Sveriges lantbruksuniversitet
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Immunohistokemi av androgenreceptorn och follikelantal i ovarier från råttor exponerade för en lågdos bisfenol A

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SUMMARY

Bisphenol A (BPA) is one of the most produced chemicals in the industry today, and humans are exposed to it every day. BPA has a week oestrogen-like effect and binds to different receptors in the body. One of these is the androgen receptor (AR) that has an important role in the follicular development in the ovary. The aim of this study was to examine the ovaries of rats exposed to low dose BPA during embryonic and foetal stage and neonatal period and to examine the androgen receptor expression and the number of primary and secondary follicles in 52 weeks old rats.

Pregnant rats were exposed to two different doses of BPA (0.5 or 50 μg/kg/day) in the drinking water, from gestation day 3.5 to postnatal day 22. The pups were therefore exposed *in utero* and via lactation and were then kept unexposed to BPA for 52 weeks. The ovaries were then prepared for immunohistochemistry and the AR was stained. The primary and secondary follicles were counted, and the AR expressions in the secondary and antral follicles were evaluated.

No significant differences were found between the BPA dosage groups and the negative control, which may indicate that BPA does not interfere with either the follicle assembly or the AR expression in the follicles. However, it has previously been observed that the same ovaries had a higher weight after BPA treatment, and the reason for this is still not explained. Since BPA is a chemical constantly present in our everyday life, more studies have to be made in order to answer the question if low doses of BPA affect the ovary.

SAMMANFATTNING

Bisfenol A (BPA) är en av världens mest producerade kemikalier och människor exponeras för den dagligen. BPA är en kemikalie med en svag östrogenliknande effekt och binder till olika receptorer i kroppen. En av dessa är androgenreceptorn (AR) som är viktig i utvecklingen av folliklar i ovariet. Syftet med den här studien var att undersöka ovarier från 52 veckor gamla råttor, som har varit exponerade för låga doser av BPA under embryo- och fosterstadiet samt neonatalperioden, och utreda om det har blivit någon skillnad i AR-uttrycket och antalet primära och sekundära folliklar.

Dräktiga råttor exponerades för BPA i två olika doser (0,5 eller 50 μg/kg/d) via dricksvattnet, från dräktighetsdag 3,5 till postnatal dag 22. Råttungarna exponerades alltså för BPA *in utero* och via laktation. Råttorna hölls därefter oexponerade för BPA tills de var 52 veckor. Ovarierna preparerades för immunohistokemi och AR färgades in. Primära och sekundära folliklar räknades och uttrycket av AR utvärderades i sekundära och antrala folliklar.

Inga signifikanta skillnader kunde påvisas mellan grupperna som hade blivit behandlade med BPA och kontrollgruppen. Detta tyder på att BPA inte påverkar antalet folliklar och inte heller AR-uttrycket. Däremot har tidigare studie av samma ovarier visat att de exponerade ovarierna hade en högre vikt jämfört med kontrollgruppen, men orsaken till denna viktökning är fortfarande inte förklarad. Eftersom BPA är en kemikalie som finns i vår vardag och som vi ständigt exponeras för, behövs fler studier för att undersöka om låga doser av BPA påverkar ovariet.

CONTENT

INTRODUCTION

Bisphenol A (BPA) is a synthetic oestrogen-like chemical and was known to affect the female reproductive system in rats as early as in 1936 (Dodds 1936: see Rochester 2013). BPA is globally one of the most produced industry chemicals and is used mainly in production of polycarbonate- and epoxy plastics (Beronius & Hanberg, 2011; Plastics Europe, 2017). Humans are exposed to BPA for example via food cans, beverage bottles, indoor dust, CD- and DVD discs, electronic equipment, thermal paper (used in recipes and tickets). All these merchandise and many other consumer products are used on a daily basis (Rudel *et al.,* 2003; Beronius & Hanberg, 2011) and the chemical is frequently detected in human blood and urine (reviewed in Vandenberg *et al.*, 2007).

BPA is an endocrine-disrupting chemical (EDC) since it has been proved to bind different receptors like the androgen-, oestrogen- and thyroid receptor and can for example inhibit or activate gene transcription depending on which receptor it binds to (Moriyama *et al*., 2002; Teng *et al*., 2013). The androgen receptor is expressed in the ovary and is proven to be important for the follicular development (Walters & Handelsman, 2017). BPA is an antagonist to the androgen receptor (Teng *et al*., 2013), and animals exposed to BPA have previously been shown to cause an alteration in the expression of the androgen receptor in the ovary (Rivera *et al*., 2015; Santamaria *et al*., 2016).

In Sweden, BPA is prohibited in food packages to children under three years of age and in the EU in baby bottles, and it will be banned in thermal paper in 2020. In January 2017 BPA was added to the Candidate List of ECHA (European Chemicals Agency) among other "Chemicals of Concern" that are estimated to be particularly serious and hazardous with a persistent and prolonged effect on the environment or the human health (The Swedish Chemicals Agency, 2016; EFSA, 2017). Epidemiological studies in humans have reported associations between exposure to BPA and many different diseases including; cardiovascular disease, type-2 diabetes, obesity, altered immune function, disturbed neurodevelopment in children, asthma and infertility in humans (reviewed in Rochester, 2013).

