



Journal homepage: www.sciencearchives.org

ISSN: 2582-6697



Original Research Article

<http://dx.doi.org/10.47587/SA.2021.2207>



Effect of Bisphenol S (BPS) on the contraction of duodenal visceral smooth muscle *ex vivo* in rat

Debarati Roy, Mousumi Dutta, Mukti Mondal, Kaushik Sarkar, Goutam Paul*

Molecular Neurotoxicology Laboratory, Department of Physiology,
University of Kalyani, Kalyani, Nadia-741235, West Bengal, India

*Corresponding author: goutampaul.ku@gmail.com

Received: May 31, 2021 / Revised: June 23, 2021/ Accepted: June 27, 2021

Abstract

Bisphenol S (BPS) being a component of storage and packaging materials of foodstuffs, beverages and drinking water, contaminates them through BPS-leaching. BPS exposure to humans occurs predominantly through the consumption of BPS-intoxicated foodstuffs and subsequently BPS absorption through the small intestine into bloodstream. Though, the probable toxic effects of BPS on contractile function of small intestinal visceral smooth muscle (VSM) that provides motility to the small intestine, remains poorly understood. So, the present study aims to evaluate the effects of BPS on the efferent neurocrine mechanisms involved in the intrinsic rhythmic contraction of small intestinal VSM. Experiments were performed on duodenum segment of small intestine isolated from adult male Sprague-Dawley rats to examine the effects of BPS exposure on the movement of the duodenum, acetylcholinesterase (AChE) activity, levels of cellular oxidative stress biomarkers, and activities of cytosolic antioxidant enzymes at 100 μ M, 200 μ M, 400 μ M and 800 μ M doses of BPS for 1h duration *ex vivo* by using standard laboratory protocol. We observed significant decrease in frequency and amplitude of contraction of duodenum recorded *ex vivo* in single-dose acute exposure experiments in BPS-exposed groups compared to the control group ($P < 0.05$). Besides, acetylcholinesterase (AChE) activity in duodenal homogenate has been increased significantly along with significant alterations in both the levels of oxidative stress biomarkers and activities of antioxidant enzymes in a dose-response manner in BPS-exposed groups ($P < 0.001$). Our results indicate that BPS inhibits contraction of duodenal VSM probably by inhibiting the activity of excitatory cholinergic myenteric efferents through inducing enzymatic activity of AChE and promoting oxidative stress in duodenum.

Keywords Bisphenol S, Duodenal visceral smooth muscle, Cholinergic myenteric efferent, AChE activity, Oxidative stress.

Introduction

Bisphenol compounds are primarily utilized as plasticizer additives in plastic goods manufacturing. Foodstuff manufacturing industries rely on plastic products, for packaging and storage purposes (Yang et al., 2014). Plastic containers are used in various chemical compositions and polymeric forms for the packaging of pharmaceuticals, drinking water, beverages, and foods (Gupta and Singh, 2007). Regardless of its applicability, contact of plastics with foodstuffs is becoming serious threat to human health as leaching of bisphenols occurs from plastic containers. Among the bisphenols, use of bisphenol A (BPA) in large quantities is already well recognized in the polycarbonate - plastics and

epoxy resins manufacturing industries. The probable foodstuff contaminating sources of BPA comes from usage of tablewares, water pipes or reusable foodstuff-storing containers made up of polycarbonate-plastics and also from epoxy resin-coated inner layers of beverage bottles, food cans or glass jar lids. Above all, leaching of BPA into stored foodstuffs is also induced by temperature. Hence, unveiling of endocrine disrupting potency of BPA raised questions against its use as a biologically safe food contact material. Consequently, BPA received either complete ban or restrictions in its use limit. Subsequently, BPA-utilizing platform started to shift towards a suitable BPA-substituting entity and replacement-bisphenols rapidly emerged without being backed up by sufficient scientific information regarding

their biological safety issues, and are now being used in consumer products, probably under the tagline of “BPA-free” goods. Though, the possibility of leaching of estrogenic chemicals from these BPA-substitutes, generating adverse effects analogous to BPA exposure, still persists (Yang et al., 2014; Caballero-Casero et al., 2016; Horan et al., 2018; Bousoumah et al., 2021; Sabry et al., 2021).

Bisphenol S (BPS) (Fig.1), a structurally similar BPA-substitute, is recently replacing the BPA-usage fields. BPS has gained ‘benefit of doubt’ against BPA for its lower estrogenic activity and higher thermal stability, making its possibility of thermal leaching less probable. Though, like BPA, BPS too leaches into foodstuffs from storage containers. Existence of BPS in human urine confirms BPS-exposure to humans. Ingestion of BPS-contaminated foodstuffs is probably the major BPS-entry route to humans (Yang et al., 2014; Ullah et al., 2016; Lehmler et al., 2018; Bousoumah et al., 2021; Cao et al., 2021).

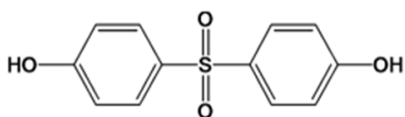


Fig. 1 Molecular structure of Bisphenol S

Though, unlike BPA, the effects of BPS exposure on biological systems are not thoroughly examined. Oral BPS exposure to experimental organisms altered a number of physiological parameters including anti-androgenic activities on reproductive organs of adult male rats and liver injury in mice (Ullah et al., 2016; Zhang et al., 2018). These reports imply that BPS being a recently emerging compound with growing industrial applicability might not be totally safe and might possess health risk to humankind through unaware oral exposure (Khmiri et al., 2020).

However, the effect of BPS on the small intestine, the organ exposed to BPS during digestion and absorption of BPS-contaminated foodstuffs is not well established and represents a knowledge gap. Though, BPA, the analogous compound of BPS, has previously been reported to suppress the contractile activity of mammalian small intestine (Sarkar et al., 2013; Sarkar et al., 2016). The focus is to enquire whether or not BPS acts as a toxicological analogue of BPA in mammalian small intestine. Hence, this study has been conducted to evaluate the effects of BPS on the contractile function of the small intestinal VSM in rats to understand the probable effects of BPS on intrinsically regulated contractile function of small intestinal VSM of humans.

Materials and Methods

Chemicals

All the chemicals including reagents and solvents used to fulfil this study were of analytical grade and were purchased from Sigma Aldrich, MO, USA; Merck Life Science Private Limited, Mumbai, India; Sisco Research Laboratories Private

Limited (SRL), Mumbai, India. BPS (Purity 98%, CAS No. 80-09-1) was procured from Sigma Aldrich, USA. Dimethyl sulphoxide (DMSO) was purchased from Merck Life Science Private Limited, Mumbai, India.

Animals

This study was conducted upon Sprague-Dawley strain of adult male albino rats, weighing around 130-150 grams. The animals were kept at the departmental animal house with an approximate 12h light-dark cycle at the room temperature of 25-27°C and were fed with a standard laboratory diet with *ad libitum* water. Experiments were performed on the animals according to the recommended guidelines of Institutional Animal Ethics Committee of the University.

