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Bisphenol A affects the maturation and fertilization competence of spermatozoa

Yoo-Jin Park[a], Md Saidur Rahman[a], Won-Ki Pang[a], Do-Yeal Ryu[a], Bongki Kim[b], Myung-Geol Pang[a,*]

[a] Department of Animal Science & Technology and BET Research Institute, Chgroung-Ang University, Anseong, Gyeonggi-do, 17546, Republic of Korea
[b] Department of Animal Resources Science, Kongju National University, Yesan, Chungnam-do, 32439, Republic of Korea

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ABSTRACT

Although there are numerous studies on bisphenol A (BPA) on the testis and spermatozoa, the effect of BPA on the physiological link between the testis and maturation of spermatozoa has not been studied. To provide an optimal environment (acidic pH) for sperm maturation in the epididymis, clear cells secrete protons and principal cells reabsorb bicarbonate and the secreted proton. Because of its crucial role in sperm maturation and fertility, functional changes in the epididymis following BPA exposure must be considered to fully understand the mechanisms of BPA on male fertility. Here, we identified the adverse effects of BPA exposure during puberty in male mice. CD-1 male mice were gavaged daily with vehicle (corn oil) and 50 mg BPA/kg-BW for 6 weeks. We determined the changes in epididymis, functional sperm parameters including motility, capacitation status, tyrosine phosphorylation, and fertility-related protein expression and in vitro and in vivo fertility rate following BPA exposure. Expression of vacuolar-type H + -ATPase is necessary for the secretion of protons by clear cells of the caput epididymis and was directly down-regulated following BPA exposure, while there were no changes in the other epithelial cell types in the epididymis. Also, pERK 1/2 signaling pathway was increased significantly in the caput epididymis following BPA exposure. Consequently, the luminal pH slightly increased, resulting in premature capacitation of spermatozoa. Moreover, there was a significant loss of the acrosomal membrane following an increase of protein tyrosine phosphorylation, while PKA activity decreased during sperm capacitation. Fertility-related proteins also showed aberrant expression upon BPA exposure. These modifications resulted in decreased male fertility in vitro and in vivo.

1. Introduction

Over the past decades, the concern about endocrine disruptors (EDs) has risen dramatically because of their indiscriminate use in the everyday products. Even though people are unaware, we are constantly exposed to EDs through a variety of sources such as plastic, air, food, and water. Bisphenol A (BPA) is one of the widely known ED and can be easily exposed to our life through plastic or polystyrene used for food packaging and epoxy resins used in the linings of cans for the packaged food (Acerini and Hughes, 2006). Therefore, concerns about BPA-mediated toxicity has been extensively studied to elucidate the risk it posed to human health. BPA works as a disruptor of normal endocrine systems by mimicking the actions of oestrogen, androgen, thyroid, and growth hormones (Rahman and Pang, 2019) and predisposes to metabolic diseases, developmental disorders, cancers, female and male reproductive abnormalities, and infertility (Matthews et al., 2001; Pike et al., 1993; Routledge et al., 2000; Wang et al., 2014, 2019).

Continuous exposure to BPA is detrimental to male reproductive health through decline in sperm count and motility, increased DNA damages and abnormal morphology of spermatozoa, ultimately leading to male infertility (Hatef et al., 2012; Meeker et al., 2010; Nordkap et al., 2012; Wang et al., 2014). In addition, BPA impairs reproductive hormones and germ cell development and disrupts spermatogenesis, causing a decline in the sperm count (Eladak et al., 2018; Jin et al., 2013; Wistuba et al., 2003). Recently, we demonstrated the alteration of fertility-related protein profiles in spermatozoa, following gestational exposure to BPA, and these alterations are linked to declining rates of fertilization and embryonic development (Rahman et al., 2015, 2017, 2018). While effects of BPA on the testis (Takahashi and Oishi, 2001; Tao et al., 2019; Yuan et al., 2019) and spermatozoa have attracted considerable attention, very little is known about the link between the testis and matured spermatozoa through sperm transition and

* Corresponding author.
E-mail address: mgpang@cau.ac.kr (M.-G. Pang).

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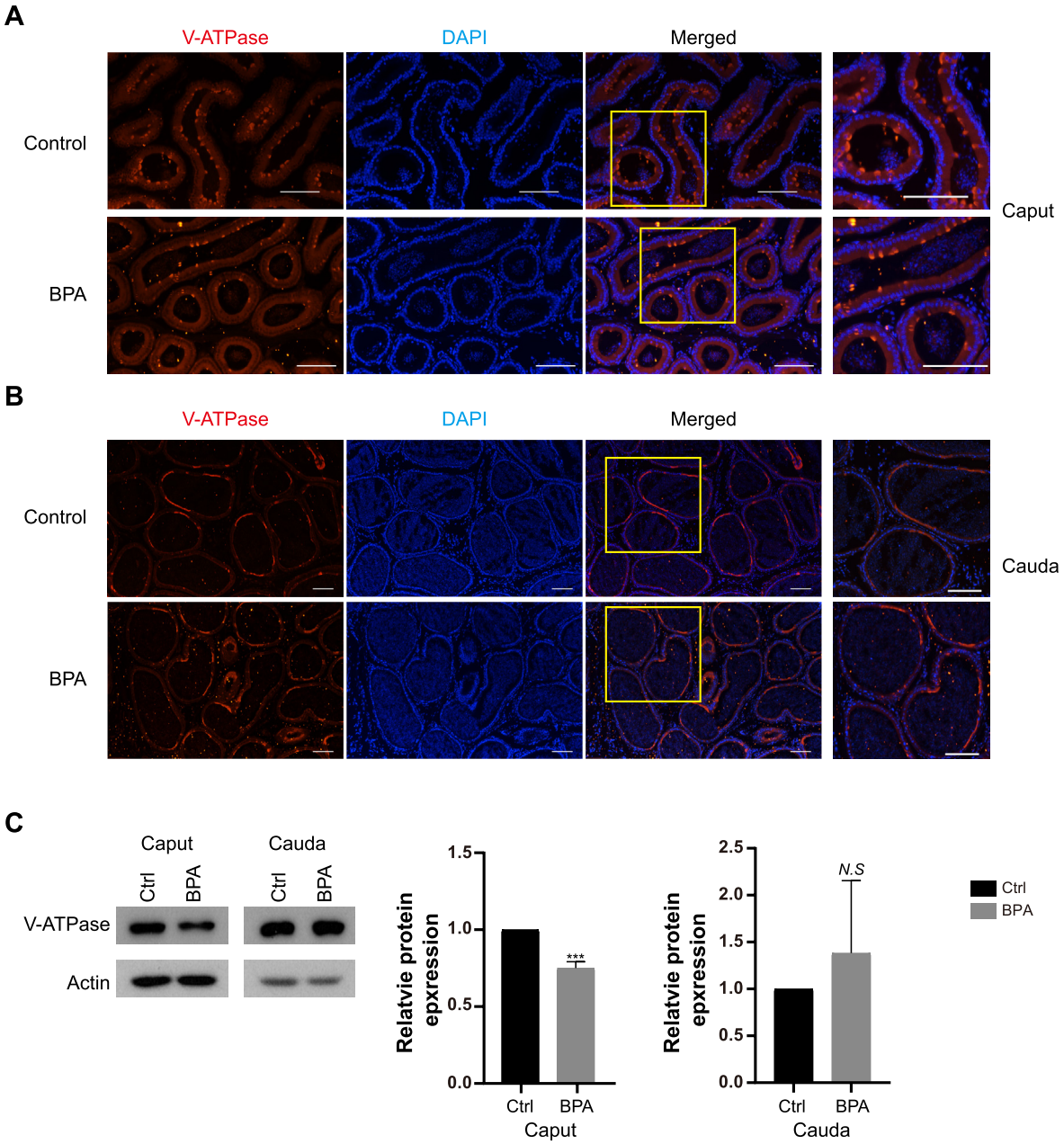


