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Inhibitory effect of Bisphenol A on *in vitro* feline uterine contractions



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ABSTRACT

Bisphenol A (BPA) is an environmental pollutant used as a plasticizer in the manufacture of many plastic products, such as packaging, containers, and water and beverage bottles. There are deleterious effects of BPA on metabolic, endocrine, nervous, and reproductive systems. This is the first study in which there was investigation of the *in vitro* effect of BPA on the spontaneous contractions of the cat uterus. The tubal uterine segments (1 cm) collected from queens in estrus were suspended in an isolated organ bath. Following tissue stabilization, spontaneous contractions were recorded for 10 min to constitute the control group. The effects of the solvent (alcohol) and BPA at different concentrations (1, 10, and 100 μ M) on uterine contractions were subsequently evaluated at 10 min intervals in terms of frequency and mean amplitude variables. The ethanol vehicle did not alter the uterine contractions compared to the control group. All concentrations of BPA used in the study resulted in a reduction ($P < 0.05$) in amplitude of uterine contractions in a dose-dependent manner, while only the largest dose of BPA decreased the frequency of contractions ($P < 0.05$). In reproductive physiology, regular uterine contractions facilitate successful fertilization, migration, implantation, and maintenance of pregnancy, as well as fetus expulsion. The results of this study indicate BPA has an inhibitory effect on spontaneous contractions of the cat uterus. It is proposed that this suppressive effect of BPA on uterine contractions might lead to queen infertility.

1. Introduction

Bisphenol A (BPA), an environmental toxic chemical, is the most popular substance of synthetic organic compounds with hydroxyphenyl groups called bisphenols (Gregoraszczyk and Ptak, 2013; Liliana et al., 2019). Although the usage of BPA is banned in many countries, including the USA, Brazil, China, Sweden, and Malaysia, according to a recent report, the annual consumption volume of BPA throughout the world is 7.7 million metric tons in 2015, and is anticipated to be 10.6 million metric tons by 2022 (Silvestre and Cimmino, 2016; Industry Experts, 2016). The BPA compound is commonly used in many plastic products, including sports equipment, household goods, toys, and especially products contacted directly with food and water, such as packaging materials, containers, water pipelines, and bottles for beverages (Liliana et al., 2019). Living beings are exposed to BPA through food intake, inhalation, or dermal contact (Vandenberg et al., 2007). The BPA compound leaches into the pet foods from cans (Kang and Kondo, 2002). Other pet exposure sources of BPA are toys and training devices (Wooten and Smith, 2013). The BPA compound has

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been detected in human serum at the concentrations of 0.2–1.6 ng/mL (Alonso-Magdalena et al., 2015) and there has also been detection in other body fluids (Azzouz et al., 2016), as well as reproductive tissues (Vandenberg et al., 2007). There is no available knowledge about the blood and tissue concentrations of BPA in cats, but daily intake in small amounts of this compound from the commercial canned cat food throughout life is thought to be a potential risk for cat health (Peterson, 2012).

