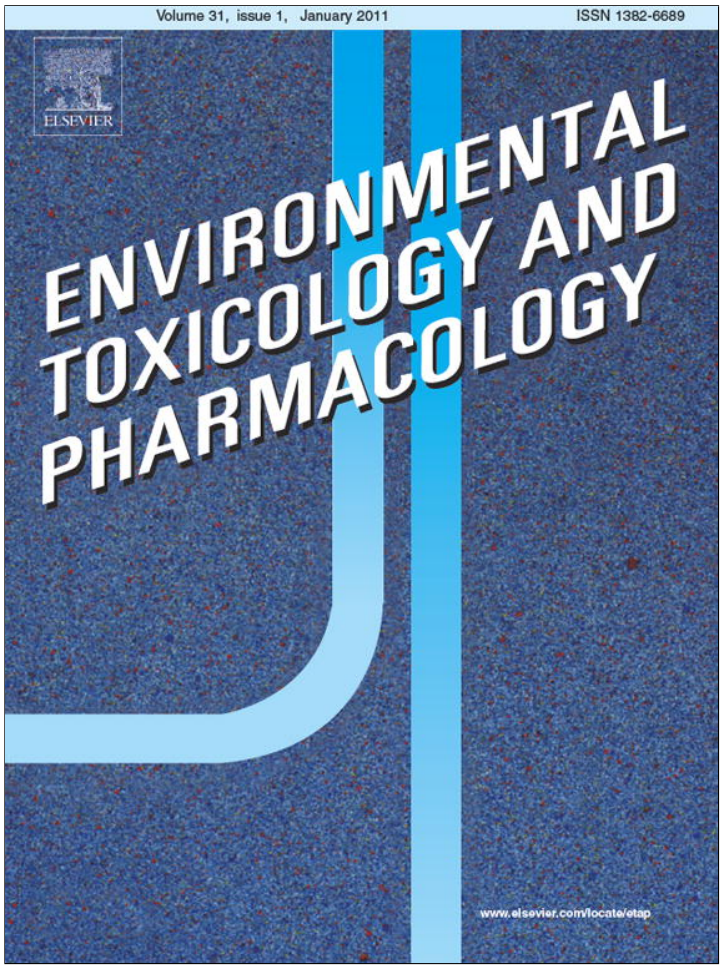

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ENVIRONMENTAL TOXICOLOGY AND PHARMACOLOGY 33 (2012) 9–15

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Differential accumulation of BPA in some tissues of offspring of Balb-C mice exposed to different BPA doses

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ARTICLE INFO

Article history:
Received 31 March 2011
Received in revised form
15 September 2011
Accepted 23 September 2011
Available online 2 October 2011

Keywords:
Endocrine disruptors
BPA
Mice
Gender difference

ABSTRACT

Pregnant adult Balb-C mice were exposed daily to two different doses of Bisphenol A (BPA) by subcutaneous injection beginning on gestational day 1 through the seventh day after delivery. The mothers were sacrificed on postpartum day 21, and the offspring were sacrificed at 3 months of age. Control mice were subjected to the same experimental protocol but received saline injections.

The liver, muscles, hindbrain and forebrain of the offspring were dissected and processed using HPLC to assess the level of BPA in the tissues and to determine its dependence on the exposure dose and gender. For comparison, the same tissues were dissected from the mothers and analysed.

We report the following results: (1) the level of BPA that accumulated in a given tissue was dependent on the exposure dose; (2) the rank order of BPA accumulation in the various tissues was dependent on the gender of the offspring; (3) the average BPA concentrations in the liver and muscle of the female offspring were higher than in the males; and (4) the average BPA concentration in the central nervous system (i.e., the hindbrain and forebrain) of the male offspring was higher than in the females.

