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Effects of Alternate Treatment of Estrogen Receptor Antagonist and Agonist on Morphology of Male Reproductive Organs of Adult Mice

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ICI 182,780 (ICI) is known as an estrogen receptor antagonist, whereas propyl pyrazole triol (PPT) is an estrogen receptor agonist. In this study, ICI or ICI added with PPT was injected into adult male mice. Body and reproductive organ weights were reduced in the ICI added with PPT group compared to the control group. Further, the ICI and ICI added with PPT groups both showed increases in luminal areas of the seminiferous tubules of the testis, whereas cell heights of efferent ductules and the initial segment of the epididymis were reduced. Sperm count in the caudal epididymis was reduced in the ICI and ICI added with PPT groups. These results show that reproductive tissues were more deeply affected in the ICI added with PPT group. We also demonstrated that treatment with ICI resulted in histological changes in the testis, efferent ductule, and epididymis. Further, alternate treatment with ICI and PPT induced abnormalities in reproductive organs. These results indicate that a high concentration of PPT together with ICI may cause histological abnormalities instead of histological restoration in reproductive organs.

Key Words: Estrogen receptor agonist, Antagonist, Testis, Efferent ductule, Epididymis

INTRODUCTION

Male reproductive organs are responsive to estrogen, which is known as a female hormone (Hess et al., 1997; Couse & Korach, 1999; Oliveira et al., 2002; Hess et al., 2011). Many researchers have suggested that estrogen plays a role in the male reproductive system (Couse & Korach, 1999; O'Donnell et al., 2001; Hess, 2003; Goyal et al., 2004; Oliveira et al., 2004; Aleem et al., 2006; Carreau et al., 2007). In male organs, the function of estrogen is mediated via estrogen receptor (ER). ER can be categorized as either ER alpha (ERα or ESR1) or ER beta (ERβ or ESR2) (Couse & Korach, 1999). Identification of ERα and ERβ has been carried out using techniques such as immunocytochemistry, in situ hybridization, and Northern blotting. However, these methods do not always provide identical results (Hess, 2003). Therefore, ER localization studies have shown disagreement between laboratories and among different species (Taylor & Al-Azzawi, 2000; Nie et al., 2002; Zhou et al., 2002; Yamashita, 2004).

ERα in the efferent ductules is related to regulation of fluid reabsorption and therefore fertility, which is estrogen's main function in the male reproductive tract, whereas the function of ERβ function is not well known (Hess, 2003). ICI 182,780 (ICI) is an antagonist of ER, whereas propyl pyrazole triol (PPT) acts as an agonist. In comparison with ERβ, the affinity of PPT for ERα has been demonstrated to be at least 410 times higher or more (Stauffer et al., 2000; Harris et al., 2002; Sun et al., 2002). As ERs are located in the testis, efferent ductules, and epididymis (Fisher et al., 1997; Zhou et al., 2002; Yamashita, 2004), these organs would be affected by ER antagonists and agonists.

The ER antagonist ICI and agonist PPT have previously been

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used to study the roles of estrogen in the male reproductive system. ICI causes seminiferous tubular atrophy in the testis, luminal dilation, decreased epithelial height in the efferent ductules, reduction of sperm number in the epididymis, and decreased fertility (Oliveira et al., 2002; Cho et al., 2003; Oliveira et al., 2003). A high dose of PPT for 8 weeks has been shown to induce seminiferous tubular atrophy, resulting in loss of spermatogenesis as well as infertility in adult male mice (Lee & Cho, 2009). In the female mouse, number of Graafian follicle and corpus luteum was reduced in PPT treatment group (Lee et al., 2009). Psychoneuroendocrine research has suggested that ERα increases aggressive behavior in male mice while ERβ decreases it (Clipperton-Allen et al., 2011). However, gonadally intact male mice are hardly affected by PPT, whereas gonadectomized male mice show increased aggression. It has been estimated whether or not administration of an ER agonist to male mice compensates for the effects of an ER antagonist. In the present study, regarding the compensation ability of PPT, the antagonist ICI was subcutaneously administered to male mice for 1 week, after which agonist PPT was applied and the alternate treatment repeated for 10 weeks. Male reproductive organs were observed by microscopy.

