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RESEARCH ARTICLE

Chronic ingestion of bisphenol A decreases the cholinergically evoked and spontaneous contractions of rat uterus *in vitro*

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ABSTRACT

Background: Bisphenol A (BPA), a chemical used in the manufacture of plastics, has toxic effects on reproductive, developmental, and metabolic systems. It is implicated as a causative factor for infertility. The uterine and tubal contractility play a vital role in fertilization and implantation of zygote. The chronic exposure to BPA on myometrial contractions is not known. **Aims and Objectives:** The aim of this study is to examine the effect of chronic ingestion of BPA on rat uterine contractions evoked by acetylcholine. **Materials and Methods:** Rats weighing 90-150 g were fed BPA (2 μg/kg/day) or placebo containing pellets (time-matched control; TMC) orally for 28 days. The contractility of the uterus was also assessed in group of rats at the beginning (initial control [IC]). After 28 days, animals showing estrous phase were chosen for *in vitro* recording of uterine contractions (35 ± 1°C). Evoked contractions to different concentrations of acetylcholine were recorded followed by the effect on spontaneous contractions. **Results:** Acetylcholine evoked uterine contractions *in vitro* in a concentration-dependent manner. The contractions were similar in magnitude in IC and TMC. The uterus from BPA-fed animal exhibited decreased responsiveness to acetylcholine-evoked contractions as compared to TMC. In the presence of acetylcholine, frequency and force of spontaneous contractions increased in a concentration-dependent manner. However, in BPA-fed group, the frequency and force of spontaneous contractions were significantly attenuated. **Conclusions:** The results indicate that chronic exposure to BPA decreased the acetylcholine-evoked contractions as well as the spontaneous uterine contractions. The decreased contractility may account for the infertility seen after exposure to BPA.

KEY WORDS: Bisphenol A; Acetylcholine; Plastic Chemical; Myometrial Contractility; Infertility

INTRODUCTION

Bisphenol A (BPA), a toxic chemical manufactured in high volumes worldwide, is used as a plasticizer. BPA produces toxic effects on endocrine and metabolic systems[1] and BPA exposure of animals and humans produce reproductive toxicity.[2] BPA has estrogenic activity and mainly binds to estrogen alpha receptors *in vitro* or *in vivo*[3] thus inhibits the activity of endogenous estrogen[4] BPA is shown to alter number of reproductive parameters such as weight of ovary, weight of uterus, ovum morphology, fertility rate, and litter size in experimental animals.[5] Women showing higher BPA concentrations are associated with infertility resulting from ovarian dysfunction.[6] Infertility can result from ovarian and extraovarian factors. In the extraovarian factors, contractility of uterus or motility of uterus assumes importance. Uterine contractility is responsible for sperm transportation, ovum mobilization for fertilization, and implantation of blastocyst.[7] Thus, the frequency and force of uterine contractions play a vital role in fertilization. Uterine contractility is governed by neuroendocrine mechanisms. The humoral factors

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(oxytocin and prostaglandin) besides producing contractions directly also sensitize the uterine tissue for the actions of acetylcholine. The neuronal mechanisms are mediated by the parasympathetic cholinergic plexuses by releasing acetylcholine to produce uterine contractions. Acetylcholine involves muscarinic receptors located on the uterine smooth muscle cell membrane.[8] Even though the myometrial contractility is an important factor for fertilization, the effect of BPA on uterine contractility is not known. Therefore, the aim of this study was to examine the effect of chronic ingestion of BPA on acetylcholine-evoked contractions and on spontaneous contractions of rat uterus *in vitro*.