1. Introduction

In recent years, our research group has primarily focused on the impact of endocrine disruptor chemicals (EDCs)—in particular, Bisphenol A (BPA)—on the environment (Diano et al., 2007; Georgieva et al., 2008, 2010; Mita et al., 2009) and on human health (Ricupito et al., 2009; Signorile et al., 2010). BPA is a well-known xenoestrogen that is contained as a monomer in polycarbonate plastics and epoxy resins.BPA

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doi:10.1016/j.etap.2011.09.008

can bind the human oestrogen receptor α and β subtypes as well as with the γ subtype that was discovered in fish (Okada et al., 2008; Vivacqua et al., 2003; Welshons et al., 2006). *In vitro* experiments revealed also significant endocrine activity of BPA through the androgen receptor (Xu et al., 2005).

Adult animals can metabolise and partially eliminate BPA. BPA is highly glucuronidated (Yokota et al., 1999) to BPA-GA by an isoform of the enzyme uridine 5′-diphosphoglucuronosyltransferase (UGT), which is expressed in the liver (Volkel et al., 2008). Recently, it was reported that BPA-GA in maternal blood crosses the placenta and is deconjugated to BPA in the foetus due to a deficiency in foetal UGT activity (Nishikawa et al., 2010).

Serious health consequences are associated with exposure to BPA and are related to foetal development, loss of reproductive capacity, and abnormal sexual behaviour. In small rodents, *in utero* exposure to 2.4 μg BPA/kg body weight/day induces premature puberty (Howdeshell et al., 1999) measured as vaginal opening (Schonfelder et al., 2002). Interference with the normal development of reproductive organs and mammary glands has also been reported following both pre- and postnatal BPA exposure in female and male mice that received 250 ng/kg body weight/day or 50 μg/kg body weight/day, respectively (Ramos et al., 2001; Takahashi and Oishi, 2000; Timms et al., 2005). Adverse effects of BPA on behavioural and mental development and function—as well as impaired sexual differentiation in exploratory behaviour—have been reported following *in utero* exposure to 10 μg BPA/kg body weight/day in mice (Kubo et al., 2003; Palanza et al., 2002).

The differential accumulation of endocrine disruptors has been reported in different animal's tissues (Bianco et al., 2011; Mita et al., 2011). For example, various BPA concentrations have been found in different tissues of different species of fish that were caught in two sites of the Tyrrhenian Sea (Mita et al., 2011). Similarly, differential accumulated levels have been found in different regions of the brains of rats that were exposed to the endocrine disruptor 4-tert-octylphenol (Bianco et al., 2011). Recently, endometriosis was reported in female offspring of Balb-C mice that were exposed during their foetal life and during lactation to two different BPA concentrations. In particular, the occurrence of endometriosis was 30 and 35% when pregnant Balb-C mice were treated from gestational day 1 through post-natal day 7 with 100 or 1000 μg/kg/day, respectively (Signorile et al., 2010).

In this study, we examined whether BPA accumulates at different levels in tissues other than the endometrium. In particular, BPA concentration was measured in tissues that are important for metabolic homeostasis and nervous functions, such as muscle, liver, cerebellum and forebrain. Furthermore, we performed our experiments in male and female offspring of Balb-C mice that were exposed during foetal life to two different BPA concentrations, to determine whether the accumulation is dependent on the dose of the exposure and/or on the gender of the exposed animals. The results are compared with those that were measured in the same organs that were isolated from the mothers.

2. Materials and methods

2.1. Animal treatment

Adult, female Balb-C mice were bred to male mice of the same strain in the animal facility at the Regina Elena Cancer Institute of Rome. The animals were housed in temperature- and light-controlled (14-h light and 10-h dark) conditions and received water and food *ad libitum*. The mice were fed standard Mouse Chow (Mucedola Srl, Milano, Italy) that had a negligible for effect on estrogenic activity as determined by us using the E-SCREEN assay. Tap water was supplied in glass bottles only. The cages and bedding also tested negative for estrogenic activity. The ethics committee of the Cancer Institute approved all of the experimental protocols that were performed in accordance with Italian regulations (116/92) and with the Guide for the Care and Use of Laboratory Animals.