The BPA compound has deleterious effects on most of the organs and physiological systems of humans and animals (Liliana et al., 2019; Gregoraszczyk and Ptak, 2013). In the nervous system, BPA inhibits the synaptic reflexes and potential action of the sciatic nerve (Pandey and Deshpande, 2012, 2015). Also, BPA can alter the neurochemical innervation of the uterus (Liliana et al., 2019). The BPA compound has a toxic effect on the liver by inducing reactive oxygen species formation (Bindhumol et al., 2003). The metabolic effects of BPA are well known, and there is a positive correlation with hyperthyroidism in cats and consumption of packaged and canned pet food (Edinboro et al., 2004; Wakeling et al., 2009). One of the most susceptible systems to the toxicity of BPA is, however, the reproductive system, which is affected by small amounts and short-term exposures (Qiu et al., 2015). The BPA compound can disrupt the homeostasis of endocrine hormones. Results of previous studies indicate increases or decreases in the concentrations of reproductive steroids, such as estrogens, progesterone, and testosterone as a result of BPA actions (Mlynarcikova et al., 2005; Zhou et al., 2008; Peretz and Flaws, 2013; Kabakci and Yigit, 2017). The toxic effect of BPA on the reproductive system is mainly due to its estrogenic activity. The BPA compound can, therefore, inhibit the activity of endogenous estrogen by binding to estrogen alpha receptors (Wetherill et al., 2007; Caserta et al., 2011). Estrogen increases the uterus activity during the estrous phase of the reproductive cycle (Gupta and Deshpande, 2017). With regard to reproductive physiology, uterine contractions have an important function in facilitating the establishment of pregnancy in terms of migration and implantation of a zygote. The contractile activity of the uterus is less during pregnancy and, thus, functions to reduce the probability of abortion or premature parturition (Chanrachakul et al., 2004), but there are changes in contractility during the various stages of the reproductive cycle. The frequency of contractions is greater during the follicular phase while the frequency and amplitude are less during the luteal phase of the reproductive cycle (Aguilar and Mitchell, 2010). Abnormalities in the frequency and force of uterine contractions may, however, result in infertility (Angioni et al., 2011). The actions of BPA are implicated in the irregularity of the estrous cycle, development of the polycystic ovarian syndrome, and infertility (Upson et al., 2014; Rutkowska and Diamanti-Kandarakis, 2016). In humans, increased BPA concentration in the urinary and maternal plasma is associated with a decrease in embryo implantation rate (Ehrlich et al., 2012) and increase in risk for preterm birth, respectively (Behnia et al., 2016). Xiao et al. (2011) reported that *in vivo* actions of BPA in mice affects the establishment of uterine embryo receptivity, which can lead to a reduction in the probability of implantation of the embryo. Results of a recent study performed in rats indicate that after 28 days of BPA ingestion, the frequency and amplitude of acetylcholine-evoked uterine contractions *in vitro* were less compared to the control group (Gupta and Deshpande, 2017). It was also reported that the actions of BPA (Gupta and Deshpande, 2018) and other endocrine disrupting compounds, such as p-nonylphenol and 4-*tert*-octylphenol lead to a reduction in the contractility of the rat uterus (Ozatic et al., 2015; An et al., 2013).

As previously described, the effect of BPA has been studied primarily in humans and laboratory animals, but not in cats. The aim of the present study, therefore, was to elucidate the effect of BPA on spontaneous uterine contractile activity of adult female cats.

2. Materials and methods

2.1. Chemicals

Krebs solution was prepared with NaCl 118 mM, KCl 4.69 mM, MgSO₄ 0.6 mM, NaHCO₃ 25 mM, CaCl₂ 2.5 mM, and Glucose 11.1 mM by dissolving in 1 L distilled water. The 2,2-bis(4-hydroxyphenyl)propane (BPA) was purchased from Sigma-Aldrich (USA), and the concentrations of BPA were used by dissolving this compound in 70% ethanol. The final concentration of ethanol in the bath was not greater than 0.7%.

2.2. Equipment

The myometrial contractions of the uterus were quantified using a force-displacement transducer (FDT 05 MAY, Commat, Turkey) and recorded using the Biopac system (MP35, USA).

2.3. Animals

We used cat uterine tissues as the experimental material in this study. Thirteen uterine tissues were obtained from six different adult queens in estrous after performing ovariohysterectomy in the Small Animal Clinics of Kirikkale University Faculty of Veterinary Medicine. The queens were mixed breeds and aged 1 to 2 years. Furthermore, the queens were free from reproductive and metabolic diseases, non-medicated (e.g., contraceptives), and had not previously been pregnant. The experimental protocol was approved by the Local Ethical Committee of Kirikkale University, Turkey (2018-E.4707).

2.4. Identification of the estrous phase

Because the spontaneous contractions of the uterus occur at regular intervals and are phasic during the estrous phase, in this study, uterine tissues of adult queens were used in this phase. The queens expressed estrous behavior with detection occurring during clinical observations, morphological examination of the ovaries, and measurement of the serum estradiol and progesterone

concentrations. Lordosis and presence of follicles on the ovaries were the criteria used to ascertain when queens were in estrus (Shille et al., 1979). Before ovariectomy, blood samples were collected from the *vena cephalica antebrachii* to quantify the estradiol and progesterone concentrations to confirm estrus. After allowing the blood to clot and following centrifugation at 1,000 g for 10 min at 4 °C, the sera were collected and stored at -20 °C until assays were conducted. Estradiol and progesterone quantification were conducted on a microplate reader (Thermo Scientific, Multiskan Go, Finland) using commercial ELISA kits (DRG, Estradiol EIA-2693, Progesterone EIA-1561, Germany). The intra-assay coefficients of variations were 6.8% and 5.4% for estradiol and progesterone, respectively. The inter-assay coefficients of variations were 7.2% and 9.8% for estradiol and progesterone, respectively.

