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RESEARCH ARTICLE

The Effect of Bisphenol A on Notch Signaling Pathway in Development of Rat Testis

Özlem ÖZDEN AKKAYA[1], Korhan ALTUNBAŞ[2], Murat TOSUN[3], Artay YAĞCI[4*]

[1,2,3] Afyon Kocatepe University, Faculty of Veterinary Medicine, Department of Histology and Embryology, 03200, Afyonkarahisar, Turkey
[4] Muğla Sıtkı Koçman University, Milas Veterinary Faculty, Department of Histology and Embryology, Muğla, Turkey

ABSTRACT

Bisphenol A (BPA) is the object of great concerns because of its widespread use, due to several millions of tons its production throughout the world each year. BPA is an endocrine disrupting chemical and is classified as a probable human carcinogen. Fetal and perinatal exposure to environmentally relevant doses of BPA induces multigenerational impairments of male fertility. Notch signaling functions to regulate cell-fate by modulating differentiation, proliferation, and survival of cells. Notch signaling system plays a significant role in male germ cell differentiation and survival. In this study, it was aimed to explore the alterations/changes in protein expression of Notch I receptor following BPA treatments during embriyonal (E) days 18 to 21, postnatal (P) days 0 to 3, and P4 to P7 time intervals in the rat testes in vivo. The results of study revealed that the used doses of BPA had'nt effect on Notch 1 protein expressions in rat testes during selected time preiods.
Key words: Bisphenol A, Notch, Rat, Testis

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Bisphenol A'nın Rat Testis Gelişiminde Fonksiyon Gören Notch Sinyal Yolağı Üzerine Etkisi

ÖZ

Endokrin bozucu kimyasallar arasında Bisphenol A (BPA) her yıl dünyada bir kaç milyon ton üretilmesi ve yaygın olarak kullanılmasıyla en çok dikkat çeken bir ajandır. BPA endokrin bozucu bir ajandır ve insanlar için karsinojen olarak sınıflandırılmaktadır. Fötal ve perinatal dönemde çevredeki dozlarla uyumlu olarak BPA'ya maruz kalmak nesiller boyunca erkek infertilitesinin bozulmasını indüklemektedir. Notch sinyal fonksiyonları hücrelerin farklılaşmasını, çoğalmasını ve canlı kalmasını düzenleyerek hücre kaderini belirler. Notch sinyal sistemi erkek germ hücrelerinin farklılaşması ve yaşamasında önemli bir rol oynamaktadır. Bu çalışmada in vivo embriyonal (E) 18-21, postnatal (P) 0-3 ve P4-7. günlerde BPA uygulamasını takiben rat testislerinde Notch I reseptör protein ekspresyonunun değişip değişmediğini araştırmak amaçlandı. Araştırmadan elde edilen sonuçlar, seçilen zaman dilimlerinde kullanılan BPA dozlarının sıçan testislerinde Notch 1 protein ekspresyonları üzerine etkisi olmadığını ortaya koydu.
Anahtar Kelimeler: Bisphenol A, Notch, Sıçan, Testis

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ORCID ID; *ÖÖA: 0000-0001-6372-9155, KA: 0000-0001-7580-3412 MT: 0000-0001-9673-9771 AY: 0000-0002-8081-9774*
*Corresponding author e-mail: artayyagci@mu.edu.tr