Vaginal plug detection was considered as day 0 of pregnancy (gestational day 0). Eighteen pregnant mice (6 per treatment group) were injected subcutaneously with 2% ethanol in physiological saline solution (control) or BPA (>99% purity; Sigma–Aldrich Inc., St. Louis, MO) that was dissolved in 2% ethanol in a physiological saline solution at a dosage of 100 or 1000 μg/kg of body weight. The injections began on gestational day 1 of gestation and continued until the seventh day after delivery (post-natal day 7). Accordingly, henceforth the treated mice and their offspring will be referred to as BPA-100 or BPA-1000, respectively. The dosages used were chosen because they were previously reported to be within the range of human exposures. In addition, the route of administration is considered relevant for assessing the potential for BPA to induce developmental effects in humans, as newborn mice do not exhibit the rapid first-pass metabolism of BPA that orally dosed adults exhibit (Yokota et al., 1999). The route of exposure is important for the effect of BPA and great differences in the reproductive and endocrine alterations can be observed following oral or parenteral (such as subcutaneous) administration. It is generally accepted that all delivery methods may be useful to reveal effects of BPA (Richter et al., 2007). Many *in vivo* studies on laboratory animals, however, currently use subcutaneous injections instead of oral administration, to ensure reproducible dosing, so avoiding BPA first-pass metabolism in the liver that results from oral exposure in adult animals.

Furthermore there is today evidence that prenatal exposure also to low doses of BPA in rodents can adversely affect the foetus, despite the efficient drug-metabolising systems of the mothers. In fact Nishikawa et al. (2010) have recently demonstrated that BPA-GA is transferred into the foetus and there deconjugated because of its vulnerable drug-metabolising system. The foetus has only a few UGT activities that glucuronidate endogenous steroid hormones and xenobiotics and consequently has low ability to glucuronidate BPA.

To minimise any potential prenatal litter effects, all of the offspring in each treatment group were pooled, separated by sex, and then fostered (with five female and five male offspring per litter) by mothers from the same treatment group. We have chosen this experimental strategy in order to have groups homogenous with identical conditions.