MATERIALS AND METHODS

Animals and Treatments

Eighteen C57BL/6 male mice aged 10 weeks were maintained under controlled conditions and randomly divided into three groups: control, ICI, and ICI added with PPT groups. For the control group, animals (n=6) were subcutaneously injected weekly with 0.08 mL of castor oil for 10 weeks. For the ICI group, animals (n=6) received subcutaneous injection of 0.1 mol ICI (Tocris Cookson Ltd., Bristol, UK) in 0.08 mL of castor oil (Sigma Chemical Co., St. Louis, MO, USA) biweekly (ICI injected during first week followed by 0.08 mL of castor oil the next week, repeatedly) for 10 weeks. For the ICI added with PPT group (ICI+PPT group), animals (n=6) received with 0.1 mol ICI in 0.08 mL of castor oil during the first week followed by 0.1 mol PPT (4,4',4"(4-propyl-[1H]-pyrazole-1,3,5-triyl)tris-phenol, Tocris Cookson Ltd.) in 0.08 mL of castor oil the next week alternately for 10 weeks. Mice were supplied with water and commercial food ad libitum. All animals were maintained under conditions of 22±2°C at 55±5% relative humidity and 12D:12L lighting cycle. Animal experiments were performed with approval from the Sunchon National University Animal Care and Use Committee.

Histological Procedure

Ten weeks after ICI administration, male mice were anesthetized with sodium pentobarbital, weighed, and perfused intracardially with 4% glutaraldehyde in 0.1 mol cacodylate buffer (pH 7.4) for 20 min. Following perfusion, the testis, distal efferent ductules, and epididymides were dissected, weighed, and placed into the same perfusion solution for further histological analyses. Fat tissue attached to each organ was carefully detached, after which organs were dried on filter paper prior to recording organ weights. For microscopy, organs were dehydrated, embedded in JB-4 (Polysciences Inc., Warrington, PA, USA), and sectioned at 2.5 μm thickness. Sections were mounted on a slide glass and stained with hematoxylin-periodic acid Schiff (PAS) solution.

Analysis of Testis, Efferent Ductules, and Epididymis Morphology

After staining and mounting, sections were photographed with a SPOT digital microscope camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA) and analyzed using SPOT Insight software ver. 4.0 (Diagnostic Instruments Inc.). Luminal areas and diameters of 30 seminiferous tubules per testis were determined and averaged. Luminal short diameters of 15 distal efferent ductules per testis were also measured. Cell height was determined from the base to the microvillus tip in 30 non-ciliated epithelial cells from non-oblique sections of distal efferent ductules per mouse. Cell height was also determined in 30 epithelial cells in the initial segment of the epididymis.

Analysis of Sperm Number in Caudal Epididymis

To determine sperm number, stained slides of distal caudal epididymis tissue were visualized with a digital camera, and sperm heads were counted in a 2,500 μm^2 (50×50 μm) area of the caudal epididymis up to 20 times per mouse. Sperm number was first measured and averaged, after which the mean and standard deviation for each group were derived from six mice per group.

Statistical Analysis

Statistical differences between control and ICI groups or between control and ICI+PPT groups were analyzed via one-way ANOVA using Microsoft Office Excel 2007 (Microsoft Corp., Redmond, WA, USA), and a p-value of <0.05 was considered to be significantly significant.

RESULTS

Body and Organ Weights

Fig. 1 shows external views of the reproductive system and urinary bladder in the control, ICI, and ICI+PPT groups. The ICI+PPT group showed a significantly altered body weight compared to the control group, whereas the ICI group did not. In the testis, the ICI group showed pattern of increasing organ weights compared to the control group, whereas the ICI+PPT group showed pattern of decreasing organ weights;

however, the differences were not significant (Table 1). Further, no significant differences in efferent ductule and epididymis weights were observed between the treatment and control groups.

