induced inhibition of AChE activity by Methylparaben

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Abstract

Methylparaben, a synthetic chemical, is used as preservative to enhance the lifespan of different food items to be stored. So, humans are often exposed to methylparaben through foods preserved with methylparaben. The aim of the study was to examine the effect of methylparaben on contractile function of duodenum, the initial part of small intestine, which helps in digestion and absorption of ingested food. In our study, we have found significant increase in amplitude and frequency of the contraction of the duodenum of rats exposed to DMSO (vehicle control). Significant inhibition of the enzymatic activity of acetylcholinesterase (AChE) in duodenal smooth muscle of exposed rats has also been observed dose dependently in all exposure durations. These results suggest that methylparaben promotes the contraction of duodenum probably by potentiating the contraction of duodenal visceral smooth muscle (VSM). The methylparaben induced potentiation of duodenal VSM might be due to inhibition of AChE activity. The activities of antioxidant enzymes (GPx and GR) have significantly decreased and LDH activity and malondialdehyde (MDA) level have significantly increased in smooth muscle homogenates of rats exposed to methylparaben in our study. These observations suggest that methylparaben inhibits the AChE activity probably by producing oxidative stress in smooth muscle cells. In conclusion, methylparaben potentiates the contractile function of duodenal VSM probably by promoting oxidative stress induced inhibition of AChE activity at myoneural junctions.

Keywords Methylparaben, Duodenal visceral smooth muscle, Oxidative stress, Acetylcholinesterase, Myoneural junction

Introduction

Methylparaben (MP), methyl ester of p-hydroxy benzoic acid, is widely used as preservative in pharmaceuticals, cosmetics, foods and beverages due to its antimicrobial activity (Matthews et al., 1956). Detection of MP in water, dust, air (Haman et al., 2015; Perez Martin et al., 2010), also in drinking water at a concentration of 12 ng/L (Carmona et al., 2014) have been reported. A considerable amount of MP in food stuffs like jam, jellies, pickles, olives, processed fruits and vegetables, grain products, frozen dairy products, in alcoholic beverages, in pharmaceuticals and personal care products (PPCPs) have also been reported (Smolinske,1992; Daughton,2016). Considering the toxicity of MP the total acceptable daily intake (ADI) suggested by the FAO/WHO joint expert committee on food additives (JECPA, 2010) was 0 to 10 mg/kg body weight (BW) for the paraben compounds. Among all the parabens, MP has been extensively used as preservatives because of its short alkyl chain that exerts low toxicity. But several studies revealed that MP has toxic effect on endocrine system as it is an endocrine disrupting chemical (Cost J.R. et al., 2017), and also resulted in maternal thyroid dysfunction on exposure (Baker et al., 2020). It is also reported that MP inhibits the early phase of folliculogenesis and steroidogenesis (Ahn et al., 2012) in neonatal rats and also inhibits the motility and viability of human spermatozoa (Samarasinghe et al., 2018).

The small intestine is directly exposed to various nutrients, substances, and/or antimicrobial agents through the food stuffs. Apart from the digestive and absorptive functions of small intestine, it has protective functions on the GI tracts and acts as a sentinel for the mucosal immunity. Prolonged consumption of food additives (preservatives) may result in
intestinal dysbiosis (Shahare et al., 2013; Van Den Brûle et al., 2015; Jawurek et al., 2017). Intestinal epithelial damage due to external sources of stress have been reported (Shen et al., 2009). Increased level of reactive oxygen species (ROS) negatively disrupts the homeostasis between pro-oxidants and antioxidant defense mechanism leading to increased oxidative DNA damage, intracellular protein damage, and lipid peroxidation (Belozerskaia and Gessler 2007; Ahmad et al., 2010; Naziroglu et al., 2010). The visceral smooth muscles, located at the wall structure of small intestine, helps in digestion and absorption through contractions of small intestine. It is assumed that MP might impair the contractile function of visceral smooth muscle found in the wall structure of small intestine by exerting toxicity on the contractile mechanism of visceral smooth muscle that provide motility to small intestine in MP exposed human beings. The study on probable toxic effects of MP on the contractile function of small intestinal visceral smooth muscle has not been reported till date. So, the aim of the present study was to examine the effect of MP on the contractile function of visceral smooth muscle (VSM) found in the wall structure of duodenum, a representative part of small intestine, that helps in the digestion and absorption of food stuffs by providing motility to the duodenum, and helps in mucosal immunity.