2.5. Preparation of uterine samples

After ovariectomy, the uterine tissues were immediately (2–5 min) transported to the laboratory in Krebs solution at 20 to 22 °C. The blood vessels and the surrounding connective and adipose tissues were removed, and approximately 1 cm tubal segments were dissected from the same part of each uterine horns, which was close to the ovaries. The uterine segments were subsequently suspended in an isolated organ bath filled with 10 mL Krebs solution bubbled with 95% O₂ and 5% CO₂ at 39 ± 1 °C, and 1 g pre-tension was applied for equilibration.

2.6. Experimental protocol

The tissues were allowed to stabilize in Krebs solution for 1 h by replacing with fresh Krebs every 15 min. After stabilization, the activity of tissues was assessed with 2.5 mIU/mL oxytocin, and then washed three times for 5 min (Yildirim and Macun, 2013). Before the tissues were bathed in ethanol (vehicle used for dissolving BPA) and non-cumulative concentrations of BPA (1, 10, and 100 μM), spontaneous contractions were recorded for 10 min to get control values. The concentrations of BPA used in this study were chosen considering findings in previous *in vitro* toxicological studies (Pant et al., 2011; Salleh et al., 2015; Gupta and Deshpande, 2017) and preliminary experiments for conducting the present study. When recording the contractions, the duration of tissue bathing was 10 min with the vehicle and each BPA dose. After each treatment, at least three washings with 10 mL Krebs for 5 min were performed to ensure that the effect of previous treatment was not carried over, and that tissues were having optimal contractions.

In each experiment, there was observation of contractions from at least two uterine segments. Tissues that did not respond to oxytocin were excluded from the study. At the end of the experiment, the frequency (number of contractions) and mean amplitude of uterine contractions during the 10 min period were calculated from the recordings and assessed for each treatment.

2.7. Statistical analysis

All data obtained from the experiments were calculated manually and expressed as the mean ± standard error of the mean (SEM). The statistical analyses were performed with the following statistical tests between two groups, control and each of the treatment groups, by using SPSS 15.0 statistics (IBM, USA). The parametric data (frequency of contractions) were analyzed using the Student t-test, while nonparametric data (mean amplitude of contractions) were analyzed using the Wilcoxon signed-rank test. A *P*-value of < 0.05 was accepted as significant.

3. Results

3.1. Uterine contractions

In this study, after equilibration of tissue to 1 g tension, the frequency and mean amplitude of contractility in the control group for 10 min were 17.2 ± 1.3 number and 3.0 ± 0.5 g, respectively. These values were considered to be 100%, and the percentages for the remaining treatment groups were determined by comparing the results to the control. There was BPA treatment of uterine samples at the concentrations of 1, 10, and 100 μM, and the inhibitory activity was observed based on the frequency and amplitude values for contractions in a dose-dependent manner.

The representative tracings in Fig. 1 provide details for the reaction of the tissue to treatment with 2.5 mIU/L oxytocin, spontaneous contractions of the control group, and the effects of ethanol (vehicle) and different concentrations of BPA (1–100 μM) on uterine contractions of queen cats.

3.2. Effects of BPA on the frequency of uterine contractions

Fig. 2 depicts the effects of all treatment groups on the frequency of uterine contractions. There was no significant effect of the vehicle ethanol on these frequencies. The responses at 1 μM BPA were not different from the control and vehicle groups. At 10 μM, BPA reduced the responses by 8.9%, which was not significant (*P* > 0.05). The most effective concentration of BPA in terms of the frequency of uterine contractions was 100 μM, which decreased the responses by 21.5% (*P* < 0.05).