INTRODUCTION

The endocrine disrupting chemicals are of great concerns for causing impairments in endocrine system functions of living organisims and due to their widespread prevalence in environment. Among all endocrine disrupting chemicals, Bisphenol A (BPA) [2,2-bis (4-hydroxyphenyl) propane] is the most noticeable agent due to its widely use in the world, with several millions of tons of production every year (Bigsby et al. 1999, Delfosse et al. 2014, Safe et al. 2001). BPA is a type of plasticizer which is mainly used in the production of polycarbonate plastics, as well as epoxy resins. Because of this feature, it is widely used in many products for both babies and adults (Calafat et al. 2009, Calafat et al. 2008, Calafat et al. 2005, Staples et al. 2000, Vandenberg et al. 2009). People and animals are constantly exposed to these substances on a daily basis through canned food, plastic food containers, baby nursing bottles and many sources. Especially babies are exposed to high levels of BPA due to the passage of BPA from baby food containers into formula and the use of BPA in medical devices (Calafat et al. 2009, Calafat et al. 2008, Carwile et al. 2009). As a result, BPA is classified as a possible carcinogen for humans (Besaratinia and Pfeifer 2007, World Health Organization, 2002). Studies conducted with BPA have shown that it has estrogenic properties and is transmitted to the baby through both placenta and milk (vom Saal et al. 2007, Vandenberg et al. 2009). BPA has also been detected in placental tissue, amniotic fluid, and serum of pregnant women and fetus (Ikezuki et al. 2002, Schönfelder et al. 2002).

While the potential effects of other endocrine disruptors on the development and function of reproductive organs in mammals have been noted (Albert and Je'gou 2014), there are very few studies on the toxicological and epidemiological evaluations of BPA (Rochester 2013). It has been shown that exposure to lower doses of BPA than the current reference dose determined for humans; disrupts the reproductive physiology in mice (Howdeshell et al 1999) and triggers behavioral disorders in rats (Farabollini et al. 1999). It has been emphasized that, exposure to BPA close to the dose present in the environment during perinatal development in mice, disrupts testicular development in puberty and early adulthood (Kabuto et al.2004; Kawai et al. 2003). It has been observed that, exposure to BPA in the uterus suppresses testosterone production in neonatal rats (Tanaka et al. 2006) and changes testosterone production in mice fetal testes in vitro (Huang et al. 2013).

Exposure to BPA during 16-20th days of pregnancy causes morphological changes in the development of Sertoli, Leydig cells and tubulus seminiferus contorts (Horstman et al. 2012). Researchers have also shown that BPA has an effect on the male reproductive system and reduces daily sperm production in rats (Takahashi and Oishi 2003). It has been reported that, in rats exposed to fetal BPA, the effects on the testes on the postnatal 3rd day are different than those who were exposed in the adulthood (Thuillier et al. 2009). In addition, it has been reported that oral exposure to BPA does not cause significant changes in the reproductive system in both generations of rats (Ema et al. 2001).

There are also few studies on the possible consequences of BPA exposure in the uterus on the development of the male urogenital canal. Exposure to endocrine disruptors during fetal and neonatal periods, which are critical periods of development, can affect the development of many systems, especially the reproductive system and the endocrine system. Therefore, recent studies have focused on these periods. The programming of hyphotalamus-hyphophysis-testis path occurs in neonatal period. Previous research reported that rats which exposed to estrogen during neonatal period caused reproductive disorders during early adulthood. Neonatal period is one of the most important phases that has a vital role in shaping of fertility of young individuals (Goyal et al. 2003). Also, just before the parturation, embriyonic days 18-22 is the phase which most important stages of male genital system had formed (Lupien et al. 2006).

In studies conducted so far, it has been determined that BPA exposure causes the most destructive effects on, spermatogenesis and spermiogenesis in men which were observed in the fetal and neonatal stages rather than the adulthood (Aikawa et al. 2004, Toyama et al. 2004, Toyama and Yuasa 2004). Therefore, we aimed to examine the effect of BPA on the "embryonal" and "neonatal" period, which is a critical period in the development of the reproductive system.

The "Notch" gene was first isolated in Drosophila in 1980s. The Notch signaling pathway is an evolutionarily conserved pathway that includes heterodimer transmembrane receptors, consisting of noncovalent intracellular domain (NICD) and extracellular action. These receptors are activated by two distinct families of ligands that bind to a distinct but equally conserved transmembrane. Thus, the Notch signal is generated through the cell to cell junctionss (Artavanis-Tsakonas et al. 1999, Chen et al. 2006).