Preparation of different doses of BPS

BPS was initially dissolved in DMSO, the vehicle compound used in this study to completely dissolve BPS, and double-distilled water was gradually added into it, to prepare the desired doses of BPS in a final concentration of 10% DMSO solution. Four different doses of BPS, 100µM, 200µM, 400µM and 800µM were used in this study. The doses of BPS were selected after standardizing its impact on the movement of duodenum through our initial experiment.

Group division for the study

For determining the effects of BPS exposure to duodenum, six different groups were prepared. Among them, four groups were BPS-exposure groups, one group was the vehicle control group and the remaining group was the control group. BPS-exposure groups were named as BPS100, BPS200, BPS400 and BPS800 groups and were administered with respectively 100µM, 200µM, 400µM and 800µM doses of BPS in 10% DMSO solution. The vehicle control group was prepared by administering 10% DMSO solution to separately examine the impact of the vehicle control used to dissolve BPS. The control group was not exposed to BPS or DMSO.

Animal sacrifice

Animals were kept at fasting condition for overnight, prior to sacrifice. Cervical dislocation process was performed on the animals to ensure the occurrence of painless sacrifice.

Tissue collection

Immediately after cervical dislocation, the abdominal cavity of the sacrificed animal was opened to isolate the small intestine. The small intestine was isolated after surgically dissecting it from mesentery, and then from both the stomach and large intestine by transverse incision.

After that, the proximal length of the small intestine adjacent to stomach, comprising of the duodenum portion, was separately isolated from the remaining portion of the small intestine and was collected. Only duodenum segment was selected as the representative of the small intestine because the movement pattern of duodenum is more prominent than the

rest of the small intestine. Following the removal of the duodenum segment, its lumen was instantly rinsed by gentle flushing out process in a careful manner. The careful washing of the tissue segment ensured the elimination of any luminal contents present within it. For the recording of the movement of duodenum, the isolated tissue segment was instantly mounted within the organ bath of Dale's apparatus using the lowest time duration possible, counting from the moment of animal sacrifice. For biochemical experiments, tissue segments were dried using blotting paper and were instantly used for homogenate preparation or they were stored within sterilized glass vials at -20°C until they were further used. Separate sets of duodenum were used for the movement recording study and biochemical experiments. For the movement recording study, duodenum collected from 6 different animals was used for each group (n=6). For biochemical experiments, homogenate was prepared from duodenum before incubation into separate groups.

Tissue mounting for recording of movement of duodenum

To record the spontaneous movement pattern or motility of duodenum of small intestine *ex vivo*, around 3 cm long duodenum segment was positioned vertically with the help of two metal hooks piercing through the two opposite ends of the tissue segment and was immersed into an organ bath filled with 45ml of Tyrode's solution. The composition for Tyrode's solution was 8.0g sodium chloride (NaCl), 0.2g potassium chloride (KCl), 0.2g calcium chloride (CaCl₂), 0.1g magnesium chloride (MgCl₂), 0.05g sodium di-hydrogen phosphate (NaH₂PO₄), 1.0g sodium bi-carbonate (NaHCO₃) and 1.0g dextrose into a final volume of 1L and a final pH of 7.4. Prior to proper positioning of the tissue segment, the organ bath was prepared with a facility, to be able to deliver oxygen from surrounding air through the use of an oxygen bubbler with a continuous flow of 2-3 air bubbles per second into the immediate surroundings of the tissue segment. The temperature in the immediate vicinity of the isolated tissue segment, positioned within the organ bath, was also maintained at 37±0.5°C by using an automatic thermostat machine attached with the Dale's fluid bath. For recording of the movement of vertically positioned isolated tissue segment, its lower end was attached and secured at the bottom side of the organ bath with a metal hook piercing through it and its upper end was attached in a similar manner with the lever of an isotonic transducer apparatus (IT-2245) (RMS, Chandigarh, India). Isotonic transducer, the recording apparatus for recording of the continuous movement of the isolated tissue segment, was coupled with analysing software, RMS Polyrite-D (RMS, Chandigarh, India). Continuous recording of isotonic contraction was achieved against a constant load of 0.1g. Each segment was initially permitted to stabilize for a minimum of 30mins under the given experimental set up and was washed repeatedly at 10mins interval with fresh Tyrode's solution for removal of accumulated metabolites. Finally, recording of isotonic contraction, resulting from the spontaneous rhythmic movement of the isolated tissue segment was acquired continuously for 1h following administration of different doses of BPS (BPS-exposure groups) or 10% DMSO solution

(vehicle control group) against the control group where neither BPS nor DMSO was applied.

Tissue homogenate preparation for biochemical study

For biochemical experiments, tissue homogenate of duodenum was prepared in 50mM phosphate buffer (pH 7.4), (except for the lipid peroxidation assay where 0.9% NaCl solution was used instead of phosphate buffer) in cold conditions using homogenizer (Remi Elektrotechnik Limited, Vasai, India). Duodenal tissue was weighed and 10% w/v homogenate was prepared. For acetylcholinesterase (AChE) enzyme assay, 2% w/v homogenate was used.

Incubation of tissue homogenate

The tissue homogenate sample of duodenum was separated into six groups before incubation. The control group contained only the tissue homogenate sample and phosphate buffer (50mM, pH 7.4) into a final mixture volume of 1.0ml. The four BPS-exposure groups viz. BPS100, BPS200, BPS400 and BPS800 groups were prepared in such a manner that the final incubation mixture volume of 1.0ml contained equal volumes of doses of BPS of 100µM, 200µM, 400µM and 800µM strengths respectively, along with tissue homogenate sample and phosphate buffer (50mM, pH 7.4). The vehicle control group contained tissue homogenate sample, phosphate buffer (50mM, pH 7.4) and an equal volume of 10% DMSO solution that was used to dissolve BPS, into a final volume of 1.0ml, in absence of BPS. All the six groups of incubation mixtures were mixed in a well manner and were incubated for 1h at 37°C within an incubator.

Measurement of activity of acetylcholinesterase (AChE) enzyme of duodenum

Activity of acetylcholinesterase (AChE) enzyme was measured spectrophotometrically at 412nm using a UV-VIS spectrophotometer (Genesys 10S UV-VIS, Thermo Fisher Scientific, USA) by following the method of [Ellman et al., \(1961\)](#) with some modification. A final volume of 3.12ml assay mixture contained 0.4ml of incubated sample (2% w/v homogenate) as the source of enzyme, 2.6ml of 0.1M phosphate buffer with pH 8.0, 0.02ml of 0.075M aqueous solution of acetylthiocholine iodide and 0.1ml of 0.01M dithiobisnitrobenzoic acid (DTNB) solution that was prepared by adding sodium bicarbonate and dissolving in 0.1M phosphate buffer with pH 7.0.