3.3. Effects of BPA on the amplitude of uterine contractions

The mean amplitude of the uterine contractions was not altered by treatment with the vehicle, ethanol (Fig. 3). The forces of

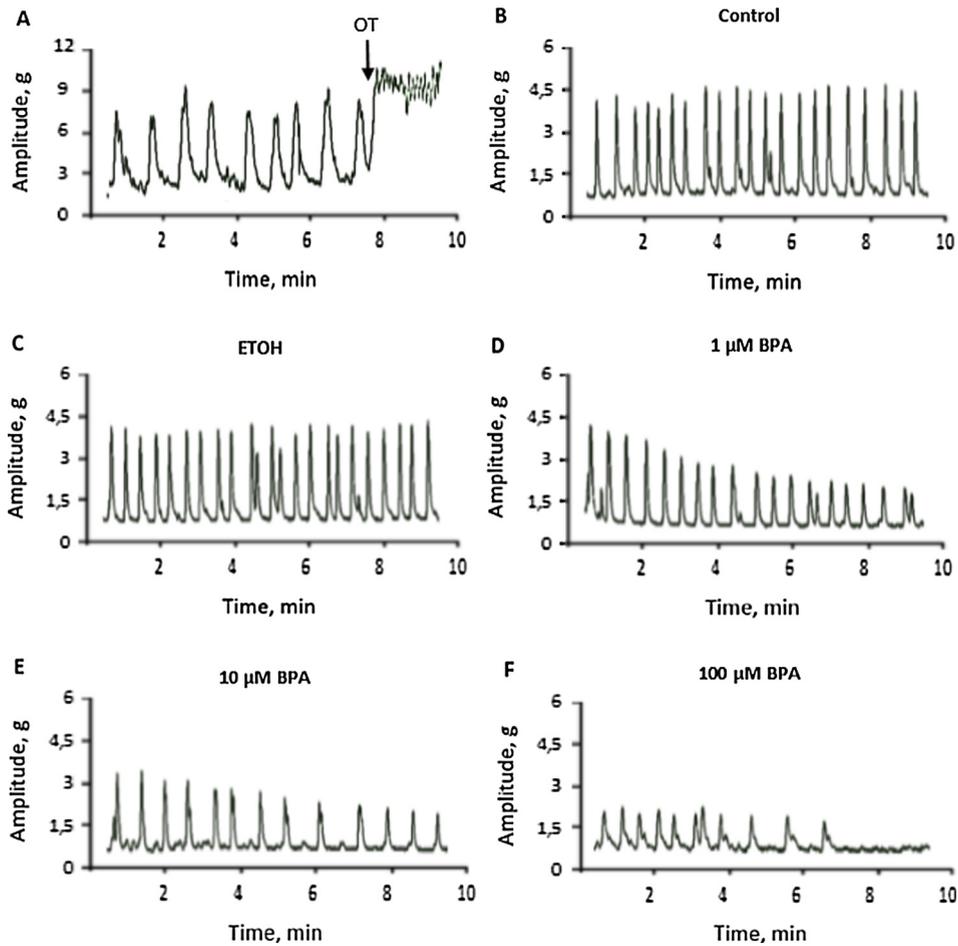


Fig. 1. (A) Representative tracing of reaction of live tissue to oxytocin (2.5 mIU/L), (B) spontaneous contractions of control group, (C) the effect of ethanol (vehicle) and different concentrations of BPA (1–100 μM) (D-F) on uterine contractions of the cat uterus; OT: Oxytocin, ETOH: Ethanol, BPA: Bisphenol A.

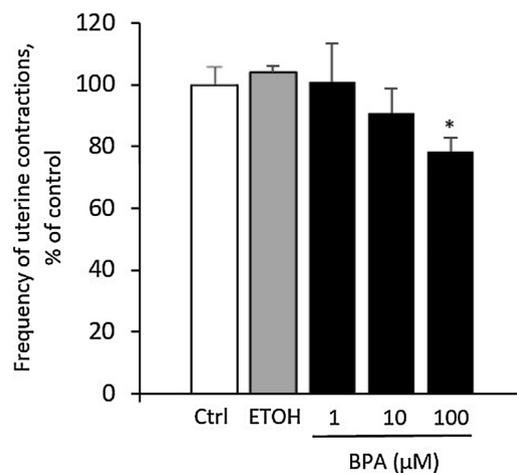


Fig. 2. Effects of vehicle (ethanol) and non-cumulative concentrations of BPA (1–100 μM) on the frequency of cat uterine contractions; Data are expressed as the mean ± SEM; All treatment groups including ethanol vehicle were compared to the control group (*: $P < 0.05$). Ctrl: Control, ETOH: Ethanol, BPA: Bisphenol A.