Notch signaling pathway members play a critical role in the cellular development of mammalian systems during embryogenesis. Notch signal family members receive extracellular signals from the cell surface and regulate gene expression in the nucleus (Baron et al. 2002). The Notch signaling pathway determines the fate of the cell (Lai 2004) and plays an important role in regulating the processes of cell proliferation (Go et al. 1998), differentiation (Mitsiadis et al. 1998) and apoptosis (Shelly et al. 1999).

In mammals, four Notch receptors (Notch 1, 2, 3, 4) and five ligands (Delta-like 1, 3, 4, Jagged 1, 2) had been identified so far. These Notch receptors are being activated after binding to one of the membrane-binding ligands, which are members of Jagged / Serrare / Delta (Baron et al. 2002, Koch and Radtke 2007). When the receptors bind to the ligands, Notch receptors release the NICD and become ready for proteolytic cleavage. NICD enters the nucleus and combines with recombination signal binding protein-JK. This complex regulates the cleavage enhancer (Hes) and Hes-related transcription factors (Hey) of the Notch target genes (Bray 2006, High et al. 2007, Shih and Wang 2007).

Notch signal has been studied in many tissues during development. Notch expression in the male genital system has also been studied by researchers. It was determined that only Notch 2 was expressed in Sertoli cells in neonatal mice (Dirami et al. 2001). It has been shown by different researchers that Notch1, 2, 3 and 4 are expressed in spermatogonia (Hayashi et al. 2001, Mori et al. 2003). In addition, it has been emphasized that the expression of Notch 1 and its ligand Jagged 2 is necessary in spermatogenesis in human and rat testes (Hayashi et al. 2001). Despite these studies, there are very few studies on the development of Notch 1 and BPA in the male genital system.

The Notch signaling system is essential for the proliferation and differentiation of germ cells in the development of the male genital system (Crittenden et al. 1994, Hayashi et al. 2001).

Disruption of Notch signaling pathway by BPA may affect early spermatogenesis in rats. Therefore, in our study, effects of BPA on spermatogenesis in embryonal and neoanatal periods and its relationship with Notch signaling pathway, which plays an important role in spermatogenesis, were investigated.

METHODS

Animal Procedures

All procedures were approved by the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-219-13; 25.04.2013). Fifty, two-month-old female Wistar rats obtained from Afyon Kocatepe University Experimental Animals Unit were used in the study. The rats were cared in a 14-h light/10-h dark cycle (lighting period from 7:00 a.m. to 9:00 p.m.) and were provided ad libitum water in BPA-free glass bottles. The animals were fed with a special soybean-free 5V01 animal feed (PMI Nutrition International, USA) ad-libitum, in order to eliminate possible effects of phyotestrogens.

5 groups were determined for the study. In each group 5 rats were used in experiments and 5 rats were spared as internal control for the same group. 50 mg / kg / day BPA was applied to the experimental groups and vehicle [Sesame oil + ethanol (SE), 9: 1] was applied to the control group. In Group 1, daily intraperitoneal (i.p.) BPA or vehicle were administered to pregnant mothers from the 18th to the 21st day of pregnancy. Neonatal rats in group 2 were subjected to i.p. injections between days P0 and 3. The first injection was made approximately 4 hours after the birth. In Group 3, injections were administered between days P4 and7. Subcutaneous (sc) injections were made in the offsprings at same doses. In the first three groups, after giving the injections in respective time intervals, the rats were euthanized on the post natal 7th day at 17.00. In group 4, pregnant rats were injected with BPA at days E18 to 21 and for the 5th group BPA injections were made on days P3 to 3 in neonatal rats. The pregnant animals in 4th group and the pups in the 5th group were euthanized at 17.00 after their last injection. The doses were named as in Table 1 according to their duration.