MATERIALS AND METHODS

Chemicals

BPA was obtained from HiMedia Laboratories Pvt., Ltd., Mumbai, India. Acetylcholine was from Sigma Chemicals CO., St. Louis, MO, USA. The stock solution (10^{-2} M) of acetylcholine was prepared in distilled water. The final dilution was made in Krebs-Ringer solution. The Krebs-Ringer solution had the following composition (mM) NaCl, 137; KCl, 3.7; $CaCl_2$, 1.02; $MgCl_2$, 0.05; NaH_2PO_4, 0.32; $NaHCO_3$, 11.9; and glucose, 5. After bubbling with 100% O_2, the pH of the solution was 7.4.

Animals

All experimental procedures were approved by the Animal Ethical Committee of Banaras Hindu University, Varanasi, India. This study was carried out using female rats (90-150 g) of Charles-Foster strain. Temperature and humidity were controlled at 25 ± 0.5°C with 12:12 hour Light: Dark photoperiod and free access to food and tap water.

Preparation of BPA Pellet

The stock of BPA-wheat flour mixture was prepared by mixing 2 mg BPA in 20 ml olive oil with 50 g of wheat flour thoroughly. Pellets of known concentration of BPA were prepared according to the weight of the rats on daily basis. BPA in the dose of 2 µg/kg/day per animal was given orally for 28 days. In case of time-matched controlled group (TMC), stock containing only olive oil and wheat flour was used (placebo).

Identification of Estrous Phase

Vaginal secretions were aspirated by Pasteur pipette and spread over the glass slide with the help of spreader. The smear was allowed to dry and stained by aqueous methylene blue (0.5%) for 10-15 min then washed thoroughly and dried. The smear was then examined under the microscope at ×40. The animals showing estrous phase (presence of irregular, non-nucleated, and cornified cells) were chosen for the experiment.

Experimental Groups

Rats were divided into 3 groups. In Group I, the experiments were performed on rats at the beginning of BPA feeding schedule and served as initial control (IC). In Group II, the animals were fed with placebo pellets for 28 days, and this group served as TMC group. In Group III, the animals were fed with pellets containing BPA (2 µg/kg/day per animal) for 28 days. They served as BPA-fed group.

Uterine Tissue Preparation and Recording of *In Vitro* Contractions

Animal showing the estrous phase were sacrificed by cervical dislocation and exsanguination. Abdomen was opened quickly by midline incision, and the uterine horn was dissected free from the surrounding tissue. Then, they were immediately placed in a Petri dish containing Krebs-Ringer solution bubbled with 100% oxygen.

A 10-12 mm segment of uterus was isolated and fastened to one end of the glass tissue holder then transferred to an organ bath. The other end of the uterine segment was fastened to a transducer through a thread. The organ bath (25 ml) was pre-filled with Krebs-Ringer solution bubbled with 100% O_2 maintained at 35 ± 1°C. An initial tension of 1 g was given to the tissue and preparation was allowed to stabilize for 30 min before taking the control (initial) recordings. The uterine contractions were recorded on a chart recorder (Biodevices, Ambala, India).

The tissue was exposed to cumulative concentrations of acetylcholine (0.1-3 µM). After stabilization, the tissue was exposed to a concentration of acetylcholine and recorded for 5 min. Subsequently, the tissue was exposed to next higher concentration of acetylcholine. The acetylcholine responses were divided into two phases. Immediate change in the force is considered as acetylcholine-evoked response. While the effect of acetylcholine on the frequency and force of spontaneous contractions over 5 min was seen as effect on spontaneous activity. At the end of each experiment, uterine segment was removed blotted gently and weighed to calculate tension g/g of tissue.

Parameters Studied

The immediate response to acetylcholine on force was measured as g tension, subsequently computed for per gram tissue. The frequency and force of spontaneous contractions were calculated from the recordings manually. The initial force and frequency of spontaneous contractions before acetylcholine exposure were taken as 100% for normalization to see the effect on various concentrations of acetylcholine.

Statistical Analysis

The mean ± standard error mean of pooled data was calculated. The cumulative concentration-response of acetylcholine

in TMC group was compared with IC group by two-way ANOVA. Furthermore, BPA-fed group was compared with the TMC group using two-way ANOVA. Student's *t*-test for paired or unpaired observations was also performed as required. $P < 0.05$ was considered statistically significant.