Measurement of cellular oxidative stress biomarkers of duodenum

The level of lipid peroxidation (LPO) in the tissue homogenate of duodenum (10% w/v in 0.9% NaCl) was measured by estimating the level of thiobarbituric acid reactive substances (TBARS) by thiobarbituric acid assay following the method of [Buege and Aust, \(1978\)](#) with some modification. Into each of the six groups of incubated tissue homogenate samples, along with the sample blank tube, thiobarbituric acid-trichloro acetic acid (TBA-TCA) reagent dissolved in diluted hydrochloric acid (HCl) was added and heated into a water bath for 20

minutes at 80°C after thorough mixing. After cooling down of the samples under running tap water at the end of the completion of the reaction, they were centrifuged using a cooling centrifuge instrument (Remi Elektrotechnik Limited, Vasai, India) and the absorbance of the pink coloured supernatants was measured spectrophotometrically against the sample blank tube at 532nm using a UV-VIS spectrophotometer. The LPO level was expressed by concentration of thiobarbituric acid reactive substances (TBARS).

The reduced glutathione (GSH) content in the tissue homogenate of duodenum (10% w/v in 20mM EDTA) was measured by following the method of Sedlak and Lindsay, (1968) with some modification. Each of the six groups of incubated tissue homogenate samples, along with the sample blank tube, were added with 10% trichloro acetic acid (TCA) and kept in a cold environment after mixing in a well manner. The mixtures were then centrifuged at cold condition and the supernatants were collected. 0.4M Tris-HCl buffer with pH 8.9 was added into each one of the collected supernatants and finally DTNB (Ellman's reagent) was mixed for colour development. After a few minutes, absorbance of the yellow coloured solutions was measured spectrophotometrically against the sample blank tube at 412nm using a UV-VIS spectrophotometer. The GSH content was then calculated using a previously prepared standard curve of reduced glutathione.

The total thiol (T-SH) content in the tissue homogenate of duodenum (10% w/v in 20mM EDTA) was measured by following the method of Sedlak and Lindsay, (1968) with some modification. Each of the six groups of incubated tissue homogenate samples, along with the sample blank tube, were added with 0.2M Tris-HCl buffer with pH 8.4 and DTNB. The preparations were kept at standing for 15mins prior to homogenization. Supernatants were collected and absorbance of the yellow coloured solutions were measured spectrophotometrically against the sample blank tube at 412nm using a UV-VIS spectrophotometer. The T-SH content was then calculated using the standard curve of reduced glutathione.

Measurement of activities of cytosolic antioxidant enzymes of duodenum

Activity of copper-zinc superoxide dismutase (Cu-Zn SOD) was measured spectrophotometrically at 420nm by following the method of Marklund and Marklund, (1974) using the pyrogallol autooxidation procedure. A final volume of 1.0ml assay mixture contained suitable quantity of incubated sample as the source of enzyme, 50mM Tris-HCl buffer with pH 8.2 and 0.2mM pyrogallol. One unit of enzyme activity was equivalent to 50% inhibition of the rate of pyrogallol autooxidation.

Activity of catalase (CAT) was measured spectrophotometrically at 240nm by following the method of

Beers and Sizer, (1952) with some modifications. Tissue homogenate was centrifuged and absolute alcohol was added into the supernatant at cold temperature. Supernatant was mixed with Triton-X and then a buffer mixture consisting of phosphate buffer with pH 7.0 containing hydrogen peroxide (H₂O₂), was added to monitor the enzyme activity.

The activity of glutathione peroxidase (GPx) was measured spectrophotometrically at 340nm by following the method of Paglia and Valentine, (1967) with some modifications. A final volume of 1.0ml assay mixture contained suitable quantity of incubated tissue homogenate as the source of enzyme, 50mM phosphate buffer containing 2mM EDTA with pH 7.6, 0.01mM NADPH, 0.15mM glutathione, 0.025mM sodium azide and 0.36mM H₂O₂.

Activity of glutathione reductase (GR) was measured spectrophotometrically at 340nm by following the method of Staal et al., (1969) with some modifications. A final volume of 1.0ml assay mixture contained suitable quantity of incubated tissue homogenate as the source of enzyme, 50mM phosphate buffer with pH 7.4, 0.05mM NADPH, 0.25mM glutathione disulfide (GSSG) and 0.4mg BSA.

The activity of glutathione-S-transferase (GST) was measured spectrophotometrically at 340nm by following the method of Habig et al., (1974) with some modifications. A final volume of 1.0ml assay mixture contained a suitable quantity of incubated tissue homogenate as the source of enzyme, 50mM phosphate buffer with pH 6.5, 0.6mM glutathione and 0.6mM dinitrochlorobenzene (CDNB).

Determination of protein content

The protein contents of tissue homogenate samples were estimated by using the method of Lowry et al., (1951).

Statistical analysis

Statistical evaluation was performed by using software (Microcal Origin version 7.0 for Windows) after repeating each experiment at least thrice. Data were presented in terms of means ± standard error of mean (SEM). Significance between the mean values of different treatment groups were analysed using Tukey's test of one way analysis of variances (ANOVA).

Results and Discussion

BPS exposure to duodenum significantly decreased both the amplitude and frequency of the contraction of duodenum recorded *ex vivo* in a dose-dependent manner in single dose acute exposure experiments in comparison to the control group (P<0.05). Whereas, compared to the control group, we did not find significant (P<0.05) alterations in the amplitude and frequency of the contraction of duodenum in response to DMSO exposure as the vehicle of BPS (Fig.2).