Testis

Spermatogenesis was observed in seminiferous tubules of the control, ICI, and ICI+PPT groups (Fig. 2). Due to decreases in the germ cell layer, luminal areas of seminiferous tubules in the ICI (5,060.2 μm^2) and ICI+PPT (6,820.9 μm^2) groups significantly increased compared to the control group (3,391.5 μm^2) (Table 1). Further, exfoliated germ cells appeared in luminal areas of the seminiferous tubules. Diameters of seminiferous tubules increased in the ICI group compared to control, whereas they decreased in the ICI+PPT group.

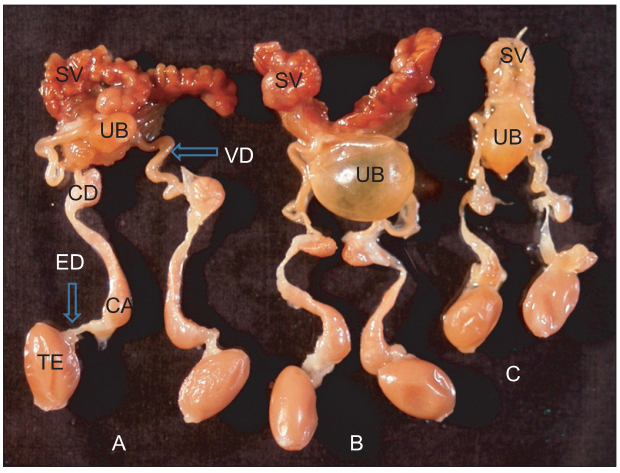

Fig. 1. Photographs of reproductive system and urinary bladder in control (A), ICI (B), and ICI+PPT (C) groups. ICI, ICI 182,780; PPT, propyl pyrazole triol; SV, seminal vesicle; UB, urinary bladder; VD, vas deference; CD, caudal epididymis; ED, efferent ductule; CA, caput region of epididymis; TE, testis.

Efferent Ductules

Weight of efferent ductules in the treatment groups decreased compared to the control group, although the decrease was not significant (Table 1). In the distal region of the efferent ductules, luminal diameters were 16.3, 41.2, and 34.0 μm in the control, ICI, and ICI+PPT groups, respectively. Efferent ductule diameters significantly increased in the treatment groups compared to the control group (Fig. 3), which showed a nearly consistent diameter. Mice treated with ICI or ICI+PPT showed decreased epithelial cell heights in efferent ductules compared to control (Table 2). Further, there were numerous PAS-positive granules in the supranuclear cytoplasmic region of non-ciliated epithelial cells in the control group, whereas they were poorly located in the treatment groups (Fig. 3).

Epididymis

Epididymis weight increased in the ICI group compared to control, whereas it decreased in the ICI+PPT group; however, the changes were insignificant. Cell heights of the initial segment region decreased in the treatment groups compared to the control group (Fig. 4). Further, there were PAS-positive granules from the supranuclear cytoplasm to the tip cytoplasm of principal cells of the initial segment in the control group, whereas the number of the granules decreased in the treatment groups. Sperm was highly concentrated in tubules of the caudal epididymis (Fig. 5). Sperm number in the caudal area

Table 1. Comparative body and reproductive organ weights in control, ICI, and ICI+PPT groups

Weight	Control	ICI	ICI+PPT
Body (g)	27.9±1.0	28.7±0.9	21.8±3.0*
Testis (mg)	99.3±17.3	115.3±8.2	79.4±12.5
Efferent ductule (mg)	2.6±1.0	2.2±0.7	2.4±0.8
Epididymis (mg)	40.4±6.1	48.5±5.7	28.0±12.7

Values are presented as mean±standard deviation. ICI, ICI 182,780; PPT, propyl pyrazole triol. *p<0.05 compared with control group.