Methods and Materials

Chemicals and reagents

All of the reagents used in this study were of analytical grade. Methylparaben, the test chemical was purchased from Sigma-Aldrich. Oxidized and reduced glutathione (GSSG and GSH), 5, 5'-dithiobis 2-nitrobenzene (DTNB or Ellman's reagent), NADPH,Na4 pyruvate were procured from Sisco Research Laboratories (SRL), India. Sodium chloride (NaCl), potassium chloride (KCl), calcium chloride (CaCl2), magnesium chloride (MgCl2), sodium bicarbonate (NaHCO3), disodium hydrogen phosphate (Na2HPO4), glucose, acetyl thioucrilic acid, trichloroacetic acid (TCA), BSA, NaOH, DMSO, triton X-100, EDTA, CuSO4, sodium-potassium tartarate, phosphoric acid, 2-thiobarbituric acid (TBA), HCl, sodium carbonate (Na2CO3), potassium dihydrogen phosphate(KH2PO4), ethanol, chloroform, sodium azide were obtained from E-merck, India.

Animal selection and care

Adult 3-4 months old male Albino rats of Sprague Dawley strain weighed between 110-130 g were selected to perform the study. The animals were maintained in Animal House as per guidelines of Animal Ethics Committee of Kalyani University framed as per National Guidelines. Animals were fed laboratory chow and water and kept in equal light-dark cycle (12L :12 D) at a room temperature of 25±2°C.

Experimental design

The animals were randomly distributed into eight groups (each group contains ten animals) after one week of acclimatization to the laboratory environment (Table 1). Selected doses were given below the LD50 (Bionetics, L., 1974) level of intoxication and animals were sacrificed by cervical dislocation on the 24th hour after completion of last doses of chronic treatment.

Table 1. Showing the divisions of experimental rats, dose received by each rat/ day in each group, and durations of exposure to Methylparaben (MP) and DMSO.

| Group | Received | Duration |
|-------|----------|----------|
| I     | distilled water for 14 days and 28 days respectively. | 14 days and 28 days |
| II    | 50% DMSO (Vehicle Control) | 28 days |
| III   | 0.6 g/kg BW/day (i.e., approximately 10% of LD50 of MP) | 28 days |
| IV    | 1.2 g/kg BW/day (i.e., approximately 20% of LD50 of MP) | 28 days |

Tracings of duodenal movement

After fasting for overnight, the rats were sacrificed by cervical dislocation. The duodenal segments (3cm each) were removed by transverse incision on immediate opening of the duodenum and used for recording of duodenal motility according to our standard laboratory protocol (followed by Dayton et al., 1970). The isolated duodenal segment was placed in tyrode solution consisting of (in gL−1): 8.0 NaCl, 0.2 KCl, 0.2 CaCl2, 0.1 MgCl2, 1.0 NaHCO3, 0.05 NaH2PO4 and 1.0 Glucose (pH-7.4). The luminal content of the segment was gently washed out and the duodenal part was placed longitudinally in a 50 mL organ bath consisting of tyrode solution and continuously supplied with 95% O2 and 5% CO2, and the temperature of the bath was controlled within a range of 37±0.5°C. The continuous recording of the duodenal movement was achieved with an isotonic transducer (IT-2245) linked to RMS-Polyrite-D (RMS Chandigarh, India). Each experimental preparation was equilibrated for 45 min and during this time repeated and prolonged washes of the duodenal preparation was performed with tyrode solution for avoiding any accumulation of metabolites in the organ bath (Sarkar et al., 2013).