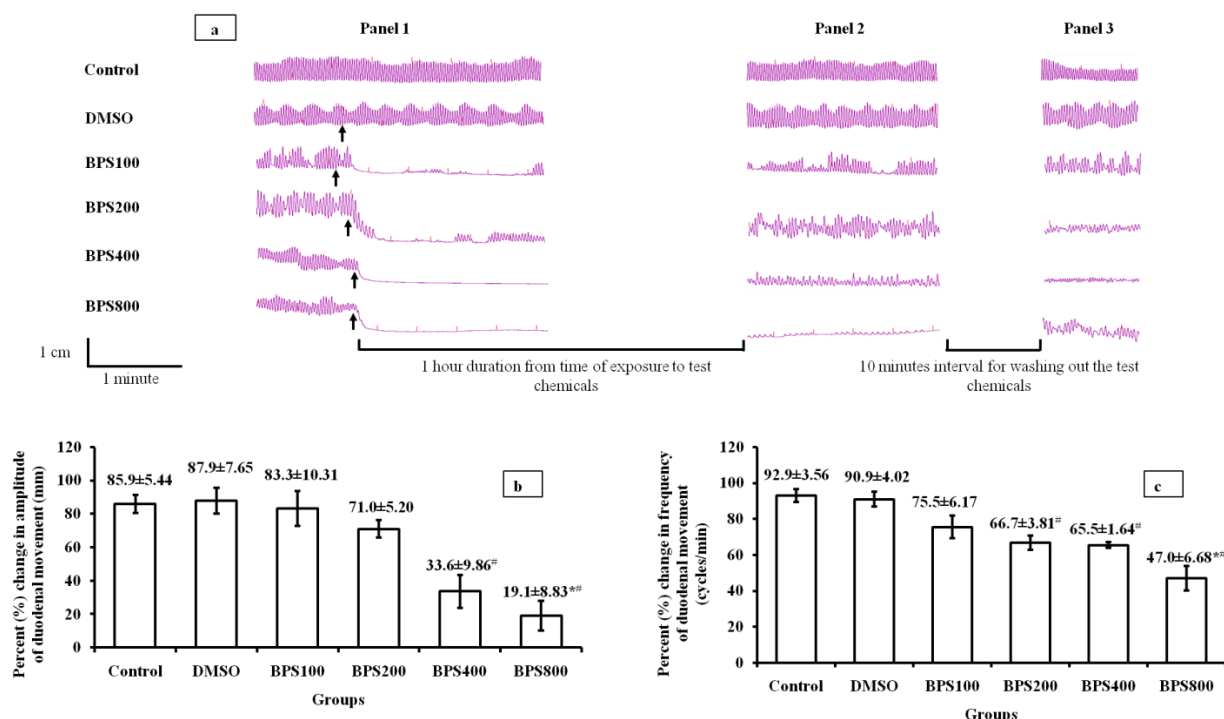


Fig. 2 Movement of isolated duodenum of small intestine recorded from different groups of rats. Representative records show contractile pattern of isolated duodenum at the time of exposure to test chemicals (panel 1), after one hour from exposure to test chemicals (panel 2) and after ten minutes of removal of test chemicals by repeated washing out process (panel 3). Arrow head indicates point of exposure to test chemicals (a). Graphical representations of percent changes in amplitude of contractions (b) and frequency of contractions (c) of isolated duodenum exposed to test chemicals for one hour duration. Results are expressed as Mean±SEM (n=6). Level of significance compared to control value at *P<0.001 and [#]P<0.05 is tested using ANOVA. Control represents control group, not exposed to test chemicals; DMSO represents vehicle control group exposed to 10% DMSO solution; BPS100, BPS200, BPS400 and BPS800 represent BPS-exposure groups exposed to respectively 100µM, 200µM, 400µM and 800µM doses of BPS dissolved in 10% DMSO solution.

AChE activity in duodenal homogenates significantly increased in a dose-dependent manner in BPS-exposure groups compared to the control group; whereas, DMSO exposure did not alter it significantly in comparison to the control group (P<0.001) (Table 1).

Table 1. Acetylcholinesterase enzyme activity measured in duodenal homogenate

| Groups | Acetylcholinesterase (AChE) activity (Units/min/mg of protein) |
|---------|--|
| Control | 10.592±0.116 |
| DMSO | 10.589±0.225 |
| BPS100 | 11.207±0.293 |
| BPS200 | 11.863±0.131 |
| BPS400 | 12.435±0.241* |
| BPS800 | 13.194±0.249* |

Data presented as mean±SEM. Values bearing asterics (*) indicate significant difference compared to control value at P<0.001 level.

Compared to the control group, TBARS level in BPS-exposure groups of duodenal homogenates significantly increased; whereas, GSH and T-SH contents significantly decreased in a dose-dependent manner (P<0.001). DMSO-exposure to duodenal homogenate did not produce significant alterations in the levels of oxidative stress biomarkers (P<0.001) (Table 2). Compared to the control group, activities of Cu-Zn-SOD and CAT significantly increased; and activities of GPx, GR and GST significantly decreased in BPS-exposure groups of duodenal homogenates in a dose-dependent manner (P<0.001). Activities of the antioxidant enzymes did not alter significantly in duodenal homogenate by DMSO-exposure in comparison to the control group (P<0.001) (Table 3).

Small intestine performs digestion and absorption of ingested food materials for generation of metabolic energy by body tissues and prevents bacterial overgrowth inside its lumen by propelling luminal contents towards aboral direction. All these motor functions are assisted by motility or movement of small intestine provided by co-ordinated inherent rhythmic contraction of visceral smooth muscle (VSM) cells, arranged in inner circular and outer longitudinal orientations in the muscularis externa layer of wall structure of small intestine, under the regulation of intrinsic myenteric efferents. Motility

of small intestine mixes luminal contents with digestive juices within small intestinal lumen, propels the chyme in forward direction and facilitates absorption of digested end products into blood stream through intestinal wall structure (Andrews & Blackshaw, 2010, pp. 1643-1658). Absorption of orally ingested BPS from gastrointestinal lumen to

systemic circulation also occurs through small intestinal wall. As a consequence, BPS might affect VSM and hence the functions of small intestine. Therefore, the present study was designed to elucidate the probable neurocrine mechanisms involved in BPS-induced alterations in the contractile function of small intestinal VSM.

Table 2. Oxidative stress biomarker levels measured in duodenal homogenate

| Groups | Lipid peroxidation level (nM TBARS/mg of protein) | Reduced glutathione (GSH) content (µM GSH/mg of protein) | Total thiol (T-SH) content (µM T-SH/mg of protein) |
|---------|---|--|--|
| Control | 0.315±0.002 | 2.237±0.026 | 23.918±0.164 |
| DMSO | 0.316±0.000 | 2.141±0.033 | 23.375±0.029 |
| BPS100 | 0.318±0.002 | 0.878±0.023* | 21.789±0.096* |
| BPS200 | 0.345±0.002* | 0.822±0.025* | 20.588±0.100* |
| BPS400 | 0.364±0.002* | 0.733±0.028* | 18.482±0.138* |
| BPS800 | 0.432±0.005* | 0.224±0.013* | 14.399±0.061* |

Data presented as mean±SEM. Values bearing asterics (*) indicate significant difference compared to control value at P<0.001 level.

Table 3. Cytosolic antioxidant enzyme activities measured in duodenal homogenate

| Groups | Copper-zinc superoxide dismutase (Cu-Zn-SOD) (Units/min/mg of protein) | Catalase (CAT) (µM H ₂ O ₂ consumed/min/mg of protein) | Glutathione peroxidase (GPx) (Units/min/mg of protein) | Glutathione reductase (GR) (Units/min/mg of protein) | Glutathione-S-transferase (GST) (Units/min/mg of protein) |
|---------|--|--|--|--|---|
| Control | 0.118±0.002 | 15.109±0.065 | 0.145±0.000 | 0.092±0.002 | 0.087±0.001 |
| DMSO | 0.120±0.003 | 15.219±0.022 | 0.139±0.002 | 0.091±0.002 | 0.085±0.002 |
| BPS100 | 0.192±0.004* | 15.619±0.111 | 0.108±0.002* | 0.084±0.000 | 0.075±0.000* |
| BPS200 | 0.208±0.003* | 16.821±0.082* | 0.090±0.002* | 0.081±0.000* | 0.070±0.000* |
| BPS400 | 0.225±0.003* | 18.334±0.051* | 0.068±0.002* | 0.075±0.001* | 0.065±0.000* |
| BPS800 | 0.247±0.003* | 21.166±0.191* | 0.055±0.001* | 0.057±0.001* | 0.057±0.000* |

Data presented as mean±SEM. Values bearing asterics (*) indicate significant difference compared to control value at P<0.001 level.

