# Modification of Gene Expression of Connexins in the Rat Corpus Epididymis by Estradiol Benzoate or Flutamide Exposure at the Early Neonatal Age

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**ABSTRACT :** Cell-cell direct communication through channel-forming molecules, connexin (*Cx*), is essential for a tissue to exchange signaling molecules between neighboring cells and establish unique functional characteristics during postnatal development. The corpus epididymis is a well-known androgen-responsive tissue and involves in proper sperm maturation. In the present research, it was attempted to determine if expression of *Cx* isoforms in the corpus epididymis in the adult is modulated by exposure to estrogenic or anti-androgenic compound during the early postnatal period. The neonatal male rats at 7 days of age were subcutaneously injected by estradiol benzoate (EB) at low-dose (0.015 µg/kg body weight) or high-dose (1.5 µg/kg body weight) or flutamide (Flu) at low-dose (500 µg/kg body weight) or high-dose (50 mg/kg body weight). The corpus epididymis collected at 4 months of age was subjected to evaluate expressional changes of *Cx* isoforms by quantitative real-time PCR. Treatment of low-dose EB resulted in increases of *Cx32, Cx37*, and *Cx45* transcript levels, while exposure to high-dose EB decreased expression of *Cx26, Cx30.3, Cx31, Cx31.1, Cx32, Cx40, Cx43*, and *Cx45*. Treatments of Flu caused significant decreases of expression of all examined *Cx* isoforms, except *Cx37* and *Cx43* shown no expressional change with high-dose Flu treatment. These findings imply that expression of most *Cx* isoforms present in the corpus epididymis would be transcriptionally regulated by actions of androgen and/or estrogen during postnatal period.

**Key words :** Connexin, Estradiol benzoate, Flutamide, Gene expression, Corpus epididymis

## INTRODUCTION

The epididymis plays important roles in male reproduction, including sperm maturation, transport, and storage (Robaire & Hermo, 1988). The epididymis has a tubular structure covered by an epithelial layer, consisting of several cell types (Arrotéia et al., 2012). Except the initial segment at the most proximal region of the epididymal region, the epididymis is divided into three parts, caput (head), corpus (body), and cauda (tail) epididymis, depending on functional, morphological, and molecular biological characteristics (Robaire & Hermo, 1988). The most predominant type of the epithelial cells in the epididymis is principal cells which are columnar shape with stereocilia protruding into the lumen (Robaire & Hermo, 1988). The number of principal cells gradually decreases from the caput to the cauda epididymis (Robaire & Hermo, 1988). However, the epithelial content of narrow cells increases from the caput to the corpus epididymis, while clear cells are relatively equally distributed through the entire epididymis (Arrotéia et al., 2012). It is generally considered that different cell types in the epididymis play specific functions on

the establishment of unique epididymal microenvironment (Robaire et al., 2006). Thus, it is reasonable to consider that the different ratios of cell population in the epididymal segment would partly contribute into creation of discrete functional epididymal unit.

Exchanges of signaling molecules, ions, and even RNAs through connexin (*Cx*)-based gap junction between neighboring cells is a type of direct cell-cell communication ways (Goodenough et al., 1996). There are at least 20 *Cx* isoforms found in mammals (Meşe et al., 2007). The gap junction is composed of a complex of two hemichannels, which each is made of six *Cx* isoforms (Cyr, 2011). The presence of *Cx* isoforms in the male reproductive tract has been examined by several researchers, including our recent findings. Expression of *Cx* transcripts in the reproductive excurrent duct has been detected in the initial segment (Seo et al., 2010) and epididymis (Han & Lee, 2013; Lee, 2013). Even though expressional pattern of each *Cx* is quite distinguishable from each other isoform during postnatal development, there are nine *Cx* isoforms expressing throughout the entire epididymis, including *Cx26*, *Cx30.3*, *Cx31*, *Cx31.1*, *Cx32*, *Cx37*, *Cx40*, *Cx43*, and *Cx45* (Han & Lee, 2013; Lee, 2013). Expression of certain *Cx* isoform in the epididymis shows the segment-specific patterns (Han & Lee, 2013; Lee, 2013). For example, the highest levels of *Cx40* are detected at 45 days of age in the caput epididymis, at early than 45 days of age in the corpus epididymis, and 5 months of age in the cauda epididymis (Han & Lee, 2013; Lee, 2013). It is difficult to generalize expressional patterns of *Cx* isoforms in the epididymis during postnatal period because expression of each *Cx* isoform shows a unique pattern in each epididymal segment. Each *Cx* isoform possesses particular permeability and gating behavior to specific signaling molecules (Bruzzone et al., 1996). Therefore, it is logic to consider that expression of various *Cx* isoforms at different levels in the epididymal segments would be necessary to communicate