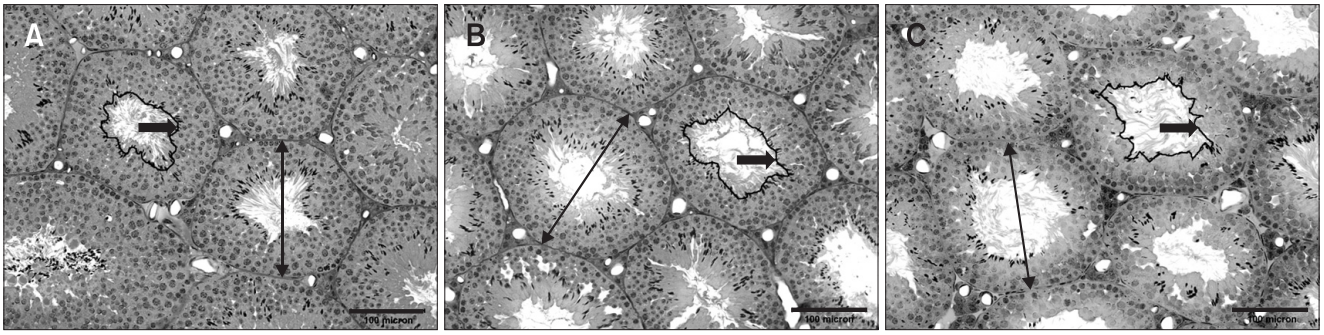

Fig. 2. Micrographs of testis in control (A), ICI (B), and ICI+PPT (C) groups. Seminiferous tubular lumen was dilated, and germinal epithelium was reduced in all tubules of the ICI+PPT group, although spermatogenesis was observed. Double arrow indicates seminiferous tubular diameter. Circled line (thick arrow) indicates luminal area of seminiferous tubule. Scale bars=100 μm. ICI, ICI 182,780; PPT, propyl pyrazole triol.