Fig. 1. Effect of pubertal BPA exposure on the distribution of V-ATPase in clear cells at adulthood. Representative images showing the distribution of V-ATPase in epididymis. Expression of V-ATPase (red, left panel) and DAPI (middle panel, blue) in the caput (A) and cauda (B) epididymis from control and BPA-exposed mice. (C) Western blot detection of V-ATPase expression in epididymis. Statistics show the relative ratio of the normalized V-ATPase expression to control. V-ATPase protein expression decreased in the caput epididymis of the BPA-exposed group compared to controls. Data represented the mean ± SEM. *** $P < 0.001$ versus control group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

maturation in epididymis.

In the epididymis, spermatozoa mature completely and acquire their fertilising ability (Breton et al., 2016; Cornwall, 2009). The epididymal lumen is maintained at acidic pH with low bicarbonate concentration, to prevent earlier activation., i.e. premature capacitation (Breton et al., 2016; Da Silva et al., 2007; Park et al., 2017; Shum et al., 2011). To establish and maintain the acidic luminal pH, narrow cells and clear cells secrete protons through the vacuolar-type H^+-ATPase (V-ATPase), while principal cells reabsorb bicarbonate in the initial segment and secrete it in the distal part via cystic fibrosis transmembrane regulator (CFTR) (Carlin et al., 2003; Leung and Wong, 2000). In addition, sodium/hydrogen exchangers (NHEs) in principal cells secrete protons to maintain acidic condition in the epididymis (Bagnis et al., 2001; Breton et al., 2016; Park et al., 2017). Meanwhile, aquaporin 9 (AQP9) in principal cells play an important role in the transport of solute and water during sperm maturation (Da Silva et al., 2006; Yeste et al., 2017). When the spermatozoa encounter the alkaline condition in the female reproductive tract, they require enormous energy to undergo capacitation, acrosome reaction, and fertilization by elevation of calcium concentration and activation of tyrosine phosphorylation (Acott and Carr, 1984; Carr and Acott, 1989; Pastor-Soler et al., 2005). Moreover, the renin-angiotensin system (RAS) plays an important role in maintaining the acidic condition in epididymis through activation of V-ATPase. Testicular angiotensin converting enzyme is released from immature spermatozoa into the lumen and it facilitates the Angiotensin II (ANG2) production from Angiotensin I (ANG1). And then, ANG2 triggers nitric oxide (NO) generation via angiotensin-II type 2 receptor (AGTR2) stimulation in basal cells. And then, NO diffused to clear cells

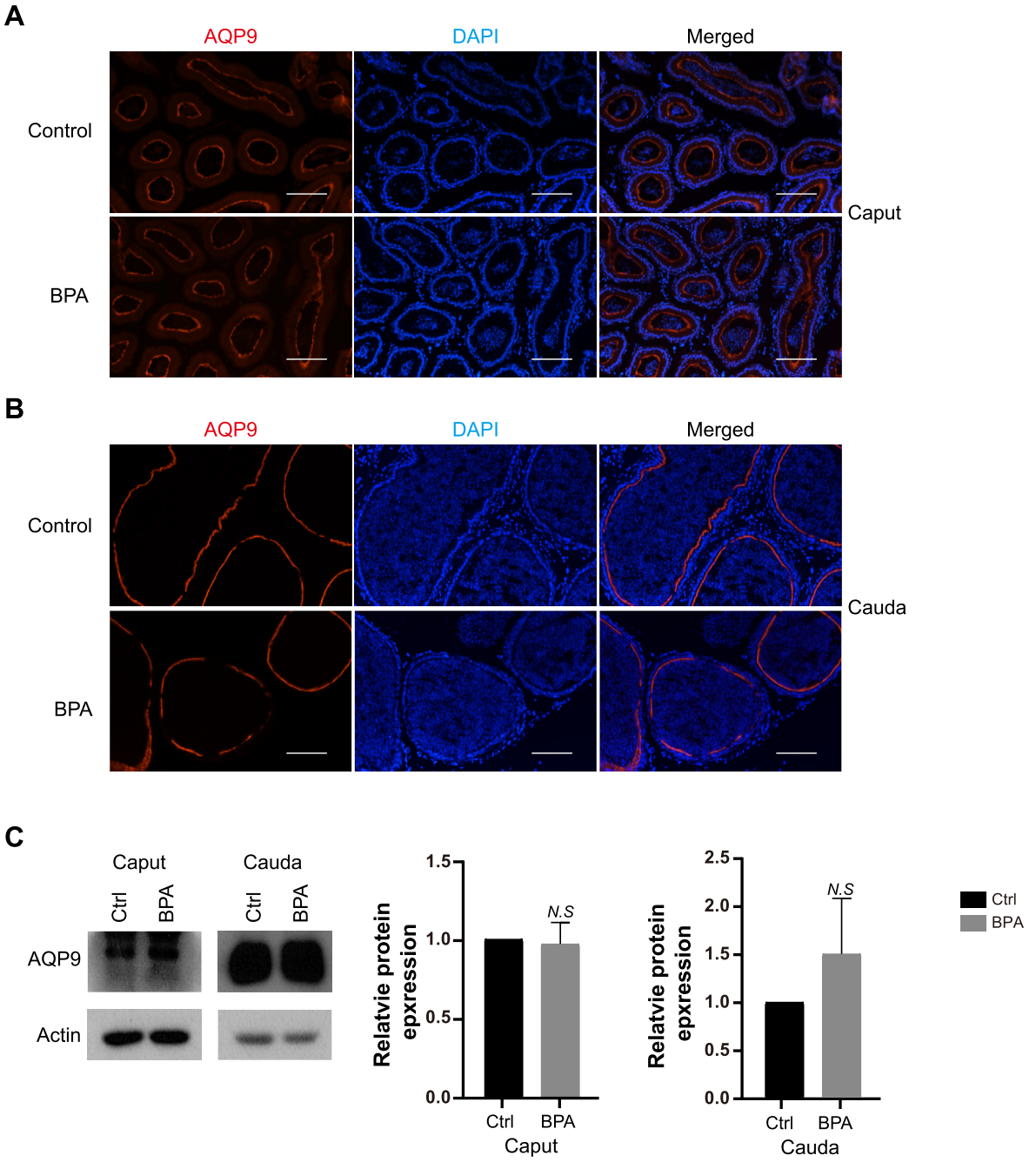


Fig. 2. Effect of pubertal BPA exposure on the distribution of AQP9 in principal cells at adulthood. Representative images showing the distribution of AQP9 in epididymis. Expression of AQP9 (red, left panel) and DAPI (middle panel, blue) in the caput (A) and cauda (B) epididymis from control and BPA-exposed mice. (C) Western blot detection of AQP9 expression in epididymis. Statistics show the relative ratio of the normalized AQP9 expression to control. BPA had no effect on AQP9 protein distribution in the caput and cauda epididymis. Data represented the mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and activates soluble guanylate cyclase, which triggers the accumulation of V-ATPase in the apical membrane (Shum et al., 2008, 2011). Towards this end, these epithelial cells work in a concerted manner to establish the acidic pH, to maintain the sperm in quiescence, and to help the spermatozoa maintain their energy before reaching the female reproductive tract and fertilization. Because of these remarkable roles of the epididymis in sperm maturation, any functional changes due to BPA exposure must be considered, to understand the effects on sperm. In the present study, we have attempted to identify the adverse effects of pubertal exposure to BPA on the process of maturation of spermatozoa in the epididymis and consequent effects on the fertility.