In our study, the motility pattern of duodenal segment recorded *ex vivo* is categorized under the fasting motor pattern and is characterized as interdigestive migrating motor complex (IDMC). IDMC observed in isolated duodenum is absolutely dependent on and regulated by intrinsic enteric

nervous system (ENS), as extrinsic inputs from brain and spinal cord were absent under the experimental conditions. In our study, a prominent decreasing tendency of contractile activity has been observed in response to BPS exposure, that is associated with an instant sudden abolishment of both the

amplitude and frequency of the contraction (contractile quiescence) in response to the immediate exposure to gradual higher doses of BPS in the representative tracings of IDMC recorded from the isolated duodenum (Fig.2a, Panel 1 and Panel 2). The decrease in amplitude of contraction of duodenum might be due to lesser force generation by the VSM cells (Fig.2b) whereas the decrease in frequency of contraction might be directly linked to reduced numbers of slow wave formation and/or spike burst generation in response to BPS exposure (Fig.2c). The transient contractile quiescence might be resulted from localized inhibitory action of BPS on optimum functioning of contractile system. The reappearance or return of contraction gets delayed with exposure to increasing doses of BPS. Though the contraction reappears after a certain period of immediate contractile quiescence, the contractile activity remains diminished throughout the BPS exposed duration in a dose-dependent manner. This phenomenon might occur due to the direct interaction of BPS molecules with the neurocrine system of intrinsic myenteric efferents and/or with the smooth muscle contractile system of the dissected segment of the small intestine. We also noticed, contractility blocking effect of BPS on isolated duodenum is slightly recovered, though remains altered from the basal activity level, with the removal of BPS containing medium from surroundings of isolated duodenum by replacement with fresh Tyrode's solution (Fig.2a, Panel 3). Hence, it might be speculated that the interaction pattern of BPS with VSM might not be fully irreversible. Though, even after removal of BPS, the functional inhibitory effect of BPS-exposure still prominently exists in VSM of isolated duodenum, in the form of executing abnormal contractile pattern with altered rhythmicity. All these observations from results indicate that BPS might inhibit the contraction of duodenal VSM probably by inhibiting the excitatory cholinergic myenteric efferents and/or stimulating the inhibitory noradrenergic or nitergic (non-adrenergic non-cholinergic, NANC) myenteric efferents innervating to VSM organized circumferentially and longitudinally in wall structure of duodenum. We hypothesized that BPS might decrease the frequency of contraction of duodenal VSM by decreasing spike burst generation or slow wave formation by duodenal pacemaker cells; and a decrease in amplitude might be due to a decrease in the concentration of free calcium ions within smooth muscle cells. Our hypothesis complies with previously reported BPA-induced inhibition of movement of the duodenum (Sarkar et al., 2013).

In small intestine, contraction of VSM is promoted by acetylcholine (ACh), released from endings of excitatory cholinergic myenteric efferents into neuromuscular junction at synapse en passant structure, in response to appropriate stimulus. Acetylcholinesterase (AChE) enzyme present in VSM cell membrane terminates action of ACh by hydrolyzing it into its precursor molecules, choline and acetate (Quinn, 1987). We speculate BPS-induced increase in AChE activity observed in our experiment (Table 1) might be responsible for enhancement of ACh hydrolysis, thereby shortening its time span of action on VSM, ultimately leading to BPS-induced decrease in duodenal VSM contraction. BPS-

induced contractile quiescence probably occurred due to hydrolysis of already existing ACh molecules in the immediate vicinity of VSM by the sudden enhanced AChE activity. Reappearance of contraction after contractile quiescence might occur due to release of excess ACh molecules from excitatory cholinergic myenteric efferent endings, above the basal level, as cope up mechanism against BPS-induced alterations. Though, excess release of ACh does not seem to be sufficiently enough to overcome BPS-induced enhanced AChE activity and thereby explains decreased contractile activity in isolated duodenum throughout the BPS exposed duration. Our observation complies with enhanced AChE activity induced by BPA exposure in duodenal tissue of rats (Sarkar et al., 2013).

Increased AChE activity has previously been reported to be associated with membrane lipid peroxidation and oxidative stress generation (Melo et al., 2003; Khadrawy et al., 2011; Moreira et al., 2012). In our study also, we observed BPS-induced enhanced AChE activity is associated with augmentation in lipid peroxidation in the form of an increase in TBARS level (Table 2). *In vitro* BPS exposure has already been reported to increase TBARS levels in rats (Ullah et al., 2019). Besides being a reliable biomarker for oxidative stress detection, augmented lipid peroxidation can induce hydroperoxide generation, leading to production of toxic intermediates that impairs important physical aspects of cell membrane functions including permeability and mobility (Zhang et al., 2018). Elevation of toxic intermediates including free radicals and reactive oxygen species (ROS) can directly damage lipid molecules (Ayala et al., 2014). We speculate BPS-induced alterations observed in our study might be associated with ROS generation, as BPS possesses ROS generating potency (Zhang et al., 2016; Ullah et al., 2019; Ijaz et al., 2020). Decreased reduced glutathione (GSH) content observed in our study (Table 2) is another prominent indicator of ROS generation that complies with our hypothesis of induction of ROS generation by BPS. Being a candidate for the antioxidant defense system, GSH protects cells against oxidative insults directly as well as by participating in catalytic cycles of glutathione-dependent antioxidant enzymes (Hassan et al., 2012). Hence, GSH depletion, by its oxidation into oxidized glutathione (GSSG) and its conjugation with xenobiotics, alters cellular oxidation-reduction status by rendering cells prone to oxidative damages (Maćczak et al., 2017). GSH also comprises a portion of cellular total thiol (T-SH) content and converts oxidized thiols into their respective reduced forms (Khadrawy et al., 2011). Hence, BPS-induced decrease in T-SH content observed in our study (Table 2) might be interconnected to decreased GSH content.

Oxidative damages to cells occur due to induction of uncontrolled oxidative stress developed by imbalance between levels of prooxidants and antioxidants, beyond the capacity of antioxidants to combat harmfulness caused by stressors including ROS molecules (Ayala et al., 2014; Vona et al., 2021). BPS exposure has been shown to be directly associated with decrease in expression of a number of antioxidant genes in zebrafish (Salahinejad et al., 2021).