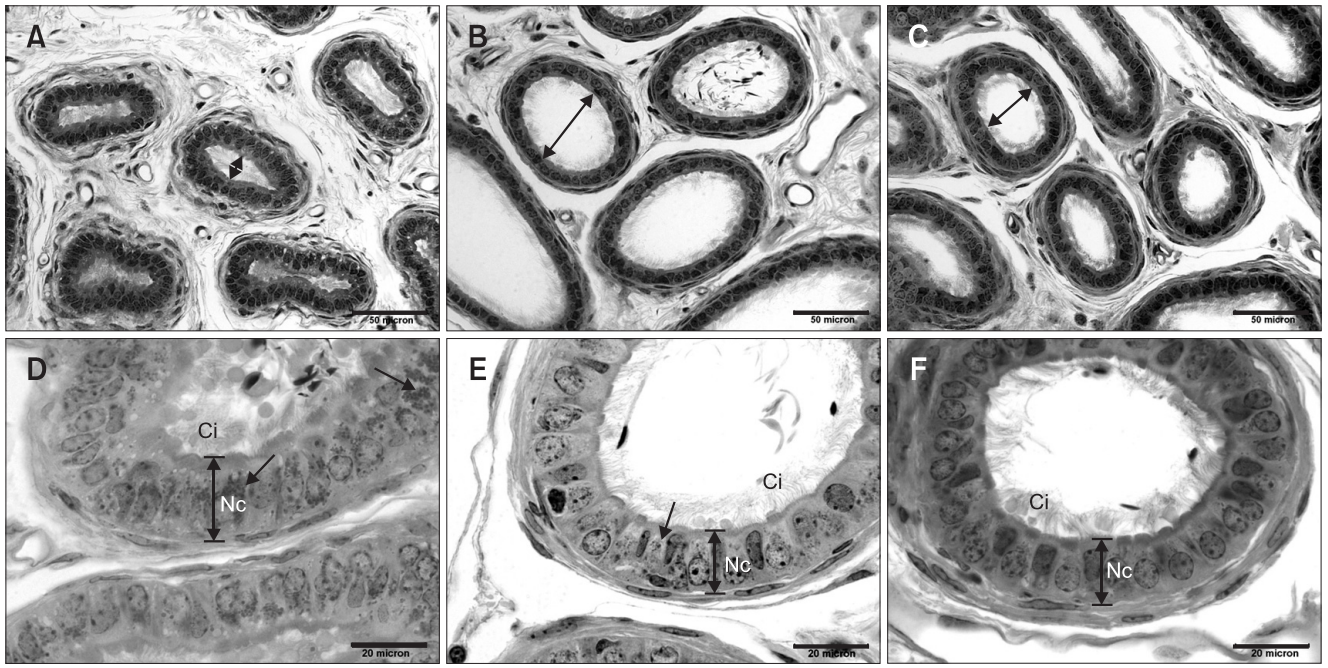

Fig. 3. Micrographs of efferent ductule in control (A and D), ICI (B and E), and ICI+PPT (C and F) groups. Efferent ductule is enlarged in D, E, and F. Luminal diameter (double arrow) increased in the two treatment groups, resulting in decreased epithelial cell height. Cilia (Ci) of ciliated cells were found in the lumen of efferent ductule. Periodic acid Schiff (PAS)-positive granules (arrows) were abundant in the supranuclear cytoplasm of non-ciliated cells (Nc) in the control group, whereas they decreased or did not appear in the treatment groups. Mice treated with ICI (E) or ICI+PPT (F) showed decreased epithelial cell heights in efferent ductules compared to control (D). Scale bars=50 μm in A-C and 20 μm in D-F. ICI, ICI 182,780; PPT, propyl pyrazole triol.

Table 2. Comparative areas, diameters, and heights of reproductive tissues in control, ICI, and ICI+PPT groups

Parameter	Control	ICI	ICI+PPT
Luminal area of seminiferous tubules (μm^2)	3,391.5±546.1	5,060.2±680.8*	6,820.9±713.3*
Seminiferous tubular diameter (μm)	185.7±5.3	188.2±5.8*	171.6±7.5*
Luminal diameter of efferent ductules (μm)	16.3±1.8	41.2±10.8*	34.0±6.9*
Cell height of efferent ductules (μm)	17.7±1.9	12.5±0.8*	11.7±0.4*
Cell height of initial segment (μm)	55.6±1.2	51.9±0.5*	44.7±0.7*

Values are presented as mean±standard deviation. ICI, ICI 182,780; PPT, propyl pyrazole triol. *p<0.05 compared with control group.

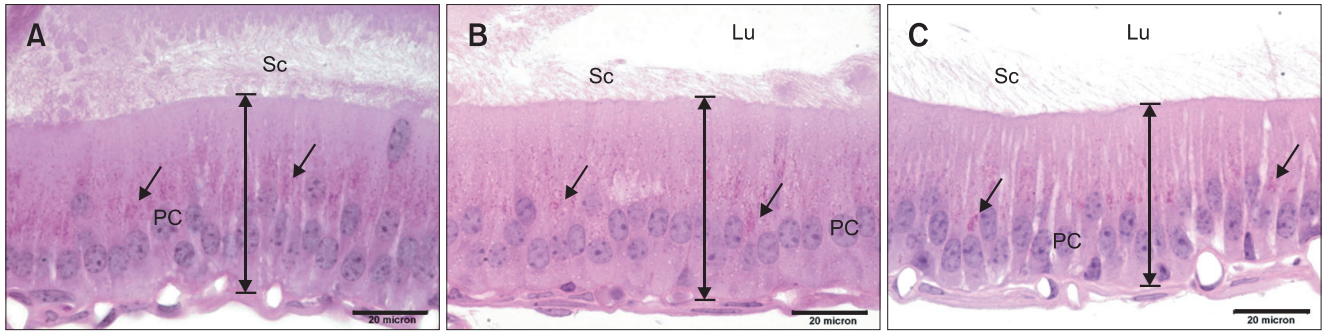

Fig. 4. Micrographs of initial segment of epididymis in control (A), ICI (B), and ICI+PPT (C) groups. Principal cells (PC) of epididymal epithelium displayed tufts of very long stereocilia (Sc) spread in the lumen (Lu). Epithelial cell height (double arrow) was reduced in the two treatment groups. Periodic acid Schiff (PAS)-positive granules (arrows) were abundant in the supranuclear cytoplasm of principal cells in the control group, although they decreased in the treatment groups. Scale bars=20 μm. ICI, ICI 182,780; PPT, propyl pyrazole triol.