Table 1. Experimental design

Groups (a= experiment, b= control)	Drug administered	**Day of euthanasia**
1a (E 18-E 21)	50mg /kg i.p.	P 7
1b (E 18-E 21)	Ethanol/sesame oil i.p.	P 7
2a (P 0-P 3)	50mg /kg s.c.	P 7
2b (P 0-P 3)	Ethanol/sesame oil s.c.	P 7
3a (P 4-P 7)	50mg /kg s.c.	P 7
3b (P 4-P 7)	Ethanol/sesame oil s.c.	P 7
4a (E 18-E 21)	50mg /kg i.p.	E 21
4b (E 18-E 21)	Ethanol/sesame oil i.p	E 21
5a (P 0-P 3)	50mg /kg s.c.	P 3
5b (P 0-P 3)	Ethanol/sesame oil s.c.	P 3

P: Postnatal; E: Embryonic

Pregnant animals were euthanized by cervical dislocation under general anesthesia (21.1 mg / kg of ketamine and 4.2 mg / kg of xylazine) (Ozden-Akkaya et al. 2017) and the offspring were decapitated. Soon after, when the pregnant rats were euthanized, the fetuses were removed from the embryonic sac.

Testes of fetuses and neonatals were fixed in Bouin's solution for 48 h at room temperature and then 5 μm parafin sections were prepared by using routine histological methods.

Immunohistochemistry for Notch 1 was performed using Universal LSAB Kits (Histostain Plus Broad Spectrum #859043; Invitrogen, Frederick, MD) according to the manufacturer's protocol. Briefly, paraffin sections (5 μm) were treated with primary antibodies against Notch 1 (Cell Signalling, D1E11, mümkünse dilüsyonu) for 30 min at 37°C followed by incubation with biotinylated secondary antibody (Histostain Plus Broad Spectrum, Invitrogen, 859043) for 30 min at room temperature (RT). Samples were incubated in streptavidin-HRP (Histostain Plus Broad Spectrum, Invitrogen 859043) for 30 min at RT. Following the sections were incubated in 3,30-diaminobenzidine (DAB) (Vector 4100) for 5 min to reveal positive signals. Counterstaining was carried out with haematoxylin (Merck, 70225752). Sections were placed first in 95% and then in absolute alcohol. In the sequel of dehydration with graded alcohols, sections were cleared in xylene and mounted with Entellan®.

Immunohistochemical evaluations were made by examining whether the target tissue was stained or not. The stained tissue structures and the intensity of the staining were also taken into consideration.

Values from 0 to 3 were given by two independent observers according to the characteristics as non-stained (-), weakly stained (+), moderately stained (++), and intensely stained (+++).

RESULTS

In order to see the fetal and neonatal effects, the effect of BPA administration in 5 different periods on Notch 1 protein in the testis was examined.

Notch 1 protein expression was detected in cytoplasms of cells. Notch 1 immunoreaction was also detected in peritubular myoid cells, vascular endothelial cells and vascular smooth muscle cells in testicular tissues of different periods. When all periods were examined, no reaction was observed in Sertoli cells, spermatogonia and Leydig cells of any experiment groups. The semi-quantitative evaluation results of the severity of Notch 1 immunostaining in the testes of the rats in the control and experimental groups in different periods are presented in Table 2.

E18-21 (7) period: BPA administrations were applied to pregnant mothers at days E18 to21. Testicular tissues of male puppies were examined by immunohistochemistry at postnatal day 7. A moderate Notch 1 immunization was observed in myoid cells and vascular smooth muscle cells. Vascular endothelial cells, on the other hand showed strong immunization in both control and experiment groups. (Figures 1A, 1B).

P0-3 (7) period: After BPA administration, the testicular tissues of the male pups were taken on the postnatal 7th day and the tissue sections were evaluated immunohistochemically. In both control and the experimental groups, a weak Notch 1 reaction was detected in myoid cells and vascular smooth muscle cells while the vascular endothelial cells showed strong positivity (Figures 2A, 2B).

P4-7 (7) period: The testicular tissues were collectedon the postnatal 7th day following the end of the experiment. A weak Notch 1 immunization was found in myoid and vascular smooth muscle cells in control and experimental group samples. In both groups a strong reaction was visible in vascular endothelial cells (Figures 3A, 3B).

E18-21 period: BPA was applied to pregnant rats during embryonal days 18-21. Testicular tissues of male offspring were collected on the embryonal 21st day. In both groups, a weak Notch 1 immunization was observed in myoid cells and vascular smooth muscle cells while a moderate reaction was visualised in vascular endothelial cells (Figures 4A, 4B).