In 2015, the European Food Safety Authority (EFSA) lowered the tolerable daily intake (TDI) of BPA in food from 50 to 4 μg/kg/day and estimated the highest daily exposure of BPA to humans to be 1.5 μg/kg (EFSA CEF Panel, 2015). The discussion today is if BPA is hazardous in even lower doses than earlier acknowledged since it is possible that BPA is following a non-monotonic dose-response curve. Given that, there is a risk that BPA can affect humans in lower doses than the present TDI (Welshons *et al*., 2006; Vandenberg *et al*., 2012).

Ekoutsidou (2017) found, in her master thesis, that rats exposed to 50 μg BPA/kg/day, but not 0.5 μg BPA/kg/day, during embryonic and foetal stage and neonatal period had ovaries with increased weight compared to negative control. No difference was found regarding to the number of antral follicles, atretic follicles or corpora lutea (CL) that could explain the higher

weight. To continue the study of the same ovaries, the aim was to examine the androgen receptor expression and the number of primary and secondary follicles in 52 weeks old rats.

LITERATURE REVIEW

Endocrine-disrupting chemicals

The endocrine system is regulated by extremely low doses of hormones that act as ligands and binds to different receptors in order to, for example alter gene expression (Vandenberg *et al.* 2012). EDCs are described as exogenous chemicals that interfere with any part of the endocrine system and the hormone action (Zoeller *et al.*, 2012) and thereby influence the metabolism, the production and the uptake or release of hormones. Thus, EDCs may have important biological consequences (Zoeller *et al.*, 2012; Vandenberg *et al.*, 2013). Vandenberg *et al*. (2012) discuss in their review that EDCs have different effects in different doses; that low doses are physiological and may affect some endpoints, compared to high doses that are described as toxic.

Exposure to BPA in the uterus

Embryos and foetuses are in the early development almost completely protected from maternal oestrogen by a plasma protein, produced in the foetal liver, called α-fetoprotein (Gitlin *et al.*, 1972; Montano *et al*., 1995). However, α-fetoprotein does not protect the foetus from some of the EDCs, because they bind inadequately to the protein, and may therefore harm the foetus during the sensitive phases of development. BPA is one of those EDCs, and has been reported to bind weakly to α-fetoprotein from rat amniotic fluid compared to oestradiol (Milligan *et al*., 1998; Vandenberg 2012).

It has also been shown that BPA binds to the oestrogen-related receptor γ that is highly expressed in the placenta (Okada *et al*., 2008; Poidatz *et al*., 2012), which may result in an accumulation of BPA in the placenta and therefore increase the exposure of the developing foetus (Teng *et al*., 2013).

Development and the endocrine system of the rat ovary

The rat ovaries develop from the gonadal ridges and at embryonic day 13, they contain primordial germ cells. At day 16–19 all the oocytes within the ovaries develop and enters the prophase of the first meiotic division. At birth, the oocytes are still embedded within the epithelium covering the ovary. The formation of primary follicles occurs in response to follicle-stimulating hormone (FSH) and luteinizing hormone (LH) during the second week of postnatal development (Hebel & Stromberg, 1986; Erb, 2006). Thus, the total follicular differentiation in rodents does not occur pre partum in contrast to humans and sheep (Rivera *et al*., 2015). During the rat pups first week, the ovary begins to convert small amounts of oestradiol 12β from the androgen testosterone in response to FSH. Between 3 and 4.5 weeks of age, the ovary goes through a transition, but only if the ovary receives proper hormonal stimulation and the follicles

become capable of oestrogen secretion (Lohmiller & Swing, 2006). Depending on when the mammals are exposed to different toxins, the organs will express different alterations and it is therefore important to define the critical periods of development (Vandenberg *et al.*, 2012).

The weight of the ovary after exposure to BPA

In a study by Santamaria *et al*. (2016) the rats were exposed to low dose of BPA (0.5 and 50 μg/kg/day), during early development, from gestation day 9 (GD9) to postnatal day 21 (PND21) and sacrificed at PND90. They found that the ovaries had a lower weight after BPA exposure. This is contrary to what Ekoutsidou (2017) found in her master thesis, where the rats were exposed to the same doses of BPA, but the ovaries from the rats exposed to 50 μg/kg/day had a higher weight compared to the control group. Interestingly, the difference in exposure between the two studies is that the rats that had lower ovary weights were exposed from GD9 and the rats that had higher ovary weights were exposed a few days earlier, from GD3.5. However, Santamaria *et al.* (2016) only presented data from 8 rats in each group, although it was earlier stated to be 10–12 rats in each group with no explanation why not all ovaries were weighed or presented.

Kobayashi *et al.* (2012) published an article with the conclusion that BPA did not cause any important effects when rats were exposed to 50, 500 and 5000 μg BPA/kg/day in the diet from GD6 to PND21. However, at 5 weeks the female offspring had exposed in utero and by lactation, a significantly higher relative ovary weight after exposure to high doses of BPA (500 and 5000 μg/kg/day). Unfortunately, the ovaries were not examined morphologically and the higher weight could not be explained. Also, no significant alteration was registered compared to control when the ovaries were weighed at 3 months. However, the group have not stated any extra precautions to minimize environmental BPA exposure nor were the food consumption measured which makes it impossible to know the exact BPA dose.