RESULTS

Rats weighing (90 ± 4 g) were randomly divided into 3 groups. The body weight of TMC and BPA-fed animals increased to 125 ± 2.38 and 138 ± 7.22 g, respectively. The increase of body weight in BPA-fed animals was significantly greater than TMC group.

Acetylcholine-evoked Concentration-dependent Increase in Uterine Contractility

Acetylcholine evoked uterine contractions in a concentration (0.1-3μM)–dependent manner in IC group (Figure 1). At 0.1 μM of acetylcholine, no responses were seen. However, 0.3 μM of acetylcholine produced contractions of the magnitude of 99 g/g muscle and at 3 μM, the response was doubled. The concentration-response of acetylcholine in TMC group was not different from the IC group ($P > 0.05$, two-way ANOVA; Figure 1). However, the concentration response of acetylcholine-evoked responses in BPA-fed rats was significantly lesser than the TMC group ($P < 0.05$, two-way ANOVA). In BPA-fed group, 0.1 μM of acetylcholine produced threshold response whereas at 3 μM, the responses were 128 g/g tissue which was significantly lesser than the TMC group.

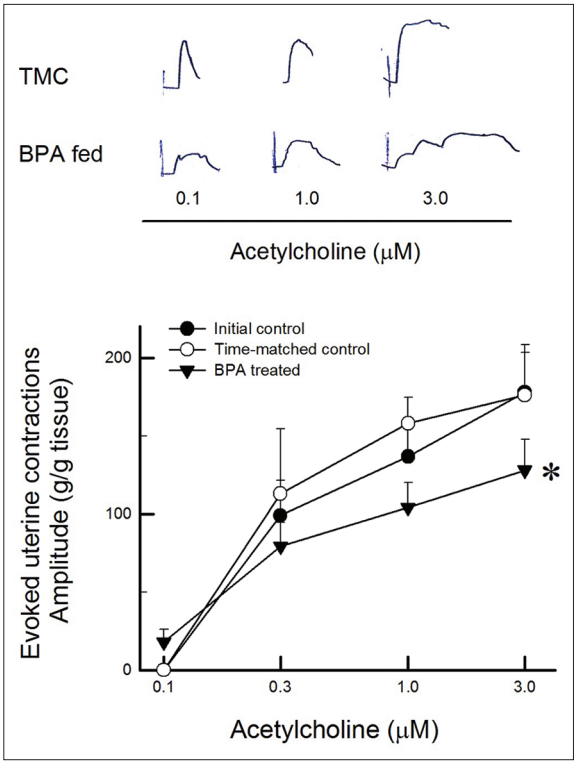

Figure 1: Chronic ingestion of Bisphenol A (BPA) attenuated the acetylcholine response in a concentration-dependent manner. The original tracings of an experiment, in time-matched control (TMC) and BPA-fed group are presented in the upper panel. The mean ± standard error mean values (from $n = 6$ for initial control and TMC; $n = 10$ for BPA treated) are shown in the line diagram. An asterisk (*) indicates significant difference ($P < 0.05$; two-way ANOVA) from BPA-treated group from TMC group

Effect of Acetylcholine on the Force of Spontaneous Contractions

The initial force of contractions before acetylcholine exposure in various groups is given in Table 1. The responses of spontaneous contractions after acetylcholine are expressed as % response before acetylcholine (initial response). Acetylcholine increased the force of spontaneous uterine contractions in a concentration-dependent manner (0.1-3 μM) in the IC group. At 0.1 μM, no change in the force of spontaneous contractions was seen; at 0.3 μM, the increase was 18%; at 1 μM, the increase was 28%; and at 3 μM, the increase was 31% (Figure 2). After 28 days of exposure to placebo in TMC group, the responses were not different from the IC group ($P > 0.05$, two-way ANOVA). In BPA-fed group, the responses at different concentrations of acetylcholine were significantly lesser than the TMC group (Figure 2; $P < 0.05$, two-way ANOVA).