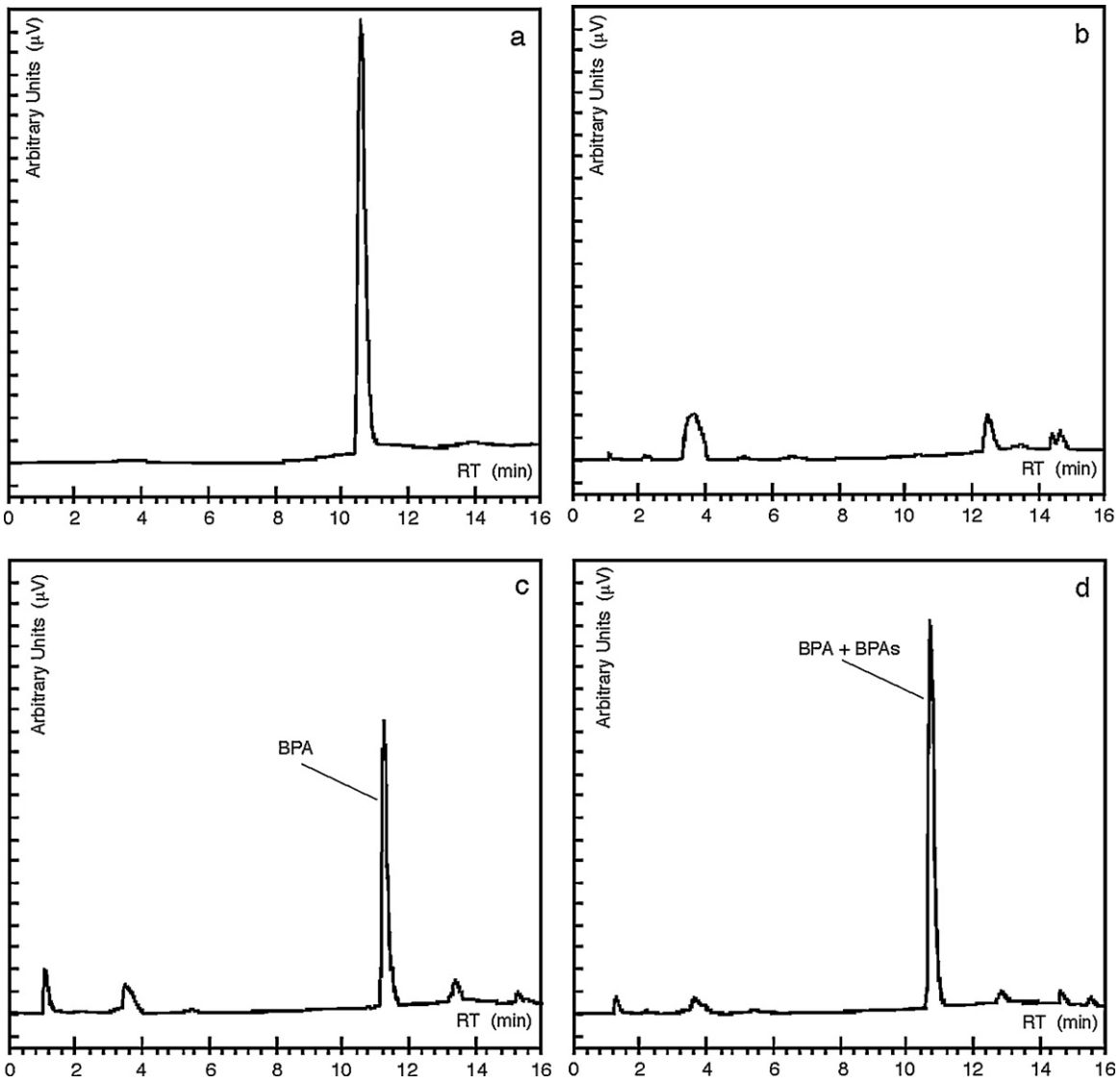

Fig. 1 – HPLC chromatograms of: (a) a BPA standard; (b) a control mice muscle; (c) a muscle of a BPA-1000 mice; (d) the same muscle in (c) with the addition of a know BPA amount.

At 21 days of age, the offspring were weaned, separated by sex, housed five per cage, and held without further treatment. At this time, the mothers were euthanised by carbon dioxide asphyxiation, and the tissues were removed for further analysis. The female and male offspring were sacrificed at 3 months of age ($n = 20$ mice per treatment group). Thus, a total of sixty animals (20 mice in each treatment groups) were available for the experiments.

2.2. *Tissue manipulation*

To assess the levels of BPA in the tissues that were isolated from the male and female offspring and their mothers, the liver, muscle, forebrain and cerebellum were dissected, immediately frozen on dry ice and stored at −80 °C in glass containers. The samples were maintained under these conditions and were thawed immediately prior to high performance liquid chromatography (HPLC) analysis.

2.3. *BPA concentration measurements*

All of the reagents were purchased from Sigma–Aldrich. Glass containers were used for storing and treating the samples. For sample analysis, the following two preparation steps were performed: homogenisation and extraction. The thawed tissues were homogenised by adding methanol to the samples and using a Diax 600 homogeniser. The choice of methanol was based on BPA's hydrophobicity. After 45 min of mechanical agitation, the samples were centrifuged to obtain two phases, with the supernatant containing the molecules of interest. The supernatant was recovered, filtered with sterile gauze and dried under a nitrogen stream. The residuals were re-suspended in acetone/hexane (at a ratio of 3:97). Solid-phase extraction was performed using a Sep-Pak Light Florisil cartridge (Waters, WAT023543). BPA was eluted with acetone/hexane (20:80). The HPLC system (Varian Prostar) was equipped with UV–vis and fluorescence detectors. BPA was detected at 220 and 280 nm. A 5-μm microsorb 300-C18 column

(250 mm × 4.6 mm) was used for the reverse-phase separations with a 20 μL sample loop. The mobile phase was prepared by mixing acetonitrile with water (30:70) in the gradient mode at a flow rate of 1 ml/min. BPA was eluted at 11 min. Using a calibration curve, the BPA concentration was calculated by taking into account the peak area. The instrumental detection limit was 10 nM. In some cases, the BPA presence in the tissues was confirmed using the GC–MS technique. The results are presented as the mean value of all samples that were analysed for each animal group and are expressed as ng of BPA per mg of tissue.