A large three-generation rat study by Tyl *et al.* (2002), sponsored by the plastic industry and influential in the EFSA evaluation on BPA (EFSA CEF Panel, 2015), with oral dosage regime from 1μg/kg/day to 500 mg/kg/day concluded that BPA is not toxic for reproductive organs in low doses (Tyl *et al.,* 2002). Although, when examining the statistics, the relative paired ovary weights were significantly reduced in the F2 generation exposed to BPA (from embryonic life until weaning and during gestation and lactation) in doses of 1 and 300 μg/kg/day, 5 mg/kg/day and 500 mg/kg/day. Disturbingly, these findings are not commented in the result or discussion section. Only the 500 mg/kg/day dosage regime, which gave lower ovary weight in all the generations where presented and discussed. They also claim that no significant histopathological findings could be observed in the ovaries, but it appears as if they were only registering atrophy and follicle cysts. It also seems like they counted the primordial follicles in the ovaries from the 500 mg/kg/day dosage group and the negative control group (and did not find any differences), but overlooked the other dosage regimes. vom Saal & Welshons (2006) are very critical to the Tyl *et al.* (2002) study in their review, pointing out that the rat model

that was used is less sensitive to oestrogens and that the rats were given an animal feed with high phytoestrogenic content. Also, as in many other BPA studies, Tyl *et al* (2002) are lacking a positive control. vom Saal & Welshons (2006) discuss in their review the importance to have a positive control when studying toxicology, for example in BPA experiments, especially when interpreting negative results. They claim that the purpose with a positive control is to demonstrate the positive effects that are being measured and to prove that the assay used is sensitive to the class of chemical that are examined. Also, if the test chemical only produces negative results, it can be compared and stated to be significantly different from the positive control.

Androgen receptor in the ovary

The AR is expressed in the ovarian stroma, most of the ovarian follicle stages and in the CL, which indicates that AR-mediated actions play a specific role in the development of the follicles. In the rat, the AR has also been located in the granulosa and theca cells and in the oocyte of preantral follicles (Walters & Handelsman, 2017). The AR has also shown to be important in the regulation of follicular growth and atresia by two different pathways in granulosa cells, which are central in the regulation of normal ovary function (Sen *et al.* 2014). It has also been demonstrated that upon activation of the AR, the FSH receptor expression increases. This will in turn induce an increased sensitivity to FSH leading to follicular growth (Sen *et al.* 2014). An AR knockout mouse strain with inactivated AR, gradually develops an increasing number of atretic follicles and is completely infertile by 40 weeks of age with no follicles in the ovary, demonstrating the importance of AR-signalling within the ovary (Shiina *et al.*, 2006).

BPA and the androgen receptor

BPA is an anti-androgen and binds competitively to the AR. It appears as if BPA does not promote any function of the AR, but unlike any other known antagonists it prevents the efficient nuclear translocation of AR (Teng *et al*., 2013). This may result in that AR requires a higher concentration of an agonist for activation or need a longer time for the translocation process (Teng *et al*., 2013). BPA has also been shown to act as an anti-androgen by inhibiting some regions in the receptor and down-regulate the AR signalling in other domains (Wang *et al*., 2017).

BPA effects on the androgen receptor in the ovary

Rats exposed orally to BPA

Santamaria *et al*. (2016) found in their study, in addition to lower ovary weight, that BPA treated animals had an increased amount of CL at PND90, a finding they interpreted as superovulation due to BPA exposure. Also, blood samples were drawn from the rats, which showed a higher level of progesterone among animals treated with BPA. AR and oestrogen receptor β (ERβ) expression in the ovaries were evaluated to gain information on which way BPA may alter the follicular development and ovulation. The expression of ERβ was

unchanged when BPA treated animals were compared to the control group. However, the rats treated with 50 μg/kg/day had a reduced expression of AR in primary-, preantral- and antral follicles and the rats treated with 0.5 μg/kg/day had a reduced expression of AR in the primary follicles and increased expression in the primordial follicles compared to negative control. They also found an increased expression of the FSH receptor in the ovaries from rats treated with 0.5 μg/kg/day. Santamaria *et al*. (2016) interprets their results as that BPA affects the ovaries ability to ovulate through different mechanisms when exposed to different dosages.

Santamaria *et al.* (2016) presents in their supplementary data that the rats were exposed to a higher dose BPA than what the study design aimed for, the 0.5 μg/kg/day group actually got about 0.7 μg/kg/day, and the 50 μg/kg/day group got about 64 μg/kg/day. Ekoutsidou (2017) also presented the average oral doses, showing that the 0.5 μg/kg/day group got 0.4 μg/kg/day and the 50 μg/kg/d got 40 μg/kg/day. However, the doses were calculated continuously during the total exposure time and this revealed that the rats got a lot higher dose after parturition than during gestation. The higher dose can be explained by higher water consumption when lactating than during gestation and since the BPA was supplemented through the water, the rats got a higher dose when drinking more (Ekoutsidou, 2017).

Rats exposed subcutaneously to BPA

To control the exact dose the rats were exposed to, Rodríguez *et al.* (2010) injected 20 mg BPA/kg/day or 50 μg BPA/kg/day subcutaneous on PND1, 3, 5 and 7. At PND8 the pups were sacrificed and the ovaries were studied. Rats exposed to 20 mg BPA/kg/day had a decrease in primordial follicles and an increase in primary follicles compared to negative control. These changes could not be found in the 50 μg/kg/day group. They also studied the expression of steroid receptors, and found AR mostly located in granulosa, theca and stroma cells with the highest levels in the preantral follicles. However, contrary to Santamaria *et al.* (2016), the AR expression was not significantly altered by the exposure of BPA.