BPS-induced alterations in the activities of cytosolic antioxidant enzymes observed in our experiment indicate towards generation of oxidative stress (Table 3). BPS-induced GPx activity decrease (Table 3) might be resulted from GSH depletion, as GSH is necessary for GPx activity. Decreased GPx activity probably diminishes hydrogen peroxide (H₂O₂) degradation, leading to accumulation of toxic H₂O₂, a relatively stable form of ROS (Hassan et al., 2012). As affinity for H₂O₂ of GPx is higher than CAT, GPx may decompose minute levels of H₂O₂ even before CAT (Ho et al., 1998). Increased lipid peroxidation might also be associated with decreased GPx activity observed in our study, as GPx is involved in degradation of lipid hydroperoxides (Maćczak et al., 2017). BPS-induced decreased GR activity observed in our study (Table 3) is speculated to decrease regeneration of GSH from its oxidized counterpart GSSG and complies with our observation of BPS-induced GSH depletion (Dutta and Paul, 2019). BPS-induced decreased GST activity observed in our study (Table 3) might also be resulted from GSH depletion, as GSH is utilized by GST as a conjugate for catalyzing endogenous detoxification of xenobiotics primarily derived from cellular oxidative reactions, thereby rendering cells prone to damages caused by oxidative stress (Hassan et al., 2012). BPS-induced elevation of Cu-Zn-SOD and CAT activities probably occurred to combat oxidative imbalances generated within cells (Table 3). Similar to our observation, BPS has previously been reported in literature to increase SOD activity (Ullah et al., 2019). Cu-Zn-SOD performs dismutation of harmful superoxide radicals into molecular oxygen and H₂O₂ (Hassan et al., 2012). BPS-induced

increased Cu-Zn-SOD activity might be triggered by elevated levels of superoxide radical generation thereby simultaneously increasing H₂O₂ production, ultimately leading to BPS-induced increased CAT activity observed in our study. Decreased GPx activity might also induce CAT activity increase by elevating H₂O₂ level. CAT attenuates intensity of oxidative damages by decomposing H₂O₂ into water and molecular oxygen (Maćczak et al., 2017). Similar to our observation, BPS-induced CAT activity increase has previously been reported to attenuate increased ROS molecules in hepatocytes and renal cells (Zhang et al., 2016). Furthermore, H₂O₂-induced oxidative stress in large intestine has previously been confirmed to suppress both the spontaneous phasic contractions in circular muscle of colon of rat and contractility of canine colonic smooth muscle cells (Gonzalez and Sarna, 2001; Shi et al., 2003). Probably in our experiment too, elevated H₂O₂ level might have induced oxidative stress, contributing to BPS-induced decrease in contractility of duodenum of small intestine. Moreover, oxidative stress has been reported to prominently alter motility of large intestine previously (Van Der Vliet et al., 1989). We here report a similar observation in the small intestine following BPS exposure, through our experiment.

So, our study reports here for the first time, the effects of BPS exposure on contractile function of duodenal VSM by giving emphasis on probable neurocrine and biochemical stress mechanisms involved and is summarized here (Fig.3). Though, further experiments are required to understand detailed molecular mechanisms behind BPS-induced alterations.

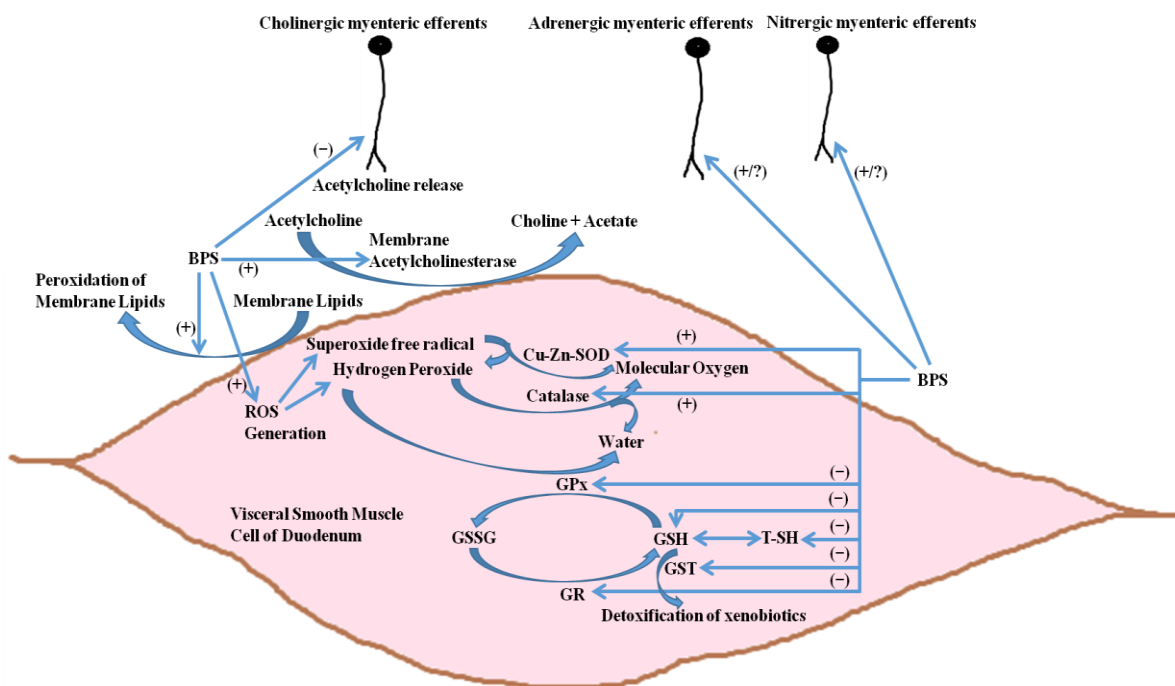


Fig. 3 Schematic representation showing the probable cholinergic myenteric and smooth muscular oxidative stress mechanisms involved in BPS induced inhibition of the contraction of small intestinal visceral smooth muscle. (+) indicates stimulated; (-) indicates inhibited; (+/?) indicates might be stimulated. BPS= Bisphenol S; ROS= Reactive oxygen species; Cu-Zn-SOD= Copper-zinc superoxide dismutase; GPx= Glutathione peroxidase; GR= Glutathione reductase; GST= Glutathione-S-transferase; GSSG= Oxidized glutathione; GSH= Reduced glutathione; T-SH= Total thiol.

Conclusion

The present study concludes that replacement of BPA by BPS might not be a wise and safe choice as BPS exhibits the potency to diminish and inhibit spontaneous rhythmic contraction of visceral smooth muscle found in small intestine and thereby induces dysmotility behavior. Inhibition of contraction occurs partly due to BPS-induced increase in enzymatic activity of acetylcholinesterase, and also through the BPS-induced generation of oxidative stress associated with alterations in the antioxidant enzyme activities, thereby hampering redox homeostasis in smooth muscle and/or myenteric neurons. BPS-induced dysmotility of small intestine may inhibit digestion and absorption of ingested foodstuffs, and propulsion of chyme within lumen of small intestine as well as may also promote bacterial overgrowth inside small intestinal lumen.