Preparation of duodenal VSM homogenates

The VSM of the segments of the duodenum of 0.2 g weight were isolated from the vehicle control and exposed groups of rats and minced in a phosphate buffer saline solution (0.1M, pH-7.4). The homogenates were obtained through centrifugation at 8000 rpm for 10 min and thereafter at 12000 rpm for 5 min at 4°C. The supernatant was removed and kept in microfuge tubes at 25°C for the estimation of Malondialdehyde (MDA) and the activities of Glutathione peroxidase (GPx), Glutathione reductase (GR), Lactate dehydrogenase (LDH) and total protein content.
Estimation of AChE activity in duodenal VSM homogenates

The activity of AChE of duodenal VSM homogenates was measured by the method of Ellman et al., 1961 with slight modifications. MP exposed and vehicle control duodenal VSM homogenates of 0.4 mL were added to a test tube having 1.2 mL 0.1 M phosphate buffer (pH 8.0) and 0.1 mL of DTNB. Then absorbance was measured at 412 nm until the optical density had stopped increasing. Thereafter, 20 µL of 2-acetyl thiococholine iodide, the substrate, was added to respective test tubes and change in absorbance was recorded for 10 min at an interval of 2 min. The AChE activity has been expressed in moles substrate hydrolysed per minute per gram of tissue.

Biochemical estimations of oxidative stress related enzymes from duodenal VSM homogenates

Glutathione peroxidase (GPx) was determined according to the method of Rotruck et al., 1973 with slight modification. Activity of the enzyme was expressed as µmol of GSH₃ consumed/min/mg protein. The Glutathione reductase (GR) activity of the tissue homogenates were measured following the method of Staal et al., 1969 and the enzyme activity were expressed as µmol of NADPH oxidized/min/mg protein. Total protein content of the duodenal tissue was measured by the method of Lowry et al., (1951) with bovine serum albumin as the standard.

Determination of MDA in duodenal VSM homogenates

The degree of Lipid peroxidation was determined by measuring the amount of Malondialdehyde (MDA), a marker of Lipid peroxidation, produced due to oxidative stress. The amount of MDA formed was calculated by measuring the absorbance at 535 nm using molar co-efficient of 1.56 ×10⁵ mol⁻¹cm⁻¹ and expressed as µmol/mg protein (Devasagayam and Tarachand, 1987).

Estimation of LDH activity of duodenal VSM homogenates

LDH activity was measured slightly modifying the proposed method of Wróblewski and Ladue (1955). Phosphate buffer of 2.4 mL was mixed with 0.1 mL of tissue homogenate and 0.1 mL of reduced Diphosphonucleotide (NADPH, 2.5 mg/mL) was added to it and then incubated for 20 min. Thereafter, 0.1 mL of sodium pyruvate (2.5 mg/mL) was mixed and the respective absorbance was measured for the 5 min at an interval of 30 sec at 340 nm. One unit LDH activity was expressed as decrease in OD of 0.001 per unit per mL under the described condition.

Statistical analysis

In this study all data were expressed as mean ± SEM. Statistical analysis between the vehicle control and the treated groups for 14 and 28 days durations were performed using one way ANOVA followed by Turkey's multiple comparisons. p≤0.05 value less than 0.05 was set as the level of significances for the group's mean comparison. GraphPad Prism version 8.4.3 (686), GraphPad Software Inc. software was used to perform all the analysis.