The route of administration of BPA in experimental designs has been discussed thoroughly by different scientists and reviewed in many articles. Since the major route of exposure in humans is through the diet, many consider oral exposure to be the only proper design in animal experiments to be able to extrapolate the results to humans (Richter *et al.*, 2007; Hengstler *et al.*, 2011; Beronius & Hanberg, 2012; Thigpen *et al.*, 2013). If the same dose is given through subcutaneous administration, it will avoid the first pass hepatic metabolism and the circulating BPA is therefore likely to end up in higher levels and with more bioactivity than via oral route (Pottenger *et al.*, 2000).

Lambs exposed to subcutaneous BPA

In a study by Rivera *et al*. (2015), they injected 0.5 μg BPA/kg/day and 50 μg BPA/kg/day subcutaneously in young lambs from birth to PND30 and examined the ovaries. As a positive control they used diethylstilbestrol (DES), which also is a synthetic oestrogen but it has much

stronger bioactivity than endogenous oestradiol (McLachlan *et al.*, 1984: see Rivera *et al*. 2015). Some of the lambs were also given FSH in multiple doses for two days after PND 30, to demonstrate the response of the ovaries and to detect dysfunctions in the growing follicles. Rivera *et al.* (2015) showed that early postnatal BPA exposure to lambs led to decreased follicular development with lower number of follicles and lower oestradiol production, compared to negative control, which were interpreted as inability of the ovaries to respond to FSH treatment after BPA exposure. They also studied the expression of steroid receptors in the ovary. There were no changes in the ER levels, but the AR level was altered after BPA exposure in both doses. The small antral follicles had a lower AR expression after treated with FSH compared to the negative control. The authors interpreted the lower AR protein result after BPA exposure as an explanation to the depressed follicular development (Rivera *et al*., 2015).

Although the authors were able to control the exact dose of BPA and DES, they did not control the environmental oestrogen-like compounds that the animals were exposed to. For example, the mother ewes were held at pasture with low amount of clover, and the exact amount of the phytoestrogens were not evaluated. Neither were any specific assurances made for minimum exposure of BPA from the environment, for example the water system. This is unfortunate since the negative control is supposed to be totally negative and all the results are interpreted as a change from the negative control. Also, the study does not appear to have been blinded, which is unfortunate, especially since the immunostaining was evaluated in a graded scale and not by quantified measurement, resulting in possible bias.

Rivera *et al.* (2011) studied ovaries from lambs exposed to 50 μg BPA/kg/day subcutaneously from PND1 to PND14. They found fewer primordial follicles and an increase of transitional and primary follicles at PND30 after BPA treatment. Rivera *et al.* (2011) also studied the steroid receptor expression, but contrary to later findings by Rivera *et al.* (2015) and Santamaria *et al.* (2016) they did not find any alteration in the expression of AR or ER comparing BPA exposed lambs to negative control, nor were the oestrogen or testosterone serum levels affected.

Summary

The articles presented demonstrate different results, were animals of different species were exposed during different time periods and routes and examined at different ages. High doses of BPA have been reported to have toxic effects on the ovary (Tyl *et al.* 2002; Kobayashi *et al.* 2012; Delclos *et al.* 2014), but it is still unclear if and in what way low doses of BPA affect the ovary.

MATERIAL AND METHODS

The rats were housed and exposed to BPA at an Uppsala University animal facility, Sweden, with the overall aim to investigate effects of BPA on bone and adipose tissue. For details, see Lejonklou *et al*. (2017). Scientists from the Department of Clinical Sciences, Division of Reproduction in Swedish University of Agricultural Sciences, collected the ovaries from these rats.

Chemicals

Bisphenol A, $(CH_3)_2C(C_6H_4OH)_2$ (≥99% purity, CAS 80-05-7, Sigma Aldrich) was dissolved in tap water and ethanol (1% of final solution) to defined concentrations.

Animals

The study was approved by the Uppsala Ethical Committee on Animal Research (C26/13), following guidelines by the European Union Legislation (Convention ETS123 and Directive 2010/63/EU).

Forty-five 9 weeks old pregnant female Fischer (F344/DuCrl) rats were housed individually in enriched polysulfone cages (Euro Standard IV) with glass water bottles, to avoid background BPA exposure. Feed and water intake were registered and available *ad libitum.* The diet was analysed by the manufacturer and the content of phytoestrogens were below the Organisation for Economic Co-operation and Development's (OECD's) upper limit.

Exposure

The pregnant rats were divided into three dosage groups randomly, lower dose: 0.5 μg BPA/kg/day (n=12), higher dose 50 μg BPA/kg/day (n=15) and control (water with 1% of ethanol) (n=17). The dams were exposed *per os* via drinking water from GD3.5 to PND22. Thus, the pups were exposed via *in utero* and via lactation. The water consumption was measured and the actual individual exposure was calculated (supplemental table 2, Lejonklou *et al.* 2017).

At PND4 the litters were adjusted to 3 males and 3 females per dam and on PND22 the dams were sacrificed. The pups were moved to a new cage with three offspring from different mothers to avoid litter effects, but with same sex and exposure group. Altogether, there were 27 female offspring whereof 11 controls, 8 in the 0.5 μg BPA/kg/day group and 8 in the 50 μg BPA/kg/day group. The rats were sacrificed at 52 weeks of age at dioestrus and the ovaries were collected, although one of the control rats was found to be in oestrus and was therefore excluded. The ovaries were weighed and fixed in 4% buffered formalin.