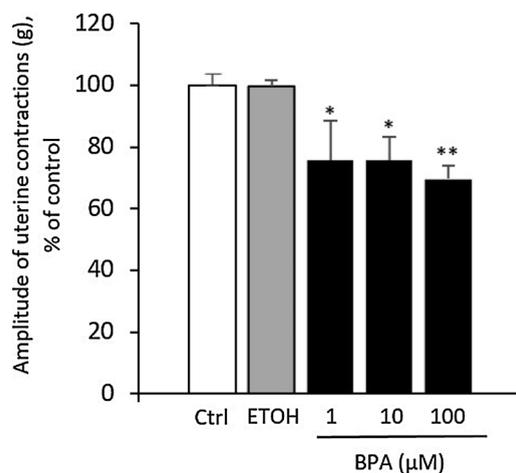


Fig. 3. Effects of vehicle (ethanol) and non-cumulative concentrations of BPA (1–100 µM) on the mean amplitude of feline uterine contractions; Data are expressed as the mean \pm SEM; All treatment groups including ethanol vehicle were compared to the control group (*: $P < 0.05$ and **: $P < 0.01$); Ctrl: Control, ETOH: Ethanol, BPA: Bisphenol A.

contractions of BPA at 1 and 10 µM doses were lesser compared to the control group. This reduction was around 25% and statistically significant for both groups ($P < 0.05$). At the 100 µM concentration of BPA, the amplitude of uterine contractions decreased by 30.4%, which was significant ($P < 0.01$).

3.4. Estradiol and progesterone concentrations

The serum estradiol and progesterone concentrations quantified in this study were > 20 pg/mL and < 1 ng/mL, respectively. These results are consistent with the previously determined serum steroid concentrations in cats during estrus (Verhage et al., 1976; Shille et al., 1979).

4. Discussion

The widespread use of BPA and its long-lasting presence in the environment increase the potential of humans and animals to be affected by this substance. As this potential causes public concerns, in many studies, researchers have focused on the deleterious effects of BPA on reproduction and development (Maffini et al., 2006). In the present study, there was examination of the effects of different concentrations of BPA (1, 10, and 100 µM) on the frequency and amplitude of uterine contractions *in vitro*. To the best of our knowledge, this is the first study assessing the effects of BPA on the cat uterus. Findings in the present study indicate BPA decreased the frequency and amplitude of spontaneous contractions of the cat uterus in a dose-dependent manner. The concentrations chosen for testing of BPA effects may be larger than what occurs with natural exposure, but it is known that BPA can bio-accumulate in the body and lead to adverse effects on health. Even if there is not knowledge about blood or tissue concentrations of BPA in cats, there is exposure of these animals to BPA by ingestion of canned foods, which is an important potential source of BPA contamination in pets. Also, the doses of BPA assessed in the present study are similar to many *in vitro* toxicological studies (Pant et al., 2011; Salleh et al., 2015; Gupta and Deshpande, 2017).

The adverse effects of BPA have been reported on reproductive tissues, including the oviduct and uterus (Newbold et al., 2009). There is also an association between the urinary and maternal plasma concentration of BPA and diethylhexyl phthalate in humans and reproductive disorders, such as sexual dysfunction, embryo implantation failure, and pre-term birth (Latini et al., 2003; Behnia et al., 2016). In this study, BPA at 100 µM and 1–100 µM concentrations attenuated the frequency and amplitude of uterine contractions of cats, respectively. Results in the present study are consistent with previous findings such as those of Gupta and Deshpande (2017) where after 28 days of ingestion of BPA by rats, the frequency and amplitude of spontaneous and acetylcholine-evoked uterine contractions *in vitro* were less than in the control group. There was also an effect of BPA at concentrations of 0.1–10 µM in decreasing the spontaneous contractility of the rat uterus in a dose-dependent manner (Gupta and Deshpande, 2018). An et al. (2013) reported that 10 µM of BPA attenuated the contractility of primary rat uterine cells at 48 h of the treatment period. Although the data regarding the effects of BPA on uterine contractions are scarce, findings in the present study are consistent with results in previous studies in which BPA suppressed the contractile activity of the atrium (Pant et al., 2011), duodenum (Sarkar et al., 2018), and small and large gut (Dixit et al., 2017) of rats.