Acknowledgements

Gratefully acknowledged to University Grants Commission (UGC) for Junior Research Fellowship (JRF) in Sciences [award letter no. F. No. 16-9 (June2017)/2018(NET/CSIR) dtd. 26.12.2018] for financial support to carry out the research work.

Conflict of interest

We declare no potential conflicts of interest in this research study and publication of the results of the research.

Ethical Considerations

The study was approved by the ethical committee.

Funding support

Junior Research Fellowship (JRF) in Sciences [award letter no. F. No. 16-9 (June2017)/2018(NET/CSIR) dtd. 26.12.2018]

References

Andrews, J. M., & Blackshaw, L. A. (2010). Small intestinal motor and sensory function and dysfunction. In M. Feldman, L. S. Friedman & L. J. Brandt (Ed.), *Sleisenger and Fordtran's gastrointestinal and liver disease: Pathophysiology/diagnosis/management* (pp. 1643-1658). Philadelphia: Saunders.

Ayala, A., Muñoz, M. F., & Argüelles, S. (2014). Lipid peroxidation: Production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative Medicine and Cellular Longevity*, 2014, 360438. <https://doi.org/10.1155/2014/360438>

Beers, R. F., & Sizer, I. W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, 195(1), 133-140. [https://doi.org/10.1016/S0021-9258\(19\)50881-X](https://doi.org/10.1016/S0021-9258(19)50881-X)

Bousomah, R., Leso, V., Iavicoli, I., Huuskonen, P., Viegas, S., Porras, S. P., Santonen, T., Frery, N., Robert, A., & Ndaw, S. (2021). Biomonitoring of occupational exposure to bisphenol A,

bisphenol S and bisphenol F: A systematic review. *Science of the Total Environment*, 783, 146905. <https://doi.org/10.1016/j.scitotenv.2021.146905>

Buege, J. A., & Aust, S. D. (1978). Microsomal lipid peroxidation. In S. Fleischer & L. Packer (Ed.), *Methods in enzymology, Biomembranes, Part C: Biological oxidations: Microsomal, cytochrome P450, and other hemoprotein systems* (Vol. 52, pp. 302-310). New York: Academic Press. [https://doi.org/10.1016/S0076-6879\(78\)52032-6](https://doi.org/10.1016/S0076-6879(78)52032-6)

Caballero-Casero, N., Lunar, L., & Rubio, S. (2016). Analytical methods for the determination of mixtures of bisphenols and derivatives in human and environmental exposure sources and biological fluids. A review. *Analytica Chimica Acta*, 908, 22-53. <https://doi.org/10.1016/j.aca.2015.12.034>

Cao, P., Zhong, H., Qiu, K., Li, D., Wu, G., Sui, H., & Song, Y. (2021). Exposure to bisphenol A and its substitutes, bisphenol F and bisphenol S from canned foods and beverages on Chinese market. *Food Control*, 120, 107502. <https://doi.org/10.1016/j.foodcont.2020.107502>

Dutta, M., & Paul, G. (2019). Gallic acid protects rat liver mitochondria ex vivo from bisphenol A induced oxidative stress mediated damages. *Toxicology Reports*, 6, 578-589. <https://doi.org/10.1016/j.toxrep.2019.06.011>

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, 7(2), 88-95. [https://doi.org/10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9)

Gonzalez, A., & Sarna, S. K. (2001). Different types of contractions in rat colon and their modulation by oxidative stress. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 280(4), G546-G554. <https://doi.org/10.1152/ajpgi.2001.280.4.G546>

Gupta, R., & Singh, K. K. (2007). Stability studies on a cough syrup in plastic containers. *Indian Journal of Pharmaceutical Sciences*, 69(3), 408-413. 10.4103/0250-474X.34551

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-transferases the first enzymatic step in mercapturic acid formation. *Journal of Biological Chemistry*, 249(22), 7130-7139. [https://doi.org/10.1016/S0021-9258\(19\)42083-8](https://doi.org/10.1016/S0021-9258(19)42083-8)

Hassan, Z. K., Elobeid, M. A., Virk, P., Omer, S. A., ElAmin, M., Daghestani, M. H., & AlOlayan, E. M. (2012). Bisphenol A induces hepatotoxicity through oxidative stress in rat model. *Oxidative Medicine and Cellular Longevity*, 2012, 194829. <https://doi.org/10.1155/2012/194829>

Ho, Y. S., Magnenat, J. L., Gargano, M., & Cao, J. (1998). The nature of antioxidant defense mechanisms: A lesson from transgenic studies. *Environmental Health Perspectives*, 106(Suppl 5), 1219-1228. <https://doi.org/10.1289/ehp.98106s51219>

Horan, T. S., Pulcastro, H., Lawson, C., Gerona, R., Martin, S., Gieske, M. C., Sartain, C. V., & Hunt, P. A. (2018). Replacement bisphenols adversely affect mouse gametogenesis with consequences for subsequent generations. *Current Biology*, 28(18), 2948-2954. <https://doi.org/10.1016/j.cub.2018.06.070>

Ijaz, S., Ullah, A., Shaheen, G., & Jahan, S. (2020). Exposure of BPA and its alternatives like BPB, BPF, and BPS impair subsequent reproductive potentials in adult female Sprague Dawley rats. *Toxicology Mechanisms and Methods*, 30(1), 60-72. <https://doi.org/10.1080/15376516.2019.1652873>

Khadrawy, Y. A., Nour, N. A., & Ezz, H. S. A. (2011). Effect of oxidative stress induced by paradoxical sleep deprivation on the activities of Na⁺, K⁺-ATPase and acetylcholinesterase in the cortex and hippocampus of rat. *Translational Research-Journal of Laboratory and Clinical Medicine*, 157(2), 100-107. <https://doi.org/10.1016/j.trsl.2010.11.005>

Khmiri, I., Côté, J., Mantha, M., Khemiri, R., Lacroix, M., Gely, C., Toutain, P., Picard-Hagen, N., Gayraud, V., & Bouchard, M. (2020). Toxicokinetics of bisphenol-S and its glucuronide in plasma and urine following oral and dermal exposure in volunteers for the interpretation of biomonitoring data. *Environment International*, 138, 105644. <https://doi.org/10.1016/j.envint.2020.105644>

Lehmmler, H. J., Liu, B., Gadogbe, M., & Bao, W. (2018). Exposure to bisphenol A, bisphenol F, and bisphenol S in US adults and children: The national health and nutrition examination survey