Immunohistochemistry

The left ovary was then embedded in paraffin and the ovaries were sectioned in series 8 μm thick. 4-6 sections from every ovary were put on one slide each. Three different middle sections of each ovary were used to count primary and secondary follicles and to evaluate AR expression.

The immunohistochemistry procedure was run once for testing different dilutions of the primary antibody and to optimize the test. First, deparaffinization was performed with the slides washed in xylene and ethanol. Then, antigen retrieval was done with citrate buffer in 95 °C water bath for 25 minutes. The slides were put first in hydrogen peroxide (1–1.5%) and then in 5% serum (Normal Goat Serum, ab7481, Abcam, Cambridge, UK) for blocking. Primary antibody was added to the slides: Anti-Androgen Receptor Antibody IHC-plus™ LS-B8656, Rabbit Polyclonal (IgG), 0.2 mg/ml LSBio (LifeSpan BioSciences, Inc. Seattle, WA), with a dilution of 1:40–1:55 for 1 hour and 45 minutes at room temperature. The secondary antibody (Goat Anti–Rabbit IgG H&L (HRP), ab205718, Abcam, 500 μg at 2 mg/ml), with a dilution of 1:2000 was added to the slides and kept in 37 °C for 1 hour. Chromogen (DAB substrate kit, ab94665, Abcam) was added to the slides for 10–13 minutes and then the slides were counterstained for 1.5 minutes with Mayers HTX (HistoLab, Gothenburg, Sweden). The slides were dehydrated in ethanol and xylene and then mounted in organic mounting media (Pertex, HistoLab, Gothenburg, Sweden). Positive tissues (testis) and negative control, where the primary antibody was replaced by goat serum, were included in the first runs to test the accuracy of the protocol. The immunohistochemistry procedure had to be run at three different occasions, resulting in a risk that the slides were not treated exactly the same way. To even out any errors, the slides were blinded by codes and randomly run in the procedure at the different occasions.

Three representative samples from each ovary with good quality sections were chosen from the 4-6 samples on every slide, and photographed in a microscope (Nikon Eclipse 80i, with 10x magnification objective). The micrographs were put together in Adobe Photoshop CS6 to three big photos per slide and the primary and secondary follicles were counted on the computer, blinded and by the same person. The immunostaining intensity of the AR in the secondary and antral follicles was evaluated blinded in the microscope according to the following grading: low, medium, high and very high. Each grading were based on the approximate amount of stained granulosa cells in the follicles, were low=0–30%, medium=30–60%, high=60–90% and very high=100% or slightly below 100%. Both early secondary follicles and vesicular secondary follicles (also called preantral in the literature) were evaluated, three of each if present. Three antral follicles, if present, were evaluated in each slide. The terminology of ovarian morphology was used according to Dixon *et al.* (2014), were primary follicles have a single layer cuboidal to columnar granulosa cells, early secondary follicles have ≥ 2 layers of granulosa cells and vesicular secondary follicles begin to have spaces between the granulosa cells but not a large single antrum. Antral (also called tertiary in the literature) follicles have continuous antrum between the granulosa cells.

Statistical analysis

For statistical analyses of the effect of treatment on the number of primary and secondary follicles, general linear models (GLM procedure of SAS 9.3, Milltown, USA) were performed using the mean number of follicles (from three evaluated sections). The total numbers of follicles counted in the three sections were analysed using regression model with poisson distribution and adjustment for overdispersion by adding a Pearson scale parameter. Statistical analysis of the effect of treatment on ordinal variables (i.e. AR staining intensity on early secondary, vesicular secondary and antral follicles) was analysed using the genmod procedure of SAS with multinomial distribution and cumulative logit as function link.

RESULTS

In this study, no significant difference was found between the two different dosage regimes (0.5 and 50 μg BPA/kg/day) and the negative control group, regarding the staining intensity of the AR expression in the secondary and the antral follicles. The AR expression in the different follicles varied among individuals, but was not associated with BPA treatment (Table 1). Example of the AR staining intensity in the secondary follicles, where the AR is stained using immunohistochemistry, is presented in Figure 1.

Table 1. *AR staining intensity evaluated from low to very high in secondary and antral follicles in ovaries of BPA exposed rats (0.5 and 50 μg BPA/kg/day) and negative control group (see section Immunohistochemistry in Material and methods for details). A median score of the staining intensity was calculated for every rat, from 3–6 follicles in every slide. The number of rats that were scored in each of the staining groups are presented*

	Treatment groups		
	Control	0.5 μg/kg/day	50 μg/kg/day
Secondary follicles			
Low	4	3	3
Medium	2	1	2
High	4	2	2
Very high	1	1	1
Number of rats	11	8	8
Antral follicles			
Low	9	4	5
Medium	1	3	3
High	1	0	0
Very high	0	0	0
Number of rats	11	7[a]	8