Fig. 1 shows a series of chromatograms that were obtained with the HPLC method as described above. Panel (a) shows the chromatogram of a standard sample of BPA in methanol and that the retention time occurred at 11 min. Panel (b) shows the chromatogram of a biological sample (muscle) that was taken from the control group of mice (and therefore without BPA). Panel (c) shows the chromatogram of a biological sample (muscle) that was taken from the group of offspring from the mice that were exposed to BPA at a dose of 1000 μg/kg/day. A BPA peak is visible at the retention time. To confirm the presence of BPA in the tissues, panel (d) shows the chromatogram of the same sample shown in panel (c), to which a known amount of BPA was added as an internal standard. The increase in the size of the peak at 11 min confirms the presence of BPA in the biological samples. By using the peak area of the chromatograms as in panel (c) and using a calibration curve that was obtained previously with chromatograms, such as in panel (a), it was possible to determine the concentration of BPA in the various biological samples. The absolute values of the BPA concentrations in panels (a), (c) and (d) were 0.5 μM, 0.35 μM and 0.43 μM, respectively.

Each histogram that is shown in the following figures represents the average of 20 samples of the same type of tissue and of the same gender of mice that were exposed to a given dose of BPA.

2.4. *Statistical analysis*

Statistical analyses were performed using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL). The BPA concentration in the tissues was analysed using repeated measures ANOVA. Differences with a p-value of <0.05 were considered significant. Multiple comparisons were performed with the Fisher's LSD post hoc test. Experimental values are expressed as the mean ± SEM.

3. Results and discussion

All of the control mice (including the mothers and male and female offspring in the control groups) showed no measurable trace of BPA in their tissues.

An examination of the data in Fig. 2 shows that the concentrations of BPA in the tissues that were dissected from the mothers were (i) strongly dependent on the dose of BPA and (ii) higher in the muscles and liver than in the cerebellum and forebrain. The BPA concentration in the tissues of the mothers was in the following rank order: muscle > liver > hindbrain > forebrain.

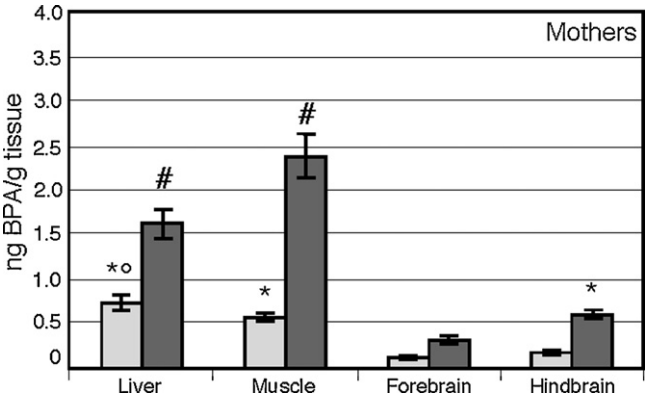

Fig. 2 – Distribution of BPA concentration in different tissues of mothers. BPA concentration is expressed as ng BPA/g tissue. Data are presented as means ± standard deviation. Legend: (▫): Exposed to 100 μg/kg body weight/day. (■): Exposed to 100–1000 μg/kg body weight/day. (*) Statistical significance relative to forebrain and hindbrain BPA-100. (°) Statistical significance relative to the forebrain BPA-1000. (#) Statistical significance relative to the other tissues BPA-100 and BPA-1000.