P0-3 period: 0Immunohistochemical evaluation was made by taking testicular tissues on the postnatal 3rd day. A weak Notch 1 immunization was observed in myoid cells and vascular smooth muscle cells. The reaction was strong in vascular endothelial cells (Figures 5A, 5B).

Table 2. When all periods were examined within themselves, there was no difference visible between the groups in terms of Notch 1 staining intensity

	E18-21(7) Group I		P0-3(7) Group II		P4-7(7) Group III		E18-21 Group IV		P0-3 Group V	
	Control	BPA	Control	BPA	Control	BPA	Control	BPA	Control	BPA
Myoid	++	++	+	+	+	+	+	+	+	+
Endothelial	+++	+++	+++	+++	+++	+++	++	++	+++	+++
Myoid cell of the vessels	++	++	+	+	+	+	+	+	+	+

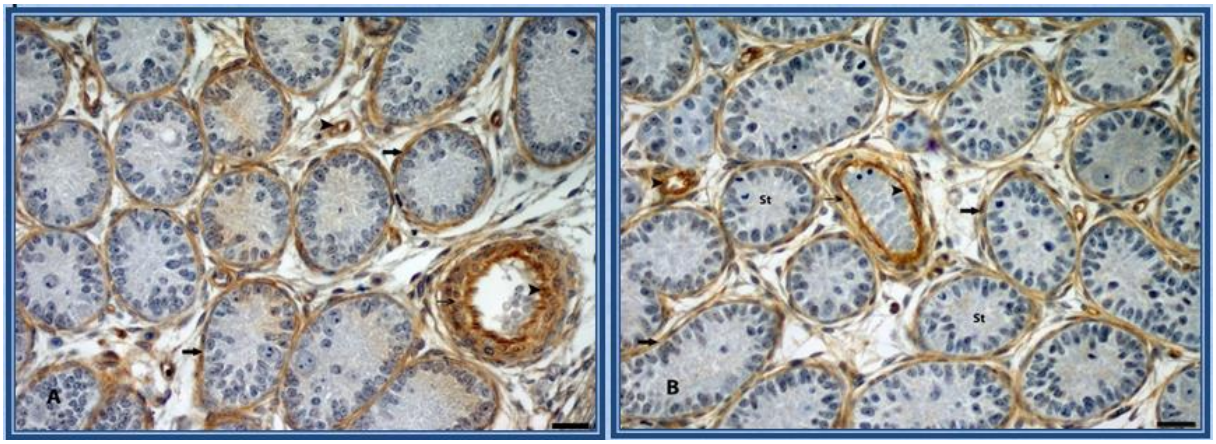

Figure 1: Notch 1 immunreaction of the group 1. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 μm

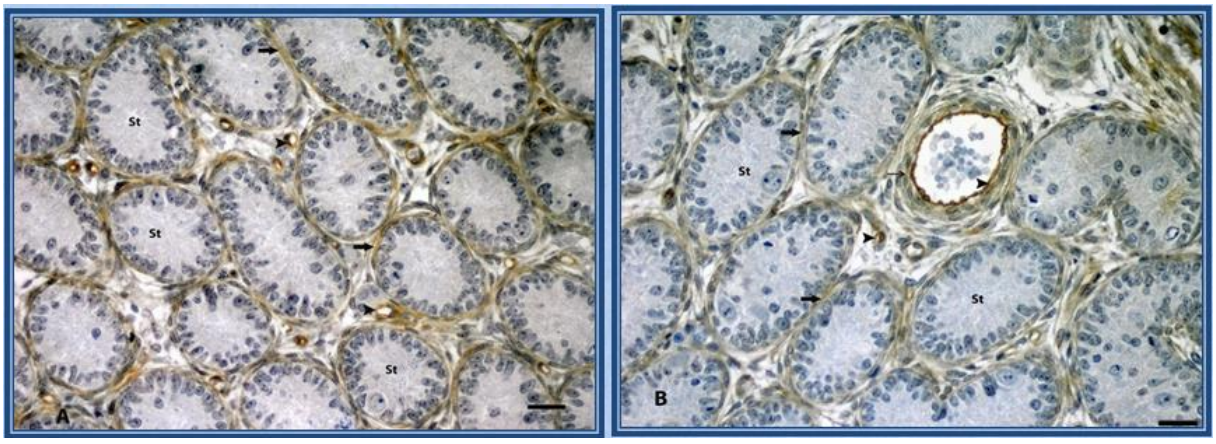

Figure 2: Notch 1 immunreaction of the group 2. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 μm