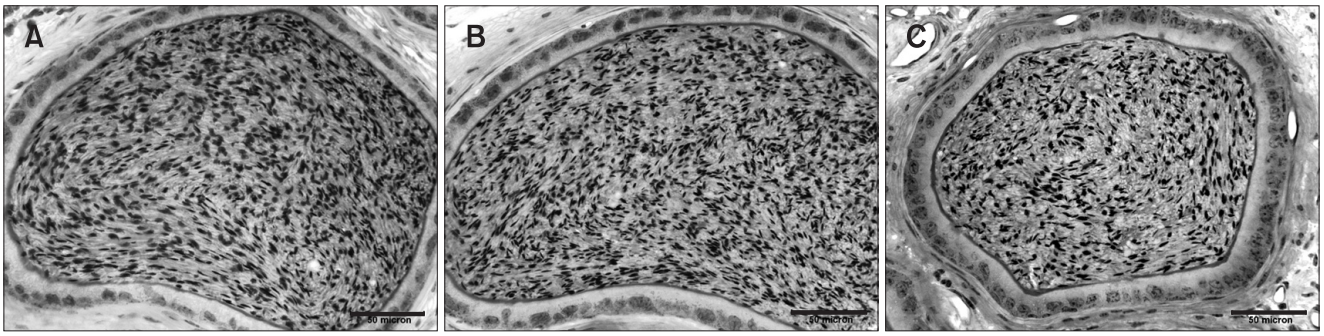

Fig. 5. Micrographs of caudal epididymis in control (A), ICI (B), and ICI+PPT (C) groups. Sperm was highly concentrated in tubules of the epididymis. Scale bars=50 μm. ICI, ICI 182,780; PPT, propyl pyrazole triol.

Table 3. Sperm numbers in caudal epididymis (area of 2,500 μm^2) in control, ICI, and ICI+PPT groups

Parameter	Control	ICI	ICI+PPT
Sperm number	91.2±1.3	89.1±2.9	80.9±1.9*

Values are presented as mean±standard deviation. ICI, ICI 182,780; PPT, propyl pyrazole triol. *p<0.05 compared with control group.

(2,500 μm^2) decreased in the ICI (89.1) and ICI+PPT groups (80.9) compared to the control group (91.2) (Table 3).

DISCUSSION

To better understand the effects of physiological estrogen in males, the effects of ER antagonist ICI or ICI added with agonist PPT were investigated in the present study. Body weights insignificantly increased in the ICI group, whereas they significantly decreased in the ICI+PPT group. The ICI group experienced insignificant increases in body weight after 8, 10, 12, 16, 25, 59, and 125 days of treatment (Cho et al., 2003). According to our results and other reports, it can be assumed that body weight is not seriously affected by ICI treatment. However, PPT treatment resulted in a 21.9% decrease in body weight in the ICI+PPT group (21.8 g) compared to the control group (27.9 g). Generally, body weight of mice decreases upon treatment with PPT (Lee & Cho, 2009) or high concentrations of 17β-estradiol (Cook et al., 1998). It has also been reported that PPT increases sensitivity of the brain to satiety hormones, resulting in reduced food consumption (Roesch, 2006). Regarding body weight, it was suggested that mice are more affected by PPT compared to ICI treatment.

Weight of testis differed depending on treatment type. The ICI group showed an increase in testis weight, whereas the ICI+PPT group showed the opposite. In ER knockout (ERKO) mice or ICI-treated mice, testis weight was shown to increase in early experiments, but recently, opposite results have been obtained (Hess et al., 1997; Cho et al., 2003). Further, increased testicular weight was related to dilation of seminiferous tubular diameter in the present study. Oliveira et al. (2002) suggested that short-term ICI treatment inhibits fluid reabsorption in the efferent ductules. Therefore, fluid-induced backpressure to the testis would indeed result in swelling of the seminiferous tubules, thereby increasing testis weight. In contrast to the ICI result, seminiferous tubular diameter insignificantly decreased in correlation with reduced testis weight in the ICI+PPT group. These results suggest that testis weight was more deeply affected by PPT compared to ICI treatment. Weights of the efferent ductules and epididymis showed similar results as that of testis weight.