2. Materials and methods

2.1. Experimental animals and BPA treatment

CD-1 male mice were obtained from Daehan BioLink® (Chungcheongbuk-do, Korea). Mice were housed individually in BPA-free polypropylene plastic cages, in animal room facilities (20–25 °C, 50%–60% humidity, and 12 h day/12 h night conditions). They were fed with commercial mouse pellets and water from glass bottles. All mice were allowed to adapt for 1 week and then divided into two groups (n = 30/group). Various guideline-compliant toxicity studies have been reported that no adverse health effects was determined at

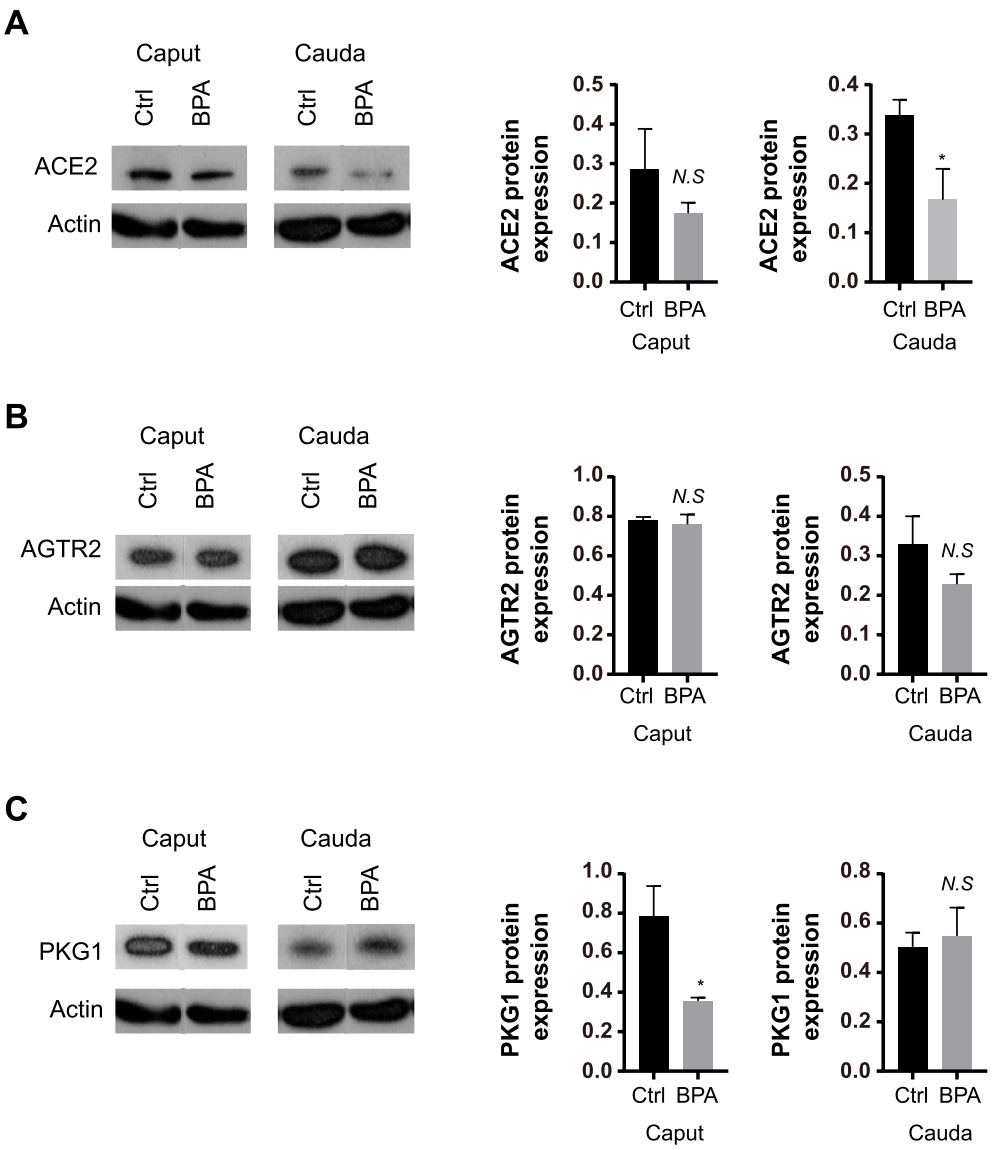


Fig. 3. Effect of pubertal BPA exposure on the renin-angiotensin signalling pathway in the epididymis. (A) ACE2 protein expression in the caput and cauda epididymis from control and BPA-exposed mice was assessed by western blotting. ACE2 protein expression decreased in the cauda epididymis of the BPA-exposed group compared to controls. (B) Western blot and quantification of AGTR2 in the caput and cauda epididymis of control and BPA-exposed mice. BPA had no effect on AGTR2 protein expression in the caput and cauda epididymis. (C) Western blot detection of PKG1 protein expression in epididymis. PKG1 protein expression decreased in the caput epididymis of the BPA-exposed group compared to controls. Data represented the mean ± SEM. ∗P < 0.05.

5 mg BPA/kg/day and reduced body and organ weights were observed at 50 mg BPA/kg/day in mice (Tyl, 2009). Therefore, we tested the effect of BPA exposure at 50 mg BPA/kg/day (lowest-observed adverse effect level) on the reproductive organ. Experimental group was exposed to BPA (≥99% pure, Sigma Aldrich, St Louis, MO, USA), dissolved in corn oil, for 6 weeks, at the dose of 50 mg/kg/day. The treatment windows were selected in order to avoid early neonatal exposure (up to 4 weeks of age), while covering the entire period of puberty/sexual maturity. Control mice were treated with appropriate volumes of corn oil, at the same time-windows. Amount of BPA to be used for exposure was calculated every day, based on the daily changes in the body weight for an individual mouse, so that the treatment dose remains constant. After the last exposure, mice were stabilized for 1 week, before sample collection at postnatal day 84.