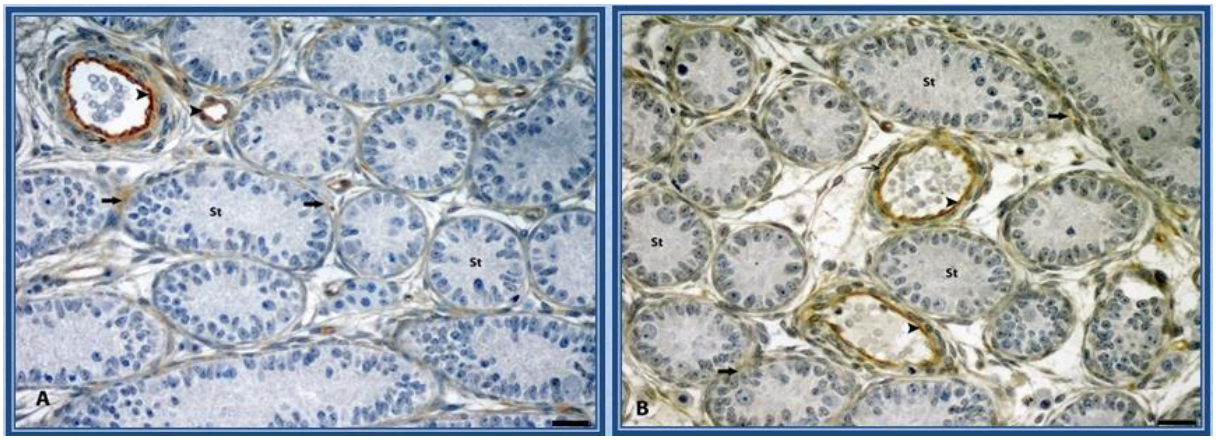

Figure 3: Notch 1 immunreaction of the group 3. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 µm

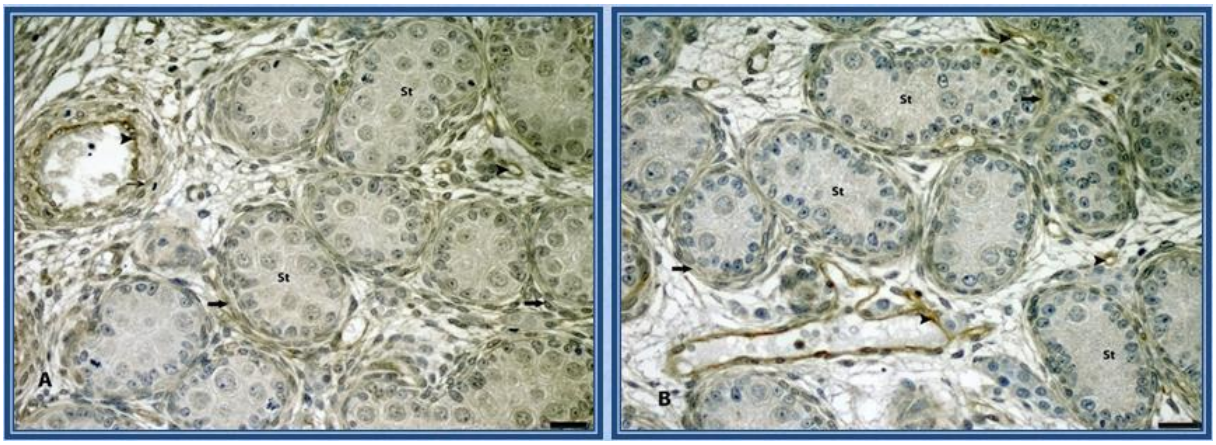

Figure 4: Notch 1 immunreaction of the group 4. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 µm