Estrogen is correlated with spermatogenesis regulation, histopathology, and changes in reproductive organ weight or fertility (Cook et al., 1998; Toyama et al., 2001; Aleem et al., 2006; Carreau et al., 2007). It has been suggested that decreased testis weight by estrogen treatment is related with interstitial cell atrophy, seminiferous tubule degeneration, and reduced sperm number (Cook et al., 1998). Further, estrogen has been shown to induce reduction of epididymal weight and sperm number in the caudal epididymis (Aleem et al., 2006). The ICI+PPT group in the present study showed decreased testis and epididymal weights as well as a reduced sperm number in the caudal epididymis. These results show that a high concentration of PPT agonist did not reverse damage to the testis induced by ICI antagonist but rather furthered the formation of abnormalities in the epididymis.

Luminal areas of seminiferous tubules increased in both treatment groups compared to that of control, whereas seminiferous tubular diameter decreased in the ICI+PPT group. Since the germinal epithelium was a little sloughed or atrophied, luminal areas of seminiferous tubules increased. Some tubules were atrophied by ICI treatment and showed abnormal spermatogenesis (Cho et al., 2003). However, treatment with 4 mg of PPT for 8 weeks has been shown to inhibit spermatogenesis in testis, resulting in lack of sperm in the caudal epididymis of adult mice (Lee & Cho, 2009). In

the present study, spermatogenesis was observed, and sperm was concentrated in the epididymal caudal region of the ICI and ICI+PPT groups. The difference between our results and those of the previous study could be attributed to the concentration of PPT, which was 0.1 mol PPT biweekly for 10 weeks (in the present study) compared to 4 mg (0.126 mol) of PPT weekly for 8 weeks (Lee & Cho, 2009). Although alternating treatment of ICI and PPT in the present study may have altered spermatogenesis in the testis compared to PPT treatment alone, the mechanism of action of ICI+PPT treatment remains uncertain.

Cell heights of efferent ductules and the initial segment of the epididymis were significantly reduced in the two treatment groups. Generally, epithelial cell height of efferent ductules and preputial gland is reduced by ICI (Cho et al., 2003) or PPT treatment (Han & Cho, 2009; Lee & Cho, 2009; Han et al., 2011). In efferent ductules, ER plays key roles in the regulation of fluid reabsorption, and ER helps to maintain fluid osmolality and pH in the epididymis (Hess et al., 2011). Therefore, in ERKO mice or ICI-treated mice, fluid reabsorption is inhibited, resulting in swollen luminal areas and decreased epithelial cell heights of ductules and epididymis. Further, a high concentration of PPT reduces cell height in ductules similar to ICI treatment (Lee & Cho, 2009). However, the mechanism by which PPT treatment reduces cell height remains unknown. An abundance of PAS-positive granules was observed in the supranuclear cytoplasm of non-ciliated cells of efferent ductules as well as principal cells of the initial segment in the control group. Granules are identified as lysosomes in relation to reabsorption of fluid (Cho et al., 2003). In the present study, the number of granules was lower in the ICI+PPT group compared to the ICI group, possibly due to reduced reabsorption in epithelial cells lining the efferent ductule tubules and initial segment.

Similar to ICI administration, a high concentration of PPT (4 mg) resulted in luminal dilation as well as reduced epithelial cell height in the efferent ductules (Han et al., 2011). By combining previous research and the present study, we observed complex damage to the weights and epithelial cell heights of reproductive organs by ICI with PPT treatment instead of a PPT recovery effect.

CONCLUSIONS

ER antagonist ICI added with ER agonist PPT group caused the severe histological changes in luminal area of seminiferous tubules, cell height of efferent ductules and initial segment, and sperm numbers comparing with ICI treatment group. These results indicate that a high concentration of PPT together with ICI induces histological abnormalities instead of histological restoration in reproductive organs including testis, efferent ductule and epididymis.

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