different cell types and thus to produce distinct microenvironment within the epididymal part. Even though a few researches show immuno-localization of some *Cx* isoforms at specific cell types within the epididymis (Cry, 2011), the function of *Cx* isoforms has not been clearly demonstrated.

Expressional regulation of *Cx* isoforms in the epididymis has not been extensively examined, in spite of the presence of strong evidence demonstrating expressional modulation of a number of genes in the epididymis by estrogens and androgens. Dubé et al (2012) have showed possible regulation of *Cx43* expression in the human cauda epididymis by epidermal growth factor. Our previous researches have demonstrated that exposure to estradiol benzoate (EB) or flutamide (Flu) at the early or weaning postnatal age results in altered expression of several *Cx* isoforms in the initial segment of the adult rat epididymis (Lee, 2014; 2015). However, scientific research showing hormonal factor(s) giving an influence on *Cx* expression in the corpus epididymis is very rare. Thus, in accordance with our earlier studies (Lee, 2014; 2015), the present research was attempted to reveal the effect of EB, an estrogenic agonist, or Flu, an antiandrogenic agent, on expression of *Cx* isoforms in the corpus epididymis. Expressional changes of *Cx* isoforms in the adult corpus epididymis followed by the subcutaneous expose to these steroidal compounds at 7 days of postnatal age were evaluated by quantitative realtime PCR analyses.

## MATERIALS AND METHODS

### 1. Preparation and treatment of estradiol benzoate (EB) and flutamide (Flu)

The EB and Flu were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). The powder of EB or Flu was dissolved in 100% EtOH at room temperature. The final stock solutions of EB and Flu were prepared with a series

of dilution in peanut oil.

A total of five pregnant Spragure Dawley rats were obtained from Samtako (OSan, Korea). Each female rat was individually kept during entire experimental period. Free access to drinking water and food was allowed. Each rat was randomly assigned to one of five experimental groups, including control (peanut oil), low-dose EB treated (EB-L), high-dose EB treated (EB-H), low-dose Flu treated (Flu-L), or high-dose Flu treated (Flu-H) group. Each pregnant rat delivered 5 to 8 male pups, which were treated with EB or Flu at 7 days of postnatal age. Subcutaneous injection of EB or Flu was carried out at the final concentration of 0.015 μg (EB-L) or 1.5 μg (EB-H) per kilogram of body weight (/kg BW) for EB or 500 μg (Flu-L) or 50 mg (Flu-H)/kg BW for Flu. Number of animals for each experiment group are as following; control (n=5), EB-L (n=7), EB-H (n=8), Flu-L (n=7), and Flu-H (n=7). The present study was carried out in accordance with the guide for the care and use of laboratory animals of National Research Council in S. Korea.

## 2. Total RNA and cDNA preparation

The epididymis was collected from the experimental animals at 4 months of age. First, the animals were anesthetized by $CO_2$ stunning in a closed chamber. The testis and epididymis were exposed through a lower abdominal incision. In cold PBS, the epididymis was separated from the testis and further dissected into initial segment, caput epididymis, corpus epididymis, and cauda epididymis. The epididymal parts were washed in fresh cold PBS for a couple of times and frozen in liquid nitrogen.