[a] One of the slides lacked antral follicles

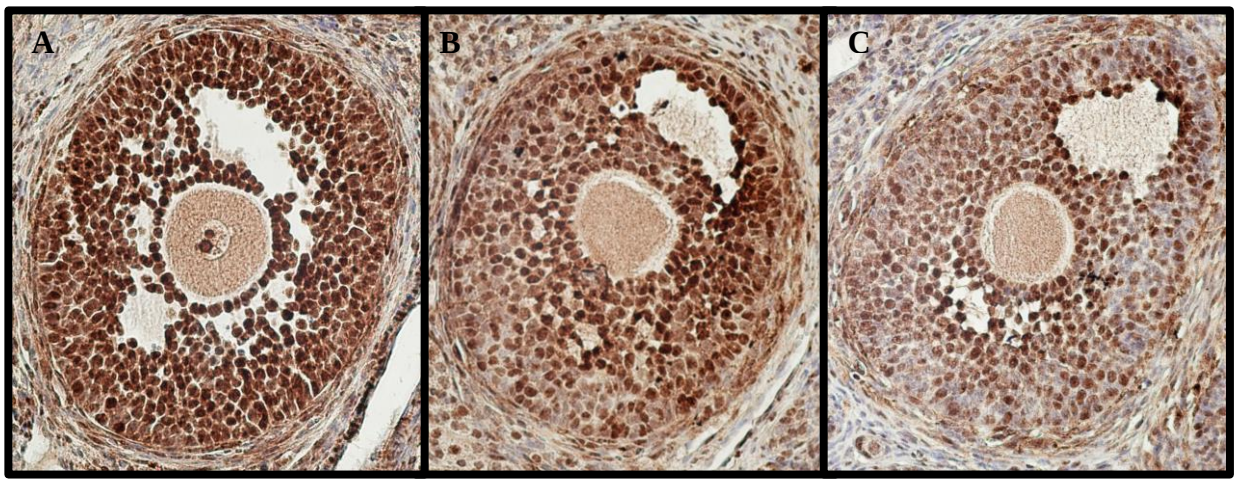

Figure 1. *Ovarian expression of androgen receptor (AR) in vesicular secondary follicles. DAP was used to develop the immunohistochemistry and Mayers HTX was used for counterstaining. The staining intensity evaluated between very high to low (see section Immunohistochemistry in Material and methods for details). A. "Very high" in ovary of rat treated with 0.5 μg BPA/kg/day. B. "High" in ovary of rat treated with 50 μg BPA/kg/day. C. "Medium" in ovary from rat in control group.*

No significant difference was found when comparing the number of primary and secondary follicles in the two dosage groups (0.5 and 50 μg BPA/kg/day) to the negative control group (Figure 2).