Fig. 3 shows the BPA concentrations in the various tissues obtained from females born from mothers exposed to the two doses of BPA as mentioned above. A strong correlation in BPA concentration between the mothers and the female offspring was observed (with the exception of the liver). These results suggest that in the liver of 3-month-old female offspring BPA was extensively metabolised, whereas the metabolism of BPA was low in the mothers that were sacrificed approximately 3 months earlier than their offspring. Remarkably, the concentration of BPA in the female offspring was (i) different in the

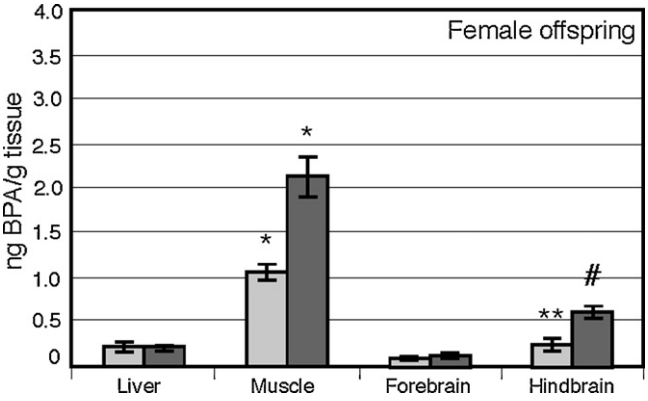

Fig. 3 – Distribution of BPA concentration in different tissues of female pups. BPA concentration is expressed as ng BPA/g tissue Data are presented as means ± standard deviation. Legend: (▫): Exposed to 100 μg/kg body weight/day. (■): Exposed to 100–1000 μg/kg body weight/day. (*) Statistical significance relative to the others tissue BPA-100 and BPA-1000. (#) Statistical significance relative to the others tissue BPA-100 and BPA-1000. (**) Statistical significance relative to the forebrain BPA-100.

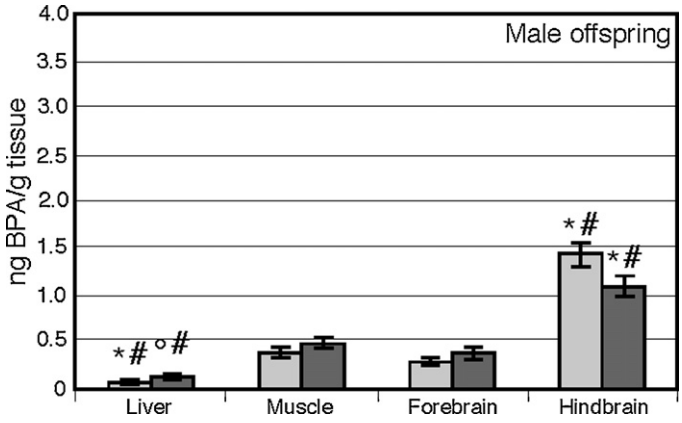

Fig. 4 – Distribution of BPA in different tissues of male pups. BPA concentration is expressed as ng BPA/g tissue. Data are presented as means ± standard deviation. Legend: (▒): Exposed to 100 μg/kg body weight/day. (■): Exposed to 100–1000 μg/kg body weight/day. (*) Statistical significance relative to the others tissue BPA-100. (#) Statistical significance relative to the others tissue BPA-1000. (°) Statistical significance relative to hindbrain, muscle BPA-100.

various types of tissues, (ii) dependent on the dose of BPA and (iii) much higher in muscle than in the other tissues.

Fig. 4 shows the concentrations of BPA that were found in the tissues of the male offspring. We found that the concentration of BPA was strikingly different in the different types of tissue compared to the female offspring and mothers. In addition, the BPA concentration was less dependent on the dose of BPA, with a reversal in the case of brain. The concentration of BPA in these animals was much higher in the hindbrain than in the other tissues. Considering the average values that are presented in Figs 3 and 4, it can be concluded that the concentration of BPA in the various tissues follows the following rank order muscle > hindbrain > liver > forebrain for females; while hindbrain > muscle > forebrain > liver in male offspring.

To confirm the significance of the above results, we performed a detailed statistical analysis of these findings.