Total RNA from the corpus epididymis was harvested with 0.5 mL of easy-Blue total RNA extraction solution (iNtRON Biotechm Seongnam, Korea). Tissue was first homogenized in the solution with a polytron homogenizer (Fisher Scientific, Pittsburgh, USA). After then, phenol-chloroform extraction protocol was applied to obtain a pellet of total RNA. Total RNA was resuspended in RNase-free $dH_2O$, and purity and concentration of total RNA were determined spectrophotometrically (Eppendorf, New York, USA).

Reverse transcription (RT) reactions to generate the first strand of cDNAs were carried out with oligo-dT primer and ImProm-II™ reverse transcription system (Promega, Madison, USA). One microgram of total RNA was utilized as a template, and RT reactions were performed at 25°C for 5 min, 42°C for 1 hr and 30 min, and 70°C for 15 min.

## 3. Quantitative real-time PCR

One microliter of 20 μL RT reaction mixtures was used to carry out real-time PCR. The PCR cocktail included 10 pmol of primer set, 10 μL of master mixture (Finnzymes, Espoo, Finland), and DNase-free water to make a final volume of 20 μL. The CFX96™ real-time PCR detection system (Bio-rad, Hercules, USA) was used for real-time PCR analysis. The specificity of each PCR was determined by the melting curve analysis. The following primers were used for quantitative real-time PCR. *Cx30.3* (NM_053984) primers: forward: CCCAATGTCTGCTATGACGA; reverse: CACAGCAGCCTTGAAGATGA, *Cx31* (NM_019240) primers: forward: TTGAGCGGTGTGAACCAGTA; reverse: TGTTGGAGATGGGGAAGAAG, *Cx31.1* (NM_019241) primers: forward: CATCGTCTGCATCCTGCTTA; reverse: ATGAGGTCGCTTGAGAGGAA, *Cx32* (NM_017251) primers: forward: AGAATCATGGTGCTGGTGGT; reverse: CCTCAAGCCGTAGCATTTTC, *Cx37* (NM_021654) primers: forward: AGTGTCTGTACCTTGGATGCC; reverse: CAGCACACTTAGCCAAGAGC, *Cx40* (NM_019280) primers: forward: ATACCATTCAGCCTGGTTGC; reverse: CGGCCTCTTTAGCTTTCTCA, *Cx43* (NM_012567) primers: forward: AGCAAGCTAGCGAGCAAAAC; reverse: GAG TTCATGTCCAGCAGCAA, *Cx45* (NM_001085381) primers: forward: GATCATCCTGGTTGCTACTC; reverse: GATC CTCTTCATGGTCCTCT, and cyclophilin A (*Ppia*) primers:

forward: GGCAAATGCTGGACCAAACAC; reverse: TT AGAGTTGTCCACAGTCGGAGATG. The PCR was performed at a cycle of 95°C for 30 sec and cycles of denaturation at 95°C for 30 sec, annealing at melting temperature for 30 sec, and extension at 72°C for 30 sec. Each PCR was completed with an additional extension at 72°C for 30 sec.

### 4. Statistical analysis

Triplicated PCRs were performed for each cDNA, and three to four cDNAs were used for each experimental group. Following densitometric analysis, signal intensities of *Cx* isoforms were normalized to internal controls (*Ppia*). One-way ANOVA was carried out to check significant differences among experimental groups, and Duncan's test, a post-hoc analysis, was performed to determine statistical significances between control and EB-treat or Flu-treated groups. When *P* value was lower than 0.05, it was considered to be significant.

## RESULTS

### 1. Expressional modification of *Cx26* and *Cx30.3* in the corpus epididymis exposed to EB or Flu at 7 days of postnatal age

Expression of *Cx26* in the adult corpus epididymis was not changed by treatment of EB at low dose (Fig. 1A). However, compared with that of control, the treatment of high-dose EB resulted in a significant decrease of *Cx26* transcript level in the adult corpus epididymis (Fig. 1A). Interestingly, regardless of dose of administration, the treatments of Flu at 7 days of age caused significant decreases of *Cx26* expression in the adult corpus epididymis (Fig. 1A). Similar findings were observed in expression patterns of *Cx30.3* (Fig. 1B). Even though there was no expressional change of *Cx30.3* by low-dose EB treatment at 7 days of age, a significant decrease of *Cx30.3* transcript