Results and Discussions

Effect of methylparaben (MP) on the contraction of duodenal visceral smooth muscle

To examine the effect of methylparaben on the contractile function of duodenal visceral smooth muscle (VSM), the contractions of duodenum, the initial segment of the small intestine, in methylparaben exposed rats and DMSO (vehicle of methylparaben) exposed rats have been recorded ex vivo. In this study, we have found significant increase in force and frequency of the contractions of duodenum in rats exposed to MP in a dose response manner for 14 and 28 days exposure durations compared to force and frequency of contractions of duodenum in rats exposed to DMSO (vehicle control) (Fig. 1 and 2).

These results suggest that methylparaben promotes the contraction of duodenum probably by potentiating the contraction of duodenal visceral smooth muscle found in the wall structure of duodenum that provides the movement of the duodenum. It has been studied that the contractile function of duodenal (or small intestinal) visceral smooth muscle found in the muscularis externa layer of the wall of duodenum are innervated by facilitatory cholinergic myenteric efferents and inhibitory nor-adrenergic (NA) and/or nitrergic (non-adrenergic non-cholinergic, NANC) myenteric efferents (Sarkar et al., 2013).

In our result, we have observed facilitatory effect of methylparaben on the contractile function of duodenal visceral smooth muscle. So, from the results, we can hypothesize that methylparaben potentiates the contraction of duodenal visceral smooth muscle probably by augmenting the activity of facilitatory cholinergic (ACh secreting neurons) myenteric efferents that promotes the contraction of duodenal VSM.

Effect of methylparaben (MP) on the enzymatic activity of AChE in duodenal visceral smooth muscle

Acetylcholine (ACh), the facilitatory neurotransmitter, released from the cholinergic myenteric efferents serve as neurotransmitter substance for transmitting excitatory signals from cholinergic axon terminals to the smooth muscle membrane in synapse en passant synaptic junctions at muscularis externa layer of the wall structure of duodenum. The lifespan of the ACh in the synaptic junction is very short i.e., ACh acts on the muscle membrane for a fraction of a second. Because, the enzyme acetylcholinesterase (AChE), an extrinsic hydrolytic enzyme found in the smooth muscle membrane, splits acetylcholine very fastly into choline and acetate to terminate the action of ACh on the muscle membrane.
Fig. 1 Representative records showing the effects of methylparaben (MP) on the movement of duodenum in rats exposed to methylparaben for 14 day exposure duration (Panel A: (a) vehicle control tracing, (b) tracing in response to 0.6 g methylparaben exposure/kg BW of rat (10% of LD$_{50}$) per day, (c) tracing in response to 1.2 g methylparaben exposure/kg BW of rat (20% of LD$_{50}$) per day); and 28 day exposure duration (Panel B: (a) vehicle control tracing, (b) tracing in response to 0.6 g methylparaben exposure/kg BW of rat (10% of LD$_{50}$) per day, (c) tracing in response to 1.2 g methylparaben exposure/kg BW of rat (20% of LD$_{50}$) per day) recorded ex-vivo through a force-displacement transducer coupled to RMS-Polyrite-D.

![Fig. 1](image)

Fig. 2 Bar diagrams showing the percent changes in the amplitude (A) and frequency (B) of contraction of the duodenum in methylparaben (MP) exposed groups (vehicle control- 50% DMSO, treated I- rats received 0.6 g/kg BW per day, treated II- rats received 1.2 g/kg BW per day for 14-day and 28-day durations) compared to vehicle control groups. The data were represented as mean ± SEM for all the groups. $^a, c p<0.0001$, 0.05 vs. vehicle control and $^b, d p<0.000$, 0.05 vs. treated I.