2.2. Preparation of epididymis for immunofluorescence labelling

Mice were killed using an intraperitoneal injection of avertin (2,2,2-tribromoethanol). The caput and cauda epididymis were collected and fixed in 4% paraformaldehyde (Boston Bioproducts, Worcester, MA), overnight, at 4 °C as described previously (Park et al., 2017). Tissues were then washed with PBS and incubated in 30% sucrose in PBS overnight at 4 °C for cryoprotection. Following this, tissues were embedded in Tissue-Tek OCT compound (Sakura Fintek, Torrance, CA, USA), mounted on a cutting block, and frozen, before cutting 10 μm sections using a microtome (Leica CM3050-S) and mounting on glass slides (Fisher Superfrost/Plus microscope slides from Fisher Scientific, Pittsburgh, PA, USA). The A subunit of the V-ATPase (ATP6V1A), specific marker of clear cells, and aquaporin 9 (AQP9), as a marker of principal cells, were localized, using immunofluorescence. Briefly, sections were washed with PBS to hydrate and incubated with DAKO target retrieval solution (pH 6.0, DAKO, Glostrup, Denmark) for 10 min at 90 °C. After 3 washes with PBS, they were blocked with 1% BSA in PBS containing 0.1% Tween-20 (0.1% PBS-T), before incubating with primary antibody against either ATP6V1A (Thermo Fisher), or AQP9 (Abcam, Cambridge, MA, USA) antibody in a moist chamber overnight at 4 °C. Excess antibody was washed off with PBS, and the sections were incubated with secondary antibody for 1 h at room temperature and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Images were captured using Nikon Eclipse Ni-U microscope, equipped with a Nikon DS-Ri2 camera and the Nikon NIS-Elements Fr software (Version 5.11, Nikon Instruments Inc., Melville, NY, USA).

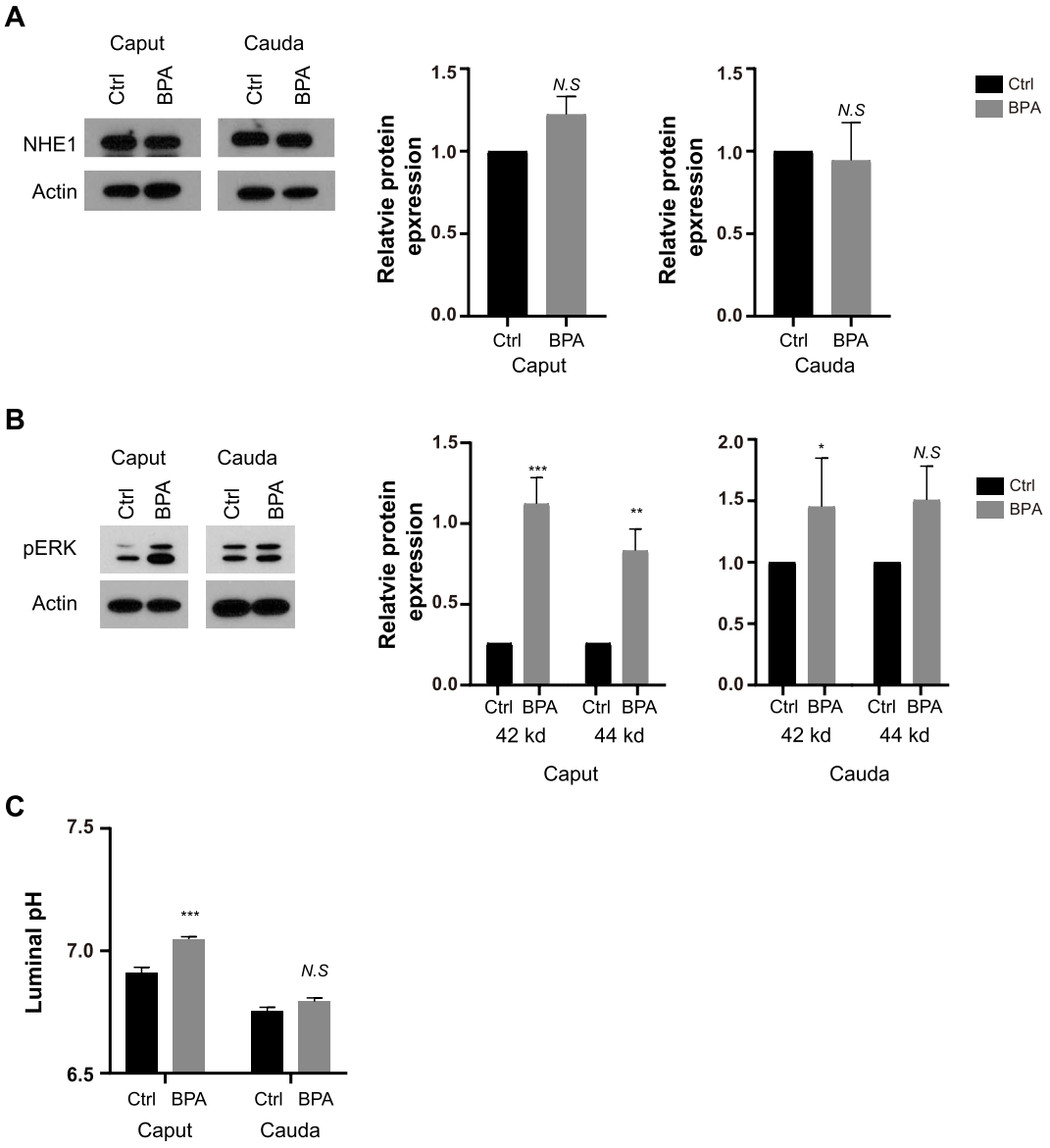


Fig. 4. Effect of pubertal BPA exposure on the NHE1 expression, phosphorylation of ERK 1/2, and luminal pH. (A) NHE1 protein expression in the caput and cauda epididymis from control and BPA-exposed mice was assessed by western blotting. BPA had no effect on NHE1 protein expression in both the caput and cauda epididymis. (B) Western blot and quantification of ERK-phosphorylation in the caput and cauda epididymis of control and BPA-exposed mice. Statistics show the relative ratio of the normalized pERK expression to control. Data represented the mean ± SEM. ∗P < 0.05, ∗∗P < 0.01, ∗∗∗ P < 0.001 versus control group. (C) Luminal pH in the caput and cauda epididymis from control and BPA-exposed mice. The slight elevation of luminal pH was detected in the caput epididymis of BPA-exposed mice as compared to control. Data represented the mean ± SEM. ∗∗∗ P < 0.001 versus control group.