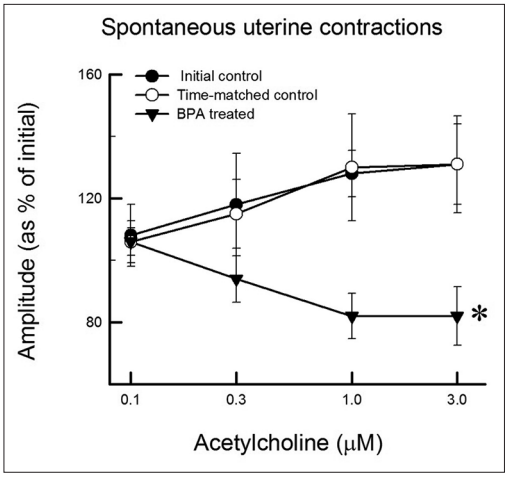

Figure 2: Increased force of spontaneous uterine contractions in the presence of acetylcholine was attenuated in BPA-treated group. The mean ± standard error mean values ($n = 6$ for initial control and time-matched control (TMC); $n = 10$ for BPA treated) are shown in the line diagram. An asterisk (*) indicates significant difference from BPA-treated group ($P < 0.05$; two-way ANOVA) from TMC

Effect of Acetylcholine on the Frequency Spontaneous Contractions

The initial frequency of contractions before acetylcholine exposure in various groups is given in Table 1. The responses of spontaneous contractions after acetylcholine are expressed as % response before acetylcholine (initial response). Acetylcholine increased the frequency of spontaneous

Table 1: Initial values of force and frequency of spontaneous contractions before exposing to acetylcholine in various groups

Groups	Force (g/g muscle)	Frequency(/min)
IC (*n*=6)	12.0±1.04	0.21±0.06
TMC (*n*=6)	14.4±1.52	0.24±0.03
BPA fed (*n*=10)	11.4±2.20*	0.41±0.07*

The values (mean±SEM) are obtained from 6 to 10 different preparations from rats showing estrous phase in various groups, An asterisk (*) indicates $P<0.05$ as compared TMC, SEM: Standard error mean, TMC: Time-matched control, BPA: Bisphenol A, IC: Initial control

contractions of uterus in a concentration-dependent manner (0.1-3 μM) in the IC group. At 0.1 μM of acetylcholine, the increase was 16%; at 0.3 μM, the increase was 24%; at 1 μM the increase was 82%; and at 3 μM, the increase was 293% (Figure 3). After 28 days of exposure to placebo in TMC group, the responses were similar to IC group ($P > 0.05$, two-way ANOVA). In BPA-fed group, the responses at different concentrations were significantly lesser than the TMC group (Figure 3; $P < 0.05$, two-way ANOVA). However, the initial frequency was significantly higher in BPA-fed group than TMC ($P < 0.05$ Students *t*-test for unpaired observations).

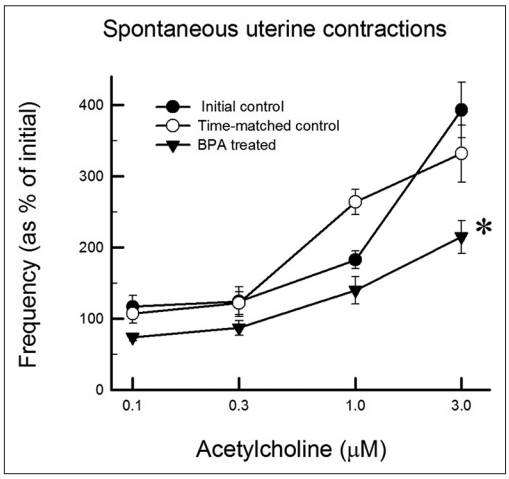

Figure 3: Enhancement of frequency of spontaneous uterine contractions in the presence of acetylcholine was attenuated in bisphenol A (BPA)-treated group. The mean ± standard error mean values ($n = 6$ for initial control and time-matched control (TMC); $n = 10$ for BPA treated) are shown in the line diagram. An asterisk (*) indicates significant difference from BPA-treated group ($P < 0.05$; two-way ANOVA) from TMC