Results of previous studies indicate the estrogenic activity of BPA can inhibit the activity of endogenous estrogen by binding to estrogen alpha receptors, thus, BPA is referred to as a xenoestrogen (Wetherill et al., 2007; Caserta et al., 2011). Polychlorinated biphenyls, another xenoestrogen compound, can stimulate the contractile activity of the myometrium of rats (Tsai et al., 1996) and cattle (Wrobel and Kotwica, 2007) functioning through an estrogen receptor. Inconsistent with these reports, p-nonylphenol (Ozatik

et al., 2015) and 4-*tert*-octylphenol (An et al., 2013) function as xenoestrogens in rats by decreasing uterine contractions. Gupta and Deshpande (2016) reported that BPA reduced the frequency and amplitude of rat uterine contractions independent of the estrogen receptor. This indicates that the estrogenic effect of BPA on the uterus remains to be defined. *in vitro* myometrial contractions are, however, associated with intracellular and extracellular calcium ion concentrations (Anwer and Sanborn, 1989; Tasaka et al., 1991). Also, BPA is reported to block voltage-activated Ca^{2+} channels (Deutschmann et al., 2012) and disrupt Ca^{2+} signaling (Alonso-Magdalena et al., 2005). Inhibition of uterine contractions by BPA may, therefore, have resulted by affecting Ca^{2+} channel functionality or signaling. Results from another study indicated the activation of nitric oxide (NO) mechanism might be responsible for the reduced contractility of uterine tissues (Gupta and Deshpande, 2018). A similar effect of BPA on the NO-cGMP mediated mechanisms regulating contractile activity of the atrium led to a decrease in the frequency and amplitude of contractions (Pant et al., 2011). Thus, the NO mechanism may be responsible for the inhibitory effect of BPA on contractions. Although the present experiment was not conducted to explain the mode of action of BPA (mechanism of action) on uterine contractility of cats, it is possible to make such inferences based on the data from previous studies. This issue, however, requires further investigation.

The results of the present study indicate that there are different patterns for the inhibitory effect of BPA on the frequency and amplitude of contractions. The BPA compound reduced the amplitude of uterine contractions in the cat at all concentrations (1–100 μM) studied, but did not affect the frequency with the smaller doses. These findings are consistent with those from an earlier study where there were similar effects of BPA on the frequency and amplitude of spontaneous rat uterus contractions. As a result of findings in the previous study, it was suggested that there was a different control mechanism responsible (Gupta and Deshpande, 2018). Similarly, the inconsistency in the effect of BPA on the frequency and amplitude of uterine contractions can be explained by the differences in the underlying mechanisms. The myometrial contractions of the uterus are stimulated by two mechanisms; electrochemical or pharmacomechanical coupling. The electrochemical mechanism is related to voltage-gated calcium channels and changing calcium permeability of the plasma membrane which leads to an action potential (Al Otaibi, 2014). A single action potential is adequate to induce the electrical activity of myometrium, but it requires multiple coordinated depolarizations for forceful and continuous contractions (Garfield and Maner, 2007). Furthermore, the number of action potentials could influence the force of contractions (Salleh et al., 2015). If BPA affects the uterine contractile activity by blocking voltage-activated calcium channels as reported previously (Deutschmann et al., 2012), this may explain why the amplitude of contractions was reduced before the frequency. The latter, pharmacomechanical mechanism is related to membrane receptor-agonist binding, which activates the secondary messengers, such as G-proteins and phospholipase C, and results in increased intracellular calcium ions (Al Otaibi, 2014). The BPA compound, an endocrine disrupter, could interfere with membrane receptors and change the influx and intracellular calcium concentration, resulting in decrease uterine contractility (Salleh et al., 2015). The effects of BPA on the contractility of uterine tissues, could be related to findings in the present study regarding the frequency and amplitude of contractions. Nevertheless, future experiments are necessary to clarify this matter.

5. Conclusions

The coordinated contractions of the uterus are important for transportation of gametes, fertilization, and implantation of an embryo and maintenance of pregnancy. Even if the use of BPA in infant and child products is avoided, it should be noted that BPA-containing materials are frequently used in pet products in many countries and that this may adversely affect the fertility of pet animals. Results of the present study indicate that BPA has a disruptive effect on the contractility of the cat uterine tissues. The findings from the current study only provide insights as to the effect of BPA on spontaneous contractions and does not clarify which mechanism is responsible for this effect or the effect of BPA on agonist-evoked contractions. This can be elucidated in further studies concerning the effects of BPA on contractility of the cat uterus.

Conflict of interest statements

The authors declare that there is no conflict of interest.

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