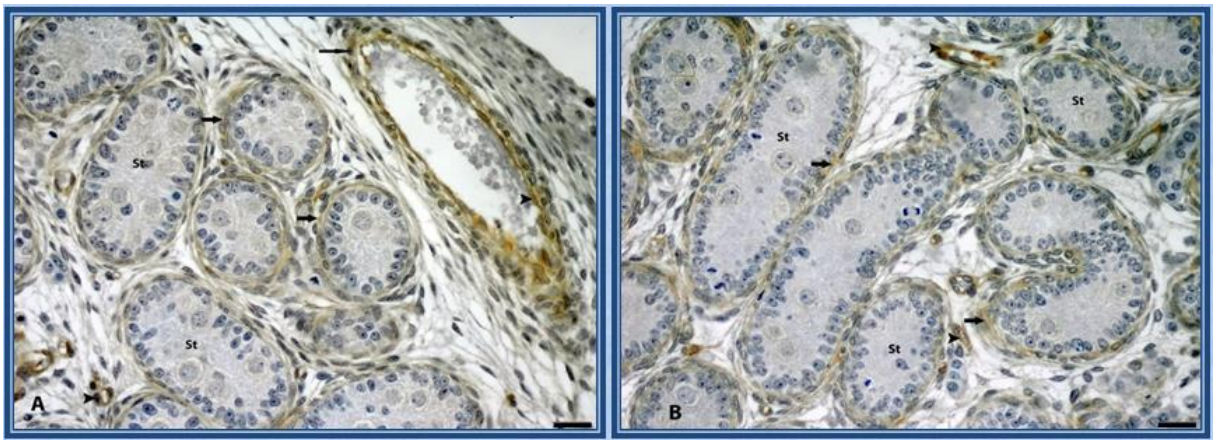

Figure 5: Notch 1 immunreaction of the group 5. A) Control group; B) BPA group; Myoid cell: (arrow); endothelial cells (arrow head); myoid cell of the vessels (thin arrow); seminiferous tubule: st, Bar= 50 µm

DISCUSSION

BPA is a conspicuous agent, which is being produced in great amounts annually in the world and is widely used in packaging, medical devices and various plastic materials (Bigsby et al. 1999). BPA has been categorized as an endocrine disruptor and in recent studies it has been elaborated that BPA affects the development of organs which are sensitive to different hormones including gonads (Munoz-de-Toro et al. 2005). BPA, which has estrogenic effects, has been reported to have destructive effects on spermatogenesis and spermiogenesis, especially when exposed in fetal and neonatal stages (Aikawa et al. 2004). Although, studies have been conducted for various effects of BPA on females and males gonads, however, the effects of embryonal and neonatal exposure to BPA are not clear entirely yet.

The Notch signaling pathway is of vital importance in the regulation of processes of cell proliferation (Go et al. 1998), differentiation (Mitsiadis et al. 1998), apoptosis (Shelly et al. 1999) and development of the male genital system (Hayashi et al. 2001). Expressions of different Notch receptors and their ligands in testes had been identified by researchers. However, the effects of embryonal and neonatal exposure of BPA on Notch 1 receptor had not been analyzed. In the recent study, the effects of embryonal and neonatal BPA exposure aimed to be investigated in 5 different stages by demonstrating Notch 1 receptor immunreactivity by immunohistochemical staining.

The Notch 1 immunoreaction was located above the endothelium and in the vessel cells. No reaction was observed in Sertoli cells, spermatogoniums or Leydig cells. No difference was found between the experimental and control groups in terms of testicular morphology. Spermatogonia and Sertoli cells were observed in closed seminiferous tubules and tubules in testes.