2.3. Western blot

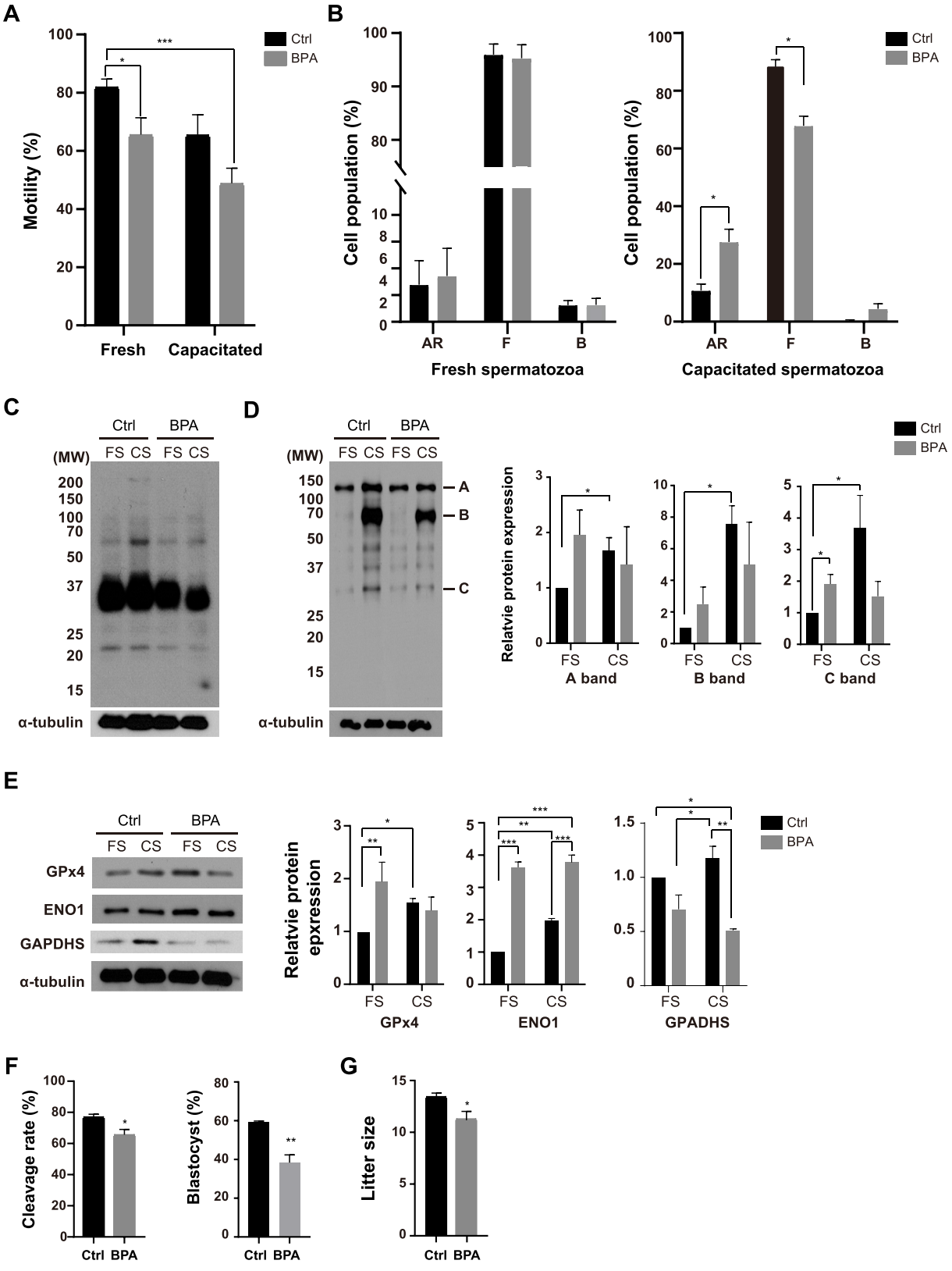
Epididymides were collected from five different animals for each group and were homogenized in RIPA buffer containing complete protease inhibitors and phosphatase inhibitor (Roche Applied Science, Indianapolis, IN), and the lysate was centrifuged at 17,000 g for 30 min at 4 ˚C to collect the supernatant. The total protein lysates were boiled with LDS sample buffer (Invitrogen, Carlsbad, CA) containing 5% β-mercaptoethanol for 5 min at 100 ˚C, cooled, loaded on LDS polyacrylamide gel and electrophoresed as described before (Kim and Breton, 2016; Park et al., 2017). The separated protein bands were electroblotted on a PVDF membrane. Antibodies against ATP6V1A, AQP9, NHE1 (Abcam), pERK (Cell Signaling Technology, Inc., Beverly, MA), angiotensin II (ANG2) type 2 receptor (AGTR2, Abcam), angiotensin II-converting enzyme 2 (ACE2, Cell signalling), protein kinase cGMP-dependent type 1 (PKG-1, Cell signalling), and β-actin (Cell Signaling Technology) were used and the labelled antigens were detected by chemiluminescence. Amount of protein in each band was quantified by Image J software (National Institutes of Health, Bethesda, USA).

2.4. Determination of luminal pH in epididymis

To evaluate the luminal pH in the epididymis obtained from four mice for each group, the caput and cauda epididymis were minced in 200 μl of distilled water and the pH of the suspension was measured within 10 s to minimize the effect of air, using LAQUAtwin Compact pH Meter (B-712, Horiba Scientific). We tried to minimize the contamination of the blood and fat.

2.5. Preparation of spermatozoa

We evaluated the fertility parameters and expression of fertility-related proteins both in freshly collected and capacitated spermatozoa



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following BPA exposure mice. Modified Tyrode's medium (97.84 mM NaCl, 1.42 mM KCl, 0.47 mM $MgCl_2.H_2O$, 0.36 mM $NaH_2PO_4H_2O$, 5.56 mM D-glucose, 25 mM $NaHCO_3$, 1.78 mM $CaCl_2H_2O$, 24.9 mM Na-lactate, and 50 μg/mL gentamycin) was prepared according to our previous study (Rahman et al., 2017) and was used as the basic medium (BM) for fresh sperm collection. Collected spermatozoa from four mice for each group were incubated for 90 min in BM containing 0.4% bovine serum albumin (capacitation media; CM) at 37 °C under 5% CO_2, as previously described (Kwon et al., 2013). We evaluated sperm motility using computer-assisted sperm analysis (CASA) (SAIS-PLUS

Fig. 5. Effect of pubertal BPA exposure on sperm function and fertility of male mice at adulthood. (A) Differences in motility of freshly collected and capacitated spermatozoa between control and BPA-exposed group. (B) Different patterns of combined Hoechst 33258/chlortetracycline fluorescence staining, such as, the acrosome-reacted (AR pattern), non-capacitated (F pattern), and capacitated (B pattern) among freshly collected and capacitated spermatozoa from control and BPA-exposed mice. (C) Western blot detection of phospho-PKA, in freshly collected (FS), and capacitated (CS) from control and BPA-exposed mice. (D) Western blot detection of phospho-tyrosine proteins in FS and CS from control and BPA-exposed mice. Density of phosphorylated proteins such as, 100 kDa band (A band), 70 kDa band (B band), and 25 kDa band (C band), in FS and CS, following BPA exposure. Statistics show the relative ratio of the normalized protein expression to control. Data represented the mean ± SEM. ∗P < 0.05. versus control group. (E) Effect of BPA on sperm fertility-related proteins in FS and CS. Density of GPx4, ENO1, GAPDHS in FS and CS after BPA exposure. Statistics show the relative ratio of the normalized protein expression to control. Data represented the mean ± SEM. ∗∗P < 0.01 and ∗∗∗ P < 0.001 versus control group. (F) The fertilization rate (cleavage rate) and embryonic development (blastocyst formation) in control and BPA-exposed group. Data represented the mean ± SEM. ∗∗P < 0.01, ∗∗∗ P < 0.001 versus control group. ∗P < 0.05 and ∗∗P < 0.01 versus control group. (G) Difference in litter size between control and BPA-exposed groups. Data represented the mean ± SEM. ∗P < 0.01 versus control group.

version 10.1; Medical Supply, Seoul, Korea), as previously described (Kwon et al., 2013). Capacitation status of spermatozoa was determined by a combined Hoechst 33258/chlortetracycline fluorescence assessment dual staining method, according to our previous study (Kwon et al., 2013). Sperm lysates (100 X 10^6/mL) were subjected to western blotting to detect the fertility-related protein expression, including, glutathione peroxidase-4 (GPx4, Abcam), enolase 1 (ENO1, Abcam), and glyceraldehyde-3-phosphate dehydrogenase (GAPDHS, Abcam).