DISCUSSION

Present observations indicate that the chronic ingestion of BPA in rats attenuated the acetylcholine-induced contractions as well as the acetylcholine effect on spontaneous contractility of the uterus *in vitro*. These observations while supporting the inhibitory effects of BPA on cultured uterine cells *in vitro*[9] further, demonstrate the decreased responsiveness of uterine contractions to acetylcholine after exposure to BPA.

It is known that BPA produces number of metabolic and endocrine abnormalities.[1] In our study, the BPA-fed animals have a greater body mass than the TMC group. This supports the metabolic abnormalities induced in BPA-fed animals. Recently, chronic ingestion high-concentration BPA (25 times the concentration used in the present study) decreased rat ileal contractions.[10] On reproductive system, BPA exerts toxic effect on uterine endometrial proliferation, uterine receptivity associated with implantation failure in animals.[6] In human beings, BPA exposure is associated with adverse birth outcome, sexual dysfunction, and impaired implantation.[11] The oviduct abnormalities have also been reported after BPA exposure.[12] Myometrium is innervated by cholinergic parasympathetic fibers and acetylcholine activates the M_2 receptors to bring about the contractions.[13] It has been shown that uterotonic activity of *Ficus asperifolia* extracts increased uterine contractility involving cholinergic (muscarinic) mechanism.[14] Our observations indicate that BPA exposure decreased. The response to acetylcholine and also attenuated the force and frequency of spontaneous contractions in the presence of acetylcholine after BPA. Thus, these findings indicate the suppression of cholinergic activity by BPA. The mechanism responsible for it is not clear. It can be attributed to the antiestrogenic activity of BPA as estrogen is known to increase the excitability of myometrium. The antiestrogenic activity of BPA is supported by the following reports. BPA is shown to inhibit the activity of endogenous estrogen and also disrupts receptor action.[4] It is shown that low dose of BPA increased testosterone and progesterone levels.[15,16] In another study, low dose of BPA decreased estradiol, testosterone, Cyp19 (aromatase), and StAR (steroidogenic acute regulatory protein).[17] Thus, antiestrogenic actions may responsible for the decreased responsiveness of uterus to acetylcholine. These findings support for the decreased uterine receptivity and decreased transport of pre-implantation embryo reported elsewhere.[18,19]

This study provides the effect of chronic exposure of BPA on uterine contractility *in vitro* as against the acute *in vitro* effects of BPA. Thus, these observations indicate *in vivo* toxicity of BPA. However, this study used only one dose of BPA. It is necessary to perform the dose-response of BPA so as to identify the threshold dose that produces the toxic effects. Further, it may be necessary to identify the uterine responsiveness to other agonists such oxytocin, prostaglandin, and histamine.

CONCLUSIONS

Chronic ingestion of BPA to adult female rats decreased the uterine contractility evoked by acetylcholine *in vitro*. The force of spontaneous contractions after BPA was decreased

significantly whereas the frequency of contractions was increased. The response of spontaneous contractility in the presence of acetylcholine at various concentrations was also lesser in terms of force and frequency. Even though the frequency of spontaneous contractions was increased significantly after exposure to BPA, but the associated decrease in force of contractions may not be able to produce effective contractions that are necessary for the transportation of ovum. These observations suggest possible modulation of cholinergic responses by BPA. All these effects support for infertility due to the failure of embryo transport, fertilization, or implantation.

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