It has been reported that the Notch signaling system is induced by the mitotic division of germ cells in Caenorhabditis elegans. In the absence of this induction, the cell leaves the mitotic cell cycle; enters meiosis and completes gametogenesis (Austin and Kimble 1987). On the contrary, excessive induction of Notch causes ectopic germ cell mitosis (Henderson et al. 1997). The first information about Notch signal expression in neonatal and adult rodents and human testes was reported in 2001 (Dirami et al. 2001).

The researchers examined rat testicular tissues for Notch receptors in 3 days intervals until day 28. The first Notch 1 immunoreaction was reported in spermatogenic cells on the 19th day. Moreover, it has been reported that meiotic spermatogoniums and Leydig cells express Notch1 in postnatal 15th day while at the end of the 1st month Notch 1 expression was reported in spermatogoniums and Sertoli cells (Murta et al. 2013). Researchers initially reported only Notch 2 expression in ertoli cells of neonatal mice (Dirami et al. 2001). But, later on Notch 2 expression was also reported in Sertoli cells (Hahn et al. 2009).

Notch 1 expression also had been demonstrated in spermatogonia during adulthood and puberty (Dirami et al. 2001, Hayashi et al. 2001, Murta et al. 2013; Sahin et al. 2005). The activity of Notch 1 reported to be higher in spermatocytes and spermatids (Mori et al. 2003). Notch 1 and its ligand Jagged 2 had been localized in the acrosomal region during the maturation of spermatids especially in the pachetene phase both in rat and human (Hayashi et al. 2001). Positive Notch 1 immunoreactivity was also demonstrated in Leydig cells, elongating spermatocytes, spermatogonia, Sertoli cells and primary spermatocytes in a study conducted in adult rats (Sahin et al. 2005). It was suggested that Notch 1 / Jagged 2 signalling contributes in acrosome formation and Notch 1 expression could have higher correlation in spermiogenesis than germ cell proliferation (Sahin et al. 2005). Since we investigated Notch 1 expression in testis until postnatal 7th day; we could'nt see any expression in Leydig and Sertoli cells in accordance with the previous reports. The expression was only visible in myoid cells, vascular endothelial cells and vascular smooth muscle cells. Myoid cells participate in the formation of the basement membrane and together with Sertoli cells, they maintain the morphology of the seminiferous tubule. While the contractions of the myoid cells are effective in sperm transport, the fluid in the seminiferous tubules flows towards the rete testis (Romano et al. 2005). In addition, myoid cells provide the nourishment of germ cells for the development by secretion and synthesis of the extracellular matrix (Liu et al. 2013).

In the experimental study we conducted, in E18-21 when the effects of BPA were examined, a moderate Notch 1 immunereaction was observed in myoid cells and vascular smooth muscle cells in both groups. The reactions in both groups were strong in vascular endothelial cells. In the E18-21 period, a weak Notch 1 immunization was detected in myoid cells and vascular smooth muscle cells while a moderate expression was detected in vascular endothelial cells in both groups. In addition, a weak Notch 1 reaction was observed in myoid cells and vascular smooth muscle cells meanwhile the expression was found to be strong in vascular endothelial cells in both groups during the P0-3 period and P4-7 period (Calafat et al. 2008). There was no difference in Notch 1 expression between the groups.

Although we studied BPA's effect on Notch 1 expressions in rat testes, investigating other Notch family members for BPA's effect could be beneficial. Also determining different time intervals in order to investigate BPA's effect during whole testis development may provide more comprehensive results.

CONCLUSION

Since Notch 1 reaction was not observed in spermatogenic cells during these periods, it was thought that the possible effects of BPA could be on myoid cells and vascular endothelium where Notch 1 reactions were observed. However, there was no difference between the experimental groups and the control groups in terms of reaction intensity.

It was concluded that the determined dose of BPA had no effect on Notch 1 in the testes during the investigated periods. It is possible that BPA can be effective on spermatogenic cells over other Notch receptors and ligands during these periods.

Conflict of Interest: The authors declared that there is no conflict of interest.

Ethical Statement: All procedures were approved by the Ethical Committee of Afyon Kocatepe University, Turkey (AKÜHADYEK-219-13; 25.04.2013).

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