2.6. In vitro fertilization

In vitro fertilization (IVF) was carried out as described in our earlier study (Rahman et al., 2019). Briefly, the cumulus–oocyte complexes (COCs) were collected carefully from at least three superovulated female mice (B6D2F1/CrljOri hybrid, 10–12 week old), and divided equally into 2 groups, viz., for spermatozoa from control males and for those from BPA-exposed males. Spermatozoa collected from both groups (four mice/group) were washed and incubated in BM containing 0.4% BSA for 90 min to induce capacitation before insemination (1×10^6 cells/ml) of the preincubated COCs in BM containing 10% FBS, under mineral oil, for 6 h at 37 °C. Following fertilization, single celled zygotes were collected and incubated in fresh, 50 μL of BM supplemented with 0.4% BSA in fresh culture dishes. The two-cell embryos (cleavage) that developed after 18 h of insemination were counted. This was considered as the rate of fertilization. Culture dishes were incubated for an additional 4 days under the same culture conditions to allow blastocyst development. The zygotes that developed into blastocyst were then counted. The final results of cleavage/blastocyst development were presented as the rate of each parameter in the control and BPA-exposed groups.

2.7. Statistical analysis

Data were analyzed using Student's two-tailed *t*-test by GraphPad Prism (Version 8.0; GraphPad Software Inc.). P-values < 0.05 indicated statistical significance, and data are expressed as mean ± SEM. For each set of data, at least four animals were used.

3. Results

3.1. Effects of pubertal exposure of BPA on distribution of epididymal epithelial cells at adulthood

We assessed the distribution of clear cells and principal cells in the epididymis from control and BPA-treated mice. The number of V-ATPase-positive clear cells was lower in the caput epididymis in BPA-exposed mice than in controls (Fig. 1A), while in the cauda epididymis, clear cell numbers were comparable in both control and BPA-exposed mice (Fig. 1B). Consistent with this observation, the expression of V-ATPase protein was also significantly lower in the caput epididymis of BPA-exposed mice than in controls (P < 0.01), while western blotting showed no significant difference in the cauda epididymis of control and BPA-exposed mice (Fig. 1C). The distribution and number of AQP9-positive principal cells in the caput and cauda epididymis from control and BPA-exposed mice were comparable in immunofluorescence (Fig. 2A and B) and western blots (Fig. 2C). To Protein expression of sodium/hydrogen exchanger 1 (NHE1), which contributes to luminal pH acidification in the caput and cauda epididymis, showed no difference between the groups, as seen on Western blot (Fig. 4A).

Next, to explore how BPA affects V-ATPase molecular functions, we determined the RAS signalling pathway that plays an important role for V-ATPase activation (Shum et al., 2008, 2011). We assessed the protein expression of ACE2, which is a major regulator of RAS signalling pathway, in the epididymis from control and BPA exposed mice. We found a significant decrease in ACE2 protein expression in cauda epididymis following exposure to BPA, while there was no difference in caput (Fig. 3A). When ACE2 is released from immature spermatozoa into the epididymal lumen, ANG1 is converted to ANG2. To maintain the acidic condition in epididymis, V-ATPase is accumulated in the apical membrane of clear cells following the activation of the NO/cGMP pathway by stimulation of AGTR2 in basal cells by ANG2 (Shum et al., 2008, 2011). Therefore, we assess the AGTR2 and PKG1 protein expression in the epididymis from control and BPA treated mice. No changes were observed in AGTR2 protein expression following BPA exposure (Fig. 3B). Fig. 3C showed that BPA significantly reduced the AGTR2 and PKG1 protein expression in both caput and cauda epididymis.

3.2. Effects of pubertal BPA exposure on phosphorylation of ERK signalling pathway and epididymis luminal pH in adulthood

Activation of MAPK/ERK pathway in the epididymis is essential for epithelial cell proliferation, differentiation, and survival (Kim and Breton, 2016; Ramos, 2008). To find out whether the abnormal distribution of epithelial cells in the epididymis due to BPA exposure is related to ERK pathway, we examined the status of ERK phosphorylation in the caput and cauda epididymis by Western blot. A significantly higher phosphorylation of ERK 1/2 was induced in the caput epididymis of BPA-exposed mice than controls (P < 0.05), but not in the cauda epididymis (Fig. 4B).

3.3. Effect of BPA on luminal pH in the epididymis

While the pH in the caput epididymis from control mice remained constant at 6.91, in BPA-exposed mice, it increased to 7.05 (P < 0.01, Fig. 4C). Meanwhile, the average pH in the cauda epididymis did not change because of BPA exposure (6.76 and 6.79 in control and BPA-exposed mice, respectively) (Fig. 4C).

3.4. Effect of pubertal BPA exposure on motility and capacitation status of spermatozoa during adulthood

Motility of freshly collected spermatozoa was significantly lower in BPA-exposed mice than in controls (P < 0.01, Fig. 4A). Sperm motility was also measured after incubation in CM, which capacitates them for IVF. In our observation, the motility of capacitated spermatozoa from BPA-exposed mice was significantly less than that freshly collected spermatozoa from control mice. The motility of capacitated