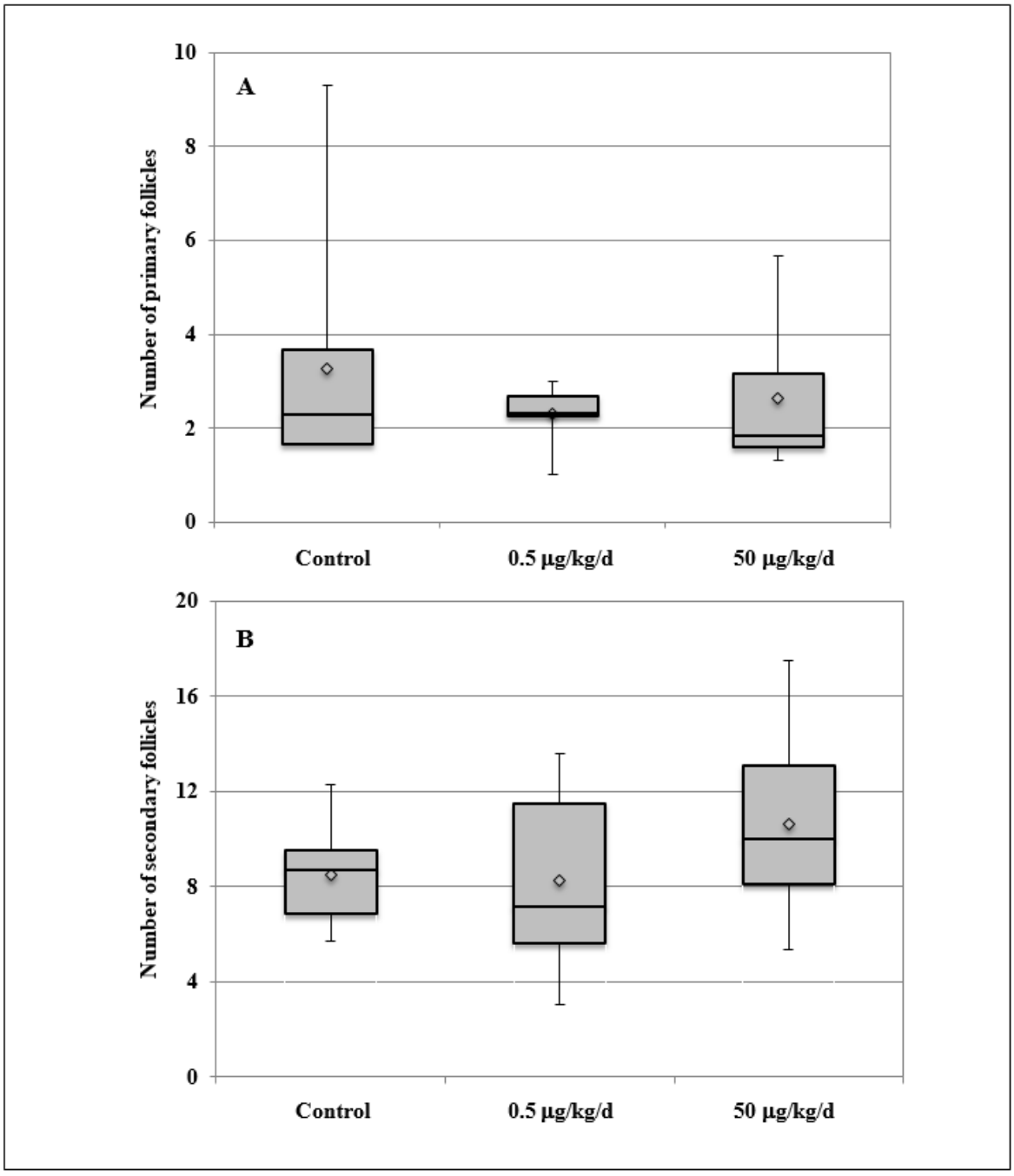

Figure 2. *Box plot demonstrating the number of follicles present in ovarian sections of the rats treated with 0.5 and 50 µg BPA/kg/day and negative control. Max, min and median values are shown and average is presented as a dot in the box. A. Number of primary follicles. B. Number of secondary follicles.*

DISCUSSION

In this study we investigated the ovaries of 52 weeks old rats that had been exposed to BPA in two low doses *in utero* and via lactation. No significant difference was found between BPA exposed rats compared to negative control, regarding the number of primary and secondary follicles and the expression of AR in the secondary and antral follicles.

Unfortunately, a positive control group was not included in this study. As previously mentioned, vom Saal & Welshons (2006) discussed the importance of having a positive control when studying toxicology and since BPA is a weak oestrogen, DES or another strong oestrogen could have been chosen. The problem with that type of positive control is that BPA is even more complex and has more effects than just oestrogenic, for example anti-androgenic. But with a positive control, it would then have been possible to compare the strong oestrogen effects in the ovaries with BPA treated rats, to see if our assay is suitable for an oestrogenic chemical. Furthermore, it would then have been possible to compare our results to the positive control and if significantly different, we would know that BPA does not affect the number of follicles or the AR expression. Today, without a positive control, we cannot be sure that BPA does not affect the ovary, just that it did not in this study. A suggestion is, in a future BPA study of the ovaries, to include a positive control group.

The expression of AR after BPA treatment has varied between studies. Santamaria *et al.* (2016) showed lower expression in primary, preantral (here called secondary) and antral follicles after treatment with 50 μg BPA/kg/day, and a higher expression in primordial follicles and lower in primary follicles after 0.5 μg BPA/kg/day. In this study, primordial and primary follicles were not evaluated in the magnification used in this study. Santamaria *et al.* (2016) quantified the expression using integrated optical density (IOD), were the images are converted into grey scale and the intensity of the grey is measured, which makes it possible to objectively differentiate small structures as well as larger. The possibility to use IOD in this study was unfortunately limited by the time available for a master thesis. Instead the intensity of staining was here evaluated using a 4-graded scale from low to very high, similarly to Rivera *et al.* (2015) who used a 5-graded scale. The problem with this type of evaluation method is the subjective judgement and possible bias. To minimize the bias, the evaluation of the staining in this study was made blinded and by one person. Rivera *et al.* (2015) only evaluated the expression in small antral follicles, presuming to be what in this thesis is called secondary follicles, but the stage is not defined in the article. They showed that AR expression was not increased in the small antral follicles in the BPA and DES treated groups, as it was in the negative control group, after injecting FSH to imitate the natural stimulation of the ovary. Rivera *et al.* (2011) got similar results as our study, with no effects on the AR expression in the ovaries from ewes treated with 50 μg BPA/kg/day subcutaneously. However, contrary to our study, they presented a change in the numbers of follicles.

Rivera *et al.* (2011) reported results of fewer primordial follicles and an increase of primary follicles after BPA treatment. Santamaria *et al*., (2016) on the other hand reported a significant decrease of the number of primary follicles in both dosage groups, together with an increased number of CL in BPA treated animals. Similarly to Rivera *et al.* (2011) Rodríguez *et al.* (2010) found a decrease in primordial follicles and in contrast an increase in primary follicles compared to negative control. However, the results are from rats treated with a much higher BPA dose than previous reports (20 mg/kg/day). In the 50 μg/kg/day group, no change in the number of follicles were reported. Likewise, no significant results could be presented in this study regarding the primary and the secondary follicles. In addition, in the initial study by Ekoutsidou (2017), no significance could be detected regarding the number of antral or atretic follicles or CL in treated rats compared to control. An important thing that separates the four studies from ours is the age of the animals when the ovaries were examined: at PND8 (Rodríguez *et al.*, 2010), PND30 (Rivera *et al.*, 2011: Rivera *et al.*, 2015), and at PND90 (Santamaria *et al.*, 2016), which all are much earlier than this study that was done at 52 weeks of age. It is possible that the age of the ovaries affect the outcome of the result, or that the time that has passed since the rats were exposed have surpassed the effect of BPA. It is also possible that BPA has affected the primordial follicles, that were not counted here, that have been present in the ovary since neonatal stage and therefore were directly affected by the BPA. To be able to count the primordial follicles a special staining or other techniques may be used.