An ANOVA with 2 between subjects factors (sex and treatment) and one within subjects factor (tissue) was performed in order to determine statistical differences in BPA concentration through the groups in the II generation. Tissue was considered as within subjects factor since the BPA concentrations from different organs could be not independent in the same rat. The results revealed significant effects for tissue [$F(3, 96) = 165.589$, $p < 0.01$], for the sex–tissue interaction [$F(3, 96) = 153.609$, $p < 0.01$], for the tissue–treatment interaction [$F(3, 96) = 17.490$, $p < 0.01$], and for the sex–tissue–treatment interaction [$F(3, 96) = 16.673$, $p < 0.01$]. The LSD post hoc test revealed the following results. (1) The female offspring of BPA-100 group showed a significantly higher BPA concentration in the muscle compared to the other tissues ($p < 0.01$) and in the hindbrain compared to the forebrain ($p < 0.05$) within the same group. A significant concentration was found in the liver compared to the muscle and hindbrain ($p < 0.01$), in the muscle compared to the other tissues ($p < 0.01$), in the forebrain compared to the muscle and hindbrain ($p < 0.01$), and in the hindbrain compared to the muscle and hindbrain ($p < 0.01$) of the female offspring of BPA-1000 group. (2) The female offspring of BPA-1000 group showed a significantly higher BPA concentration in the muscle compared to the other tissues ($p < 0.01$) and in the hindbrain compared to the liver and forebrain ($p < 0.01$) within the same group. (3) The male offspring of BPA-100 group showed a significantly higher BPA concentration in the hindbrain compared to the other tissues ($p < 0.01$) and in the liver compared to the muscle and forebrain ($p < 0.05$) within the same group; in addition, these mice showed a significantly higher concentration in the liver compared to the forebrain, muscle and hindbrain ($p < 0.01$), in the muscle compared to the hindbrain, liver ($p < 0.01$), in the forebrain compared to the hindbrain ($p < 0.01$), and in the hindbrain compared to the other tissues ($p < 0.01$) of the male offspring of BPA-1000 group. (4) The male offspring of BPA-1000 group showed a significantly higher BPA concentration in the liver and hindbrain compared to the others tissue ($p < 0.01$).

An ANOVA with 2 between subjects factors (generation and sex) and one within subjects factor (tissue) was performed in order to determine statistical differences in BPA concentration through the groups. The results revealed significant effects for tissue [$F(3, 156) = 56.981$, $p < 0.01$], for the generation-tissue interaction [$F(3, 156) = 27.171$, $p < 0.01$], for the tissue–treatment interaction [$F(3, 156) = 13.747$, $p < 0.01$], and for the generation–tissue–treatment interaction [$F(3, 156) = 6.732$, $p < 0.01$]. The LSD post hoc test showed the following results. (1) The mothers of BPA-100 group showed a significantly higher BPA concentration in the liver and muscle compared to the forebrain and hindbrain ($p < 0.01$) within the same group, in the liver compared to the liver and forebrain ($p < 0.01$) of the offspring of the BPA-100 and BPA-1000 groups and also to the muscle offspring of the BPA-1000 group; in addition, we found a significantly higher BPA concentration in the muscle compared to the liver and forebrain ($p < 0.05$) of the offspring of BPA-100 and BPA-1000 groups and the muscle and hindbrain ($p < 0.05$) of the offspring of the BPA-1000 group, as well as in the forebrain and hindbrain compared to the muscle and hindbrain ($p < 0.01$) of the offspring of the BPA-100 and BPA-1000 groups. (2) The mothers of BPA-1000 group showed a significantly higher BPA concentration in the liver compared to the muscle, forebrain and hindbrain ($p < 0.01$) within the same group, and compared to the other tissues of the other groups ($p < 0.05$ for muscle of the mothers of the BPA-1000 group), in the muscle compared to the other tissues ($p < 0.01$) within the

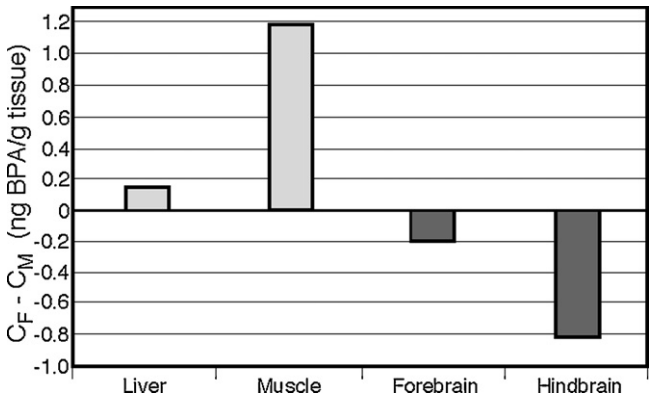

Fig. 5 – Distribution of the difference of average BPA concentrations ($C_F - C_M$) in different tissues. C_F = average BPA concentration in female offspring; C_M = average BPA concentration in male offspring.