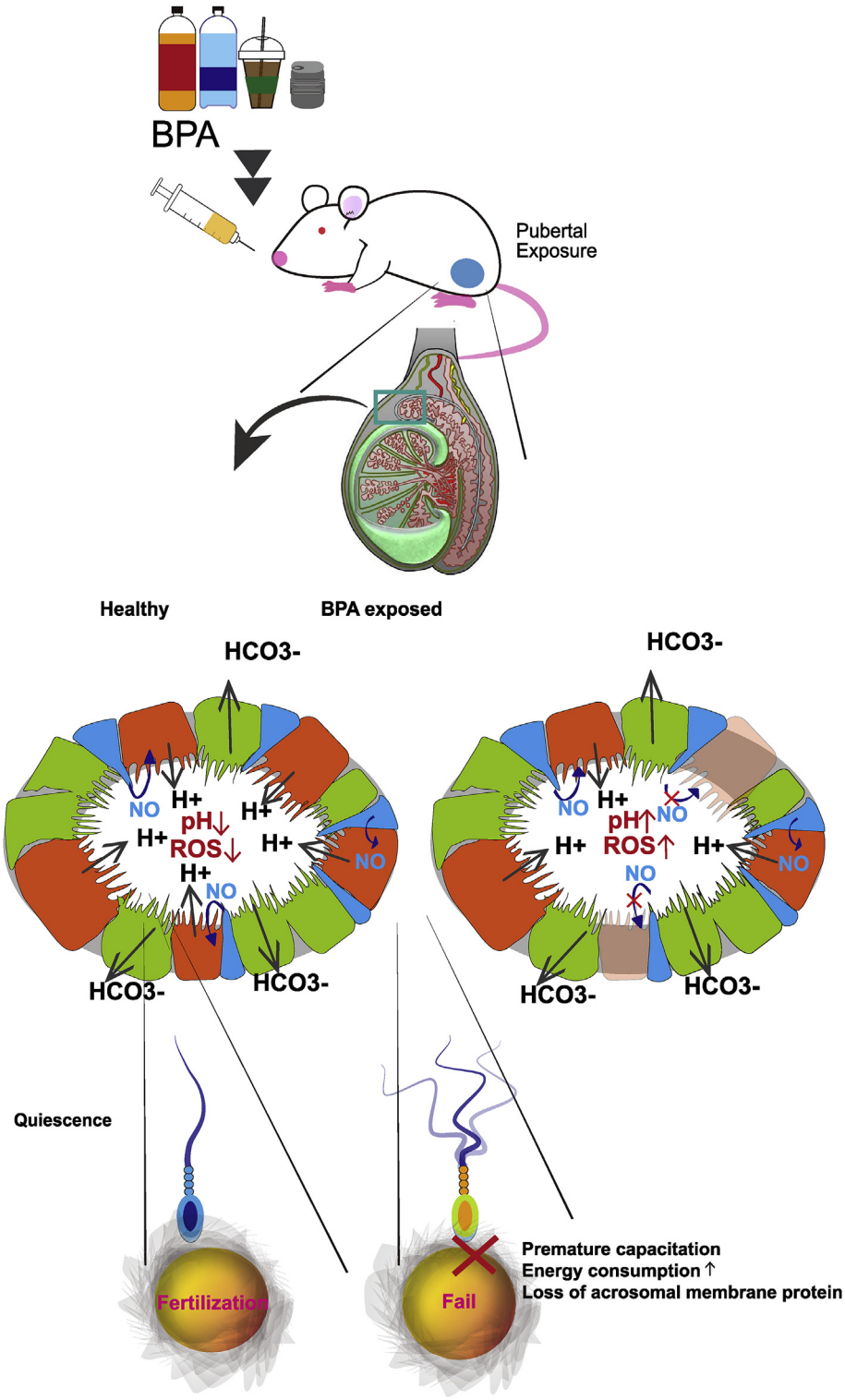

Fig. 6. Scheme of BPA action from epididymal maturation to fertilization. BPA induce decrease of clear cells population and inhibit the proton secretion by V-ATPase. Therefore, the luminal pH increased, and it leads to premature capacitation in spermatozoa during epididymal transit. When premature capacitated spermatozoa reach in female reproductive tract, they undergo the acrosome reaction while healthy spermatozoa undergo capacitation. The aberrant acrosome reaction leads to interruption of fertility-related protein works and tyrosine phosphorylation in spermatozoa. As a result of these modification, sperm fertility will be declined. The adverse effects of BPA during sperm maturation continuously maintain to fertilization.

spermatozoa was comparable between control and BPA-exposed mice (Fig. 5A). Similarly, we also examined sperm capacitation status both in fresh spermatozoa and those incubated in a CM. There were no significant differences in the number of acrosome-reacted (AR), capacitated (B), and non-capacitated (F) cells in control and BPA-exposed mice in the freshly released spermatozoa from the cauda epididymis (Fig. 5B). However, when spermatozoa were incubated in a capacitating media, AR cells were significantly higher in BPA-exposed mice (10.78% ± 2.22, $P < 0.05$, Fig. 4B) than in controls.

Next, we estimated PKA activity and protein tyrosine phosphorylation by using western blotting, to understand the molecular mechanism of BPA-induced acrosome reaction. PKA signalling was lower in BPA-exposed mice than in controls, regardless of the capacitation condition (Fig. 5C). Similarly, tyrosine phosphorylated proteins of approximately 75 kDa (B band) and 30 kDa (C band) were lower after capacitation in BPA-exposed mice than in controls (Fig. 5D). More importantly, tyrosine phosphorylated proteins in freshly collected spermatozoa were higher in BPA-exposed mice than in controls and were the same as those of capacitated spermatozoa in controls (Fig. 5D).

3.5. Effect of pubertal BPA exposure on fertility of male mice during adulthood

In addition, to evaluate the functional changes in spermatozoa following BPA exposure, expression of fertility-related protein was studied by Western blot. For this, proteins that have important roles in sperm fertility were selected (Kwon et al., 2014; Park et al., 2012, 2013; Rahman et al., 2015). Among these, GPx4 protein expression was significantly higher in non-capacitated spermatozoa from BPA-exposed mice than from controls ($P < 0.05$, Fig. 5E). However, the expression was down-regulated to a level comparable to that in the control mice during capacitation (Fig. 5E). ENO1 protein expression in spermatozoa increased significantly owing to BPA exposure, regardless of their capacitation status (Fig. 5E), while GAPDHS expression decreased slightly ($P < 0.01$, Fig. 5E) during capitation because of BPA exposure, although there was no significant difference between the BPA-exposed and control mice before capacitation. Male fertility was evaluated using both in vitro and in vivo assays. As shown Fig. 5F, rate of fertilization (cleavage rate) was significantly lower in BPA-exposed mice than in controls ($P < 0.05$). Early embryonic development (blastocyst formation) and litter size (average in control group 13.33; in BPA-exposed mice, 11.20) were also lower for BPA-exposed mice than for controls ($P < 0.01$, Fig. 5F).

4. Discussion

Previous studies have shown that gestational exposure to BPA in mice is associated with a decrease in sperm count and motility in the offspring, affecting the overall spermatogenesis (Rahman et al., 2017; Salian et al., 2009). In humans too, higher concentration of BPA in male urine was associated with fewer male birth (Bae et al., 2015)and lower sperm quality (Radwan et al., 2018). Although there were numerous studies regarding the adverse effects of BPA on spermatogenesis and sperm fertility, many questions regarding the physiological changes due to BPA exposure during epididymal maturation remain unaddressed. The changes of spermatozoa in the epididymis must be considered to understand the comprehensive physiological sperm responses to BPA. To address this, we attempted to evaluate the overall effects of BPA exposure on the whole spectrum of issues in male fertility, including sperm maturation, fertilization, and embryo development.