- 2013–2014. *ACS Omega*, 3(6), 6523-6532. <https://doi.org/10.1021/acsomega.8b00824>
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*, 193, 265-275. [https://doi.org/10.1016/S0021-9258\(19\)52451-6](https://doi.org/10.1016/S0021-9258(19)52451-6)
- Maćczak, A., Cyrkler, M., Bukowska, B., & Michałowicz, J. (2017). Bisphenol A, bisphenol S, bisphenol F and bisphenol AF induce different oxidative stress and damage in human red blood cells (*in vitro* study). *Toxicology In Vitro*, 41, 143-149. <https://doi.org/10.1016/j.tiv.2017.02.018>
- Marklund, S., & Marklund, G. (1974). Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase. *European Journal of Biochemistry*, 47(3), 469-474. <https://doi.org/10.1111/j.1432-1033.1974.tb03714.x>
- Melo, J. B., Agostinho, P., & Oliveira, C. R. (2003). Involvement of oxidative stress in the enhancement of acetylcholinesterase activity induced by amyloid beta-peptide. *Neuroscience Research*, 45(1), 117-127. [https://doi.org/10.1016/S0168-0102\(02\)00201-8](https://doi.org/10.1016/S0168-0102(02)00201-8)
- Moreira, E. L. G., de Oliveira, J., Nunes, J. C., Santos, D. B., Nunes, F. C., Vieira, D. S. C., Ribeiro-do-Valle, R. M., Pamplona, F. A., de Bem, A. F., Farina, M., Walz, R., & Prediger, R. D. (2012). Age-related cognitive decline in hypercholesterolemic LDL receptor knockout mice (LDLR^{-/-}): Evidence of antioxidant imbalance and increased acetylcholinesterase activity in the prefrontal cortex. *Journal of Alzheimer's Disease*, 32(2), 495-511. <https://doi.org/10.3233/JAD-2012-120541>
- Paglia, D. E., & Valentine, W. N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *Translational Research-Journal of Laboratory and Clinical Medicine*, 70(1), 158-169. <https://doi.org/10.5555/uri:pii:0022214367900765>
- Quinn, D. M. (1987). Acetylcholinesterase: Enzyme structure, reaction dynamics, and virtual transition states. *Chemical Reviews*, 87(5), 955-979. <https://doi.org/10.1021/cr00081a005>
- Sabry, R., Saleh, A. C., Stalker, L., LaMarre, J., & Favetta, L. A. (2021). Effects of bisphenol A and bisphenol S on microRNA expression during bovine (*Bos taurus*) oocyte maturation and early embryo development. *Reproductive Toxicology*, 99, 96-108. <https://doi.org/10.1016/j.reprotox.2020.12.001>
- Salahinejad, A., Attaran, A., Naderi, M., Meuthen, D., Niyogi, S., & Chivers, D. P. (2021). Chronic exposure to bisphenol S induces oxidative stress, abnormal anxiety, and fear responses in adult zebrafish (*Danio rerio*). *Science of the Total Environment*, 750, 141633. <https://doi.org/10.1016/j.scitotenv.2020.141633>
- Sarkar, K., Tarafder, P., Nath, P. P., & Paul, G. (2013). Bisphenol A inhibits duodenal movement in rat by increasing acetylcholinesterase activity and decreasing availability of free Ca²⁺ in smooth muscle cells. *International Journal of Pharma and Bio Sciences*, 4(2), B679-B688.
- Sarkar, K., Tarafder, P., & Paul, G. (2016). Bisphenol A inhibits duodenal movement *ex vivo* of rat through nitric oxide-mediated soluble guanylyl cyclase and α -adrenergic signaling pathways. *Journal of Applied Toxicology*, 36(1), 131-139. <https://doi.org/10.1002/jat.3154>
- Sedlak, J., & Lindsay, R. H. (1968). Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent. *Analytical Biochemistry*, 25, 192-205. [https://doi.org/10.1016/0003-2697\(68\)90092-4](https://doi.org/10.1016/0003-2697(68)90092-4)
- Shi, X. Z., Lindholm, P. F., & Sama, S. K. (2003). NF- κ B activation by oxidative stress and inflammation suppresses contractility in colonic circular smooth muscle cells. *Gastroenterology*, 124(5), 1369-1380. [https://doi.org/10.1016/S0016-5085\(03\)00263-4](https://doi.org/10.1016/S0016-5085(03)00263-4)
- Staal, G. E. J., Visser, J., & Veeger, C. (1969). Purification and properties of glutathione reductase of human erythrocytes. *Biochimica et Biophysica Acta (BBA)-Enzymology*, 185(1), 39-48. [https://doi.org/10.1016/0003-2697\(69\)90280-0](https://doi.org/10.1016/0003-2697(69)90280-0)
- Ullah, A., Pirzada, M., Jahan, S., Ullah, H., & Khan, M. J. (2019). Bisphenol A analogues bisphenol B, bisphenol F, and bisphenol S induce oxidative stress, disrupt daily sperm production, and damage DNA in rat spermatozoa: a comparative *in vitro* and *in vivo* study. *Toxicology and Industrial Health*, 35(4), 294-303. <https://doi.org/10.1177/0748233719831528>
- Ullah, H., Jahan, S., Ain, Q. U., Shaheen, G., & Ahsan, N. (2016). Effect of bisphenol S exposure on male reproductive system of rats: A histological and biochemical study. *Chemosphere*, 152, 383-391. <https://doi.org/10.1016/j.chemosphere.2016.02.125>
- Van der Vliet, A., Tuinstra, T. J., & Bast, A. (1989). Modulation of oxidative stress in the gastrointestinal tract and effect on rat intestinal motility. *Biochemical Pharmacology*, 38(17), 2807-2818. [https://doi.org/10.1016/0006-2952\(89\)90435-8](https://doi.org/10.1016/0006-2952(89)90435-8)
- Vona, R., Pallotta, L., Cappelletti, M., Severi, C., & Matarrese, P. (2021). The impact of oxidative stress in human pathology: Focus on gastrointestinal disorders. *Antioxidants*, 10(2), 201. <https://doi.org/10.3390/antiox10020201>
- Yang, Y., Yu, J., Yin, J., Shao, B., & Zhang, J. (2014). Molecularly imprinted solid-phase extraction for selective extraction of bisphenol analogues in beverages and canned food. *Journal of Agricultural and Food Chemistry*, 62(46), 11130-11137. <https://doi.org/10.1021/jf5037933>
- Zhang, R., Liu, R., & Zong, W. (2016). Bisphenol S interacts with catalase and induces oxidative stress in mouse liver and renal cells. *Journal of Agricultural and Food Chemistry*, 64(34), 6630-6640. <https://doi.org/10.1021/acs.jafc.6b02656>
- Zhang, Z., Lin, L., Gai, Y., Hong, Y., Li, L., & Weng, L. (2018). Subchronic bisphenol S exposure affects liver function in mice involving oxidative damage. *Regulatory Toxicology and Pharmacology*, 92, 138-144. <https://doi.org/10.1016/j.yrtph.2017.11.018>

This work is licensed under a [Creative Commons Attribution 4.0 International License](https://creativecommons.org/licenses/by/4.0/).



How to cite this article:

Roy, D., Dutta, M., Mondal, M., Sarkar, K., Paul, G. (2021). Effect of Bisphenol S (BPS) on the contraction of duodenal visceral smooth muscle *ex vivo* in rat. *Science Archives*, Vol.2(2),99-108. <http://dx.doi.org/10.47587/SA.2021.2207>