Interestingly, when studying the AR expression, another important factor that separates our study from Santamaria *et al.* (2016) is the stage of the oestrus cycle that the rats were in at the time of the termination. In this study the rats were in dioestrus, characterised by low hormone levels, in contrary to Santamaria *et al.* (2016) where they were in oestrus and LH and FSH had recently stimulated the ovaries and ovulation had just occurred. It has previously been shown that FSH treatment increased the AR mRNA levels in the ovaries of primates (Weil *et al.* 1999) and declined in rat ovaries after both FSH and FSH+LH treatment (Tetsuka *et al.* 1995: see Tetsuka & Hillier, 1996). It is possible that the stimulation of FSH and/or LH differentiate the AR expression when the rats have been exposed to BPA and since the rats in this study lacked the stimulation, no alterations were found. When compared to the other studies presented, the ewes studied by Rivera *et al.* (2015) and Rivera *et al.* (2011) and the rats studied by Rodríguez *et al.* (2010) were all too young to have started the oestrus cycle. Rivera *et al.* (2011) and Rodríguez *et al.* (2010) did not show any alterations in the AR expression in ovaries that were not stimulated by FSH. Remarkably, this is contrary to what Rivera *et al.* (2015) found after stimulating the ovaries with FSH injections that reduced the AR expression in BPA exposed ewes. It would be interesting to study more of the AR expression in the different stages of the oestrus cycle and if the expression is altered with low dose BPA treatment in older rats.

Ekoutsidou (2017) found in her master thesis that the ovaries had a significantly higher weight compared to control after the rats were exposed to BPA. The cause of this weight difference is still not explained. As mentioned, there was no significant difference in the number of follicles neither in this study nor by Ekoutsidou (2017). More continuous studies of these ovaries have

to be made. A suggestion is to include the end parts of the ovary, since only the middle piece of the ovaries were sectioned in this study, where the follicle morphology may be different i.e. including more slides for evaluation. Another idea is to measure the mass of the CL or the stroma of the ovary to investigate if those could be the reason for weight increase.

The human fertility rates in the world are declining (Hamilton & Ventura, 2006: England & Azzopardi-Muscat, 2017) and it is important to investigate the cause. Studies made in fertility clinics have found an association with higher total urinary BPA concentration and poorer ovary response, with lower serum oestradiol and fewer oocytes retrieved, when undergoing *in vitro* fertilization (IVF). Also, reduced maturation of oocytes and fewer normally fertilized oocytes were associated with higher urinary BPA (Mok-Lin *et al.* 2009: Eichenlaub-Ritter & Pacchierotti, 2015). These reports suggest that BPA plays a role in the increasing infertility today, but more studies have to be done to find out how. The rats in this study were only exposed to BPA in the uterus and during lactation in order to investigate the developmental effects of BPA. It would also be interesting to design a study were the rats are continuously exposed to low doses of BPA for a longer time, like humans are, and then investigate the effects of BPA in the ovaries. It would also be interesting with a generation study, to directly investigate the effects of BPA on the fertility. Then the rats would also be exposed during the sensitive fertilization period and when the genome of the embryo starts to activate, which is in the two-cell stage in rats (Zernicka-Goetz, 1994).

Humans are exposed to many different chemicals today and BPA is just one of them. They may all interfere with the different systems in the body in different ways, and even if exposure to one single substance does not induce effects, a mixture of them might. There are studies of the so called "cocktail effect", where scientists have used mixed exposures, for example Rajapakse *et al.* (2002) who studied the effect of the combination of 11 different xenoestrogenes, all below its non-observed effect-concentration, found an additive enhancement of the 17β-oestradiol action. Manikkam *et al.* (2013) studied rat ovaries, after the rats had received a mixture of three endocrine disruptors used in plastics, including BPA, and found for example polycystic ovary syndrome and primordial follicle loss. It would be interesting to further study the cocktail effect, with low dose of BPA included, to see how the chemicals affect the ovaries and the AR expression when mixed. This will more reflect what humans are exposed to every day.

It is possible that laboratory animals are exposed to a cocktail of chemicals and substances that influence the result, although the researchers are trying to avoid it. The ewes in the study by Rivera *et al.* (2011) and Rivera *et al.* (2015) were held at pasture, where phytoestrogens, which sheep are known sensitive to, may have added to the result of the BPA exposure. Likewise, in the study by Tyl *et al.* (2002) the authors were accused of using a feed with high amount of phytoestrogens and an insensitive rat model. In this study, the rats were held in BPA-free cages, with a water bottle of glass and fed with low amount of phytoestrogens, to avoid background BPA, but the animals may have been exposed anyway. In a study by Churchwell *et al.* (2014) they found levels of BPA in the serum of the rats in the negative control group, which also were

held to avoid background BPA. The levels were comparable to the serum level of the lowest BPA dosage regimes in the study (2.5 μg/kg/day) and the reason of the exposure could not be explained. Speculating, Beronius & Hanberg (2011) presented in their report different sources of exposure of BPA including tap water and indoor dust. Even though these levels are much lower than the food source, it could be a route of exposure to the rats as well. The results by Churchwell *et al* (2014) demonstrate the complexity of low-dose BPA-exposure studies where it is difficult to have a negative control to compare the results with, since the negative control also could be exposed to environmental BPA.

Conclusion

In summary, the results in this study suggest that low-dose BPA exposure during embryonic and foetal stage and neonatal period does not result in an alteration of the AR expression, nor in the number of primary and secondary follicles in 52 week old rats. However, BPA affected these ovaries causing an increased weight, (as presented in Ekoutsidou 2017), which still is unexplained. Since BPA is a chemical that is constantly present in our everyday life, more studies have to be made in order to further investigate the complexity of BPA and if low doses of BPA affect the ovary.

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