![Fig. 2](image)

To elucidate the probable mechanism of methylparaben induced potentiation of contraction of duodenal visceral smooth muscle, the effect of methylparaben on the enzymatic activity of AChE has been examined. In our study, we have found significant inhibition of the enzymatic activity of AChE in duodenal smooth muscle homogenates of rats exposed to methylparaben for all exposure durations and doses of methylparaben tested compared to vehicle control rats (Fig. 3). This result indicates that methylparaben induced potentiation of contraction of duodenal visceral smooth muscle might be due to inhibition of the enzymatic activity of AChE as a result of methylparaben induced intoxication.
Effect of methylparaben (MP) on the activities of antioxidant enzymes and the degree of lipid peroxidation in duodenal visceral smooth muscle cells

In order to understand the methylparaben induced inhibition of AChE activity, the involvement of methylparaben induced oxidative stress in duodenal visceral smooth muscles have been studied in methylparaben and vehicle exposed groups of rats. In this study, we have observed significant inhibition of the enzymatic activities of antioxidant enzymes- Glutathione peroxidase (GPx), Glutathione reductase (GR) and increase in the activity of Lactate dehydrogenase (LDH) in smooth muscle homogenates in a dose dependent manner for 14- and 28-days durations in comparison with vehicle control rats. Besides, the level of malondialdehyde (MDA), a marker of lipid peroxidation, has been increased dose dependently in smooth muscle homogenates of rats exposed to methylparaben for both durations (Fig.4). These results suggest that methylparaben promotes the oxidative stress in duodenal visceral smooth muscles probably by inhibiting the enzymatic activities of antioxidant enzymes (GPx and GR) and promoting the activity of LDH and the lipid peroxidations as the level of MDA in methylparaben exposed smooth muscle homogenates has been increased significantly. From the results, it is suggested that methylparaben probably inhibits the enzymatic activity of the AChE by promoting the oxidative stress in visceral smooth muscle cells.

Fig. 3 Bar diagram showing the effect of methylparaben (MP) on the enzymatic activity of AChE in comparison with vehicle control rats for both 14 day and 28 day exposure durations. The data were represented as mean±SEM of eight observations, b,c,d,e,f,g,h,p<0.0001, 0.001, 0.01, 0.05 vs. vehicle control and p< 0.001 vs. treated I.

Fig. 4 Bar diagrams showing the methylparaben (MP) and DMSO induced changes in the activities of GPx, GR, LDH and the amount of MDA produced in MP exposed duodenal VSM homogenates for 14- and 28-days exposure durations. The data were represented as mean ± SEM of eight observations. a,b,c,d,e,f,g,h,p< 0.0001, 0.001, 0.01, 0.05 vs. vehicle control and b,c,d,e,f,g,h,p< 0.0001, 0.001, 0.01, 0.05 vs. treated I.
**Fig. 5** Probable mechanisms of actions of methylparaben (MP) in potentiating the contractile function of duodenal visceral smooth muscle. **MP** Methyplaraben, **ACH** Acetylcholine, **ACH-E** Acetylcholine-esterase, **GR** Glutathione reductase, **GPX** Glutathione peroxidase, **GSH** Reduced glutathione, **GSSG** Oxidized glutathione, **O$_2^-$** Oxygen radical, **H$_2$O$_2$** Hydrogen peroxide, **OH** Hydroxyl radical, **LH** Lipids, **LOO•** Lipid radical, **MDA** Malondialdehyde, **LDH** Lactate dehydrogenase. (+) indicates stimulation/ augmentation and (-), indicates inhibition/ suppression.

**Conclusion**

In conclusion, methylparaben (MP) impairs the digestive and absorptive functions of duodenum probably by potentiating the contractions of visceral smooth muscle found in the wall structure of duodenum. The MP induced potentiation of the contraction of duodenal visceral smooth muscle might be mediated through the augmentation of the activity of facilitatory cholinergic myenteric efferents (intrinsic plexus neurons found in the muscularis externa layer of the duodenal wall). MP augments the activity of cholinergic myenteric efferents probably by inhibiting the activity of AChE, which splits ACh into choline and acetate very rapidly at myoneural junctions, through producing oxidative stress in smooth muscle cells.

**Conflict of Interest**

We declare no conflict of interest in publishing the research article.

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**Consent for Publication**

All the authors have given their consent for publication.

**Ethical Considerations**

The study was approved by the ethical committee.

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