same group and the other groups, in the forebrain compared to the muscle and hindbrain ($p < 0.01$) of the offspring of the BPA-100 and BPA-1000 groups, in the hindbrain compared to the forebrain ($p < 0.01$) and hindbrain ($p < 0.05$) of the mothers of the BPA-100 groups, to the muscle and forebrain ($p < 0.01$) of the offspring of the BPA-1000 group, and to the liver and forebrain ($p < 0.05$) of the offspring of the BPA-100 group.

To stress the sex-related differences in BPA concentration that we found between the tissue of the female and male offspring, the differences in the BPA concentrations (C) that were obtained in the same tissues are shown in Fig. 5, in which C_f is the mean concentration in the tissues of the female offspring, and C_m is the average concentration in the tissues of the male offspring. To simplify the presentation of these results, for each tissue, we analysed the arithmetic means of the concentrations that were measured at the two different doses. It is evident that compared with the males, the females have a high concentration of BPA in the liver and muscle, whereas the males have a high concentration of BPA in the central nervous system. This is a good proof of BPA accumulation gender dependent.

4. Conclusions

Endocrine disruptors have recently received considerable attention from the scientific community, as they are recognised as being potentially hazardous to human health. These man-made chemicals are found in abundant levels in the in residential buildings, cars, furniture, plastics, products such as baby feeding bottles, tin-food containers, and even in children's toys. These compounds can exert prominent effects during vulnerable developmental stages, such as *in utero* or in puberty, during which endocrine disruptors increase the risk of developing diseases later in life. It has been theorised that the recent insurgence of various pathologies may be due to increased exposure to endocrine disruptors during a critical window in prenatal development (Selevan et al., 2000). Studies have confirmed that exposure during the prenatal period can alter gender-specific characteristics and developmental programming and can delay the onset of puberty without the need for a second exposure (Fenton, 2006). The data in the present study confirm that BPA can readily cross the placental barrier and reach the foetus. This transport leads to a differential BPA distribution that is dependent on the sex of the foetus. This differential distribution was also found to be dependent on the type of tissue, on the exposure level, and particularly on the gender of the foetus. In particular, the high BPA levels in the nervous system of the male offspring indicate that *in utero* exposition to BPA can be more critical to males, for which development is primarily dependent on the conversion of testosterone to oestrogen via aromatase. On the other hand, female foetuses might be protected from endocrine disruptors by the presence of circulating α-fetoprotein, which can also bind oestrogen-like substances (Bakker et al., 2006; Bakker and Baum, 2008; Keller et al., 2010). Our results are in agreement with current experimental evidence showing larger effects of developmental exposure to BPA in female mice as compared to males (Cox et al., 2010; Gioiosa et al., 2007; Rubin et al., 2006).

Finally, the precise nature of BPA's effects depends on the extent to which it is taken up by the animal and on the extent to which it is degraded, excreted or metabolised. Accordingly, BPA that accumulates in animal tissues can be biologically active at concentrations that are well below what is considered to be toxic in the conventional sense. Our results—together with previous data (Signorile et al., 2010) showing the influence of perinatal BPA exposure on the onset of endometriosis—open new perspectives for research in the field of environmental toxicology, in particular related to endocrine disruptors, thus highlighting the notion that endocrine disruptors exposure must also be a "gender matter".

Conflict of interest statement

The authors declare no conflict of interest.

Acknowledgments

This work was supported by a grant from Fondazione Italiana Endometriosi under an agreement of scientific collaboration with the Interuniversity Consortium INBB, by the Italian Ministry of Health under the strategic project "Salute della Donna" and by CNR, which contributed to this research by supporting a pilot project under the general target project PIAS.

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