V-ATPases were expressed in the apical membrane of clear cells of the epididymis, which secrete protons into the epididymal lumen to maintain an acidic pH (Breton et al., 2016; Da Silva et al., 2007; Park et al., 2017). Interestingly, we found that the expression of V-ATPase in the caput epididymis was significantly down-regulated following BPA exposure, while there was no difference in its expression in the cauda epididymis. In contrast, AQP9 protein, which maintains the acidic condition in the epididymal lumen by reabsorption of bicarbonate, was not affected by BPA. This suggested that disruption of V-ATPase in the caput epididymis may result in elevation of luminal pH and this in turn may induce premature capacitation of spermatozoa during their transit through epididymis. Because the other hydrogen pumps, such as NHE1 and NHE3 (Bagnis et al., 2001; Martins et al., 2014; Park et al., 2017), present in the epididymis to regulate luminal pH, we expected that elevation of luminal pH by loss of V-ATPase expression may be compensated by a corresponding increase in NHE1 expression. However, no significant difference in NHE1 expression was observed not only in the caput but also in the cauda epididymis. We propose that the slight elevation of luminal pH in the caput epididymis was a consequence of V-ATPase disruption. Moreover, it is noteworthy that the RAS signalling pathway associated with V-ATPase activation in clear cells is also disrupted by BPA exposure. ACE2 released from epididymal spermatozoa is a key regulator of the RAS in epididymis and it is associated with male fertility (Hagaman et al., 1998; Leung and Sernia, 2003). Because the ANG2 production is regulated by ACE2, which derived from spermatozoa, we determined the ACE protein expression in epididymis following BPA exposure. We found that no differences of ACE2 and AGTR2 protein expression in caput epididymis between control and BPA exposed group. Because the ANG2 production depends on ACE2, our results indicated that ANG2 level in caput epididymis from BPA exposed mice may same as caput from control mice and the ANG2 may stimulate the AGTR2 activation to produce the NO in basal cells. Generally, NO diffuses in clear cells and it facilitates the V-ATPase activation; however, in BPA treated mice, some NO may present in the lumen or basal cells not in clear cells because BPA disrupts clear cells. Moreover, PKG1 was significantly decreased in caput epididymis from BPA exposed mice compared to control. Our results suggested that BPA is diminished the clear cells which may lead to interruption of PKG1 activation and influx of NO in clear cells. NO is known as reactive oxygen species and a higher level of NO can induce the adverse effects in spermatozoa including DNA and membrane protein disruption (Agarwal et al., 2014). Our previous study has reported that nitro-tyrosine damages were increased in F1 male mice following gestational exposure to BPA (Rahman et al., 2018). Altogether, we hypothesize that spermatozoa can be damaged by not only higher luminal pH but also ROS stress via disruption of clear cells in epididymis during maturation following BPA exposure.

The MAPK/ERK signalling pathway is associated with epithelial cell proliferation in the epididymis (Kim and Breton, 2016; Xu et al., 2010). When the initial segment of the epididymis was damaged by blocking of luminal factors, pERK signalling pathway was disrupted, leading to enhanced apoptosis (Kim and Breton, 2016; Xu et al., 2010). Activation of ERK 1/2 phosphorylation is associated with cell proliferation necessary to recover from and survive damage, not only in the epididymis but also in the heart, spleen, kidney, and brain (Kim and Breton, 2016; Maillet et al., 2008; Xu et al., 2010). Although pERK 1/2 signalling pathway is reported to be important for preventing apoptosis in the initial segment rather than in the caput region (Kim and Breton, 2016; Xu et al., 2010, 2011), our results in this study show that pERK 1/2 signaling pathway was extremely increased significantly in the caput epididymis following BPA exposure. Therefore, we assessed activation of caspases 3 and 8 by immunostaining and Western blot following BPA exposure to evaluate apoptosis in the epididymis. However, activation of caspases 3 and 8 was not detectable in both the caput and cauda epididymis from control and BPA-exposed mice (data not shown). Although there was no change in the pattern of apoptosis in the epididymis, we hypothesize that BPA exposure during puberty may impair the clear cells in the caput region, which may enhance the phosphorylation of ERK in order to recover from the damage. However, it is not yet known which cell death signalling is involved in phosphorylation of ERK and whether it is associated with recovery of clear cell disruption in the epididymis when BPA exposure is discontinued, and these issues deserve further investigation.

After epididymal maturation, spermatozoa undergo post-translational modification during transit through the female reproductive tract, collectively termed as capacitation (Naz and Rajesh, 2004; Sakkas et al., 2003; Urner et al., 2001; Visconti et al., 1995). Importantly, spermatozoa encounter an alkaline pH condition with high concentration of bicarbonate that causes intracellular alkalization of spermatozoa (Nishigaki et al., 2014; Zeng et al., 1996). Tyrosine phosphorylation in spermatozoa is enhanced by protein kinases and phosphatases during sperm capacitation, and this in turn increases sperm motility and enhances the phosphorylation of proteins in the sperm to prepare them for acrosome reaction and penetration of the oocytes (Mahony and Gwathmey, 1999; Nassar et al., 1999; Naz and Rajesh, 2004). Therefore, the acidic condition in the epididymal lumen is important in order to prevent premature capacitation of spermatozoa (Breton et al., 2016; Da Silva et al., 2007; Park et al., 2017). In this study, we found that a slight increase in the luminal pH and the RAS disruption following BPA exposure. The disruption of the RAS in epididymis may cause of presence of unnecessary NO in epididymal lumen, and it may lead to the ROS damages in spermatozoa during maturation. Especially, we found

that GPx4 protein expression was higher in freshly collected spermatozoa from BPA exposed mice than control mice. GPx4, a well-known antioxidant enzyme, is associated with repairing of oxidative damages in the spermatozoa through a decrease of oxidized phospholipids in the membrane (Ursini et al., 1997). Because the GPx4 is a major enzymatic defense system against oxidative stress and is increased in ROS damaged spermatozoa, GPx4 is considered as an oxidative stress marker in spermatozoa (Li and Zhang, 2013; Sharma et al., 2013). Therefore, the increase of GPx4 in the freshly collected spermatozoa following BPA exposure revealed that spermatozoa are in a high oxidative stress during epididymal maturation. Consequently, spermatozoa underwent acrosome reaction under capacitation conditions, i.e. loss of acrosomal membrane protein in the sperm head before reaching normal capacitation status.

Moreover, the amounts of some tyrosine-phosphorylated proteins in the freshly collected spermatozoa from BPA-exposed mice were comparable to those from capacitated spermatozoa of control mice, while after capacitation, the levels of these proteins were lower in BPA-exposed group than in controls. Interestingly, PKA-activity decreased in spermatozoa of BPA-exposed mice compared to controls, regardless of the capacitation status. Additionally, the fertility-related proteins in prematurely capacitated spermatozoa were abnormally up- or down-regulated compared to those from controls. ENO1 was more highly expressed in spermatozoa from BPA exposed mice than control regardless of capacitation conditions, while ENO1 was increased following capacitation in spermatozoa from control mice. This result indicates that spermatozoa may encounter capacitation conditions during maturation following BPA exposure and premature spermatozoa may lose their fertilising ability during capacitation and acrosome reaction. Consequently, sperm fertility decreased both in vitro and in vivo as a result of these modifications.

5. Conclusion

To our knowledge, this is the first comprehensive study about the effects of BPA exposure during puberty on sperm maturation and fertilization capacity. In summary, exposure to BPA leads to a loss of clear cells in the epididymis and elevation of luminal pH, which in turn may trigger the premature capacitation of spermatozoa during epididymal transit, and the consequent decline in sperm fertility. Moreover, NO generated by basal cells cannot diffused in clear cells and may present in the lumen, therefore, the luminal NO may act as ROS and it can induce damages in spermatozoa during epididymal maturation (Fig. 6). Further exploration of downstream ROS generation, pERK signalling, cell death, proliferation, and differentiation of epithelial cells in the epididymis following BPA exposure will be of interest, given the adverse effects of BPA in many tissues.

Ethical approval

We followed the standard guidelines for animal studies, approved by the Institutional Animal Care and Use Committee of Chung-Ang University, Seoul, Korea (IACUC Number: 2016 – 00009).

CRediT authorship contribution statement

Yoo-Jin Park: Conceptualization, Methodology, Validation, Investigation. **Md Saidur Rahman:** Validation, Writing - review & editing. **Won-Ki Pang:** Investigation. **Do-Yeal Ryu:** Investigation. **Bongki Kim:** Writing - review & editing. **Myung-Geol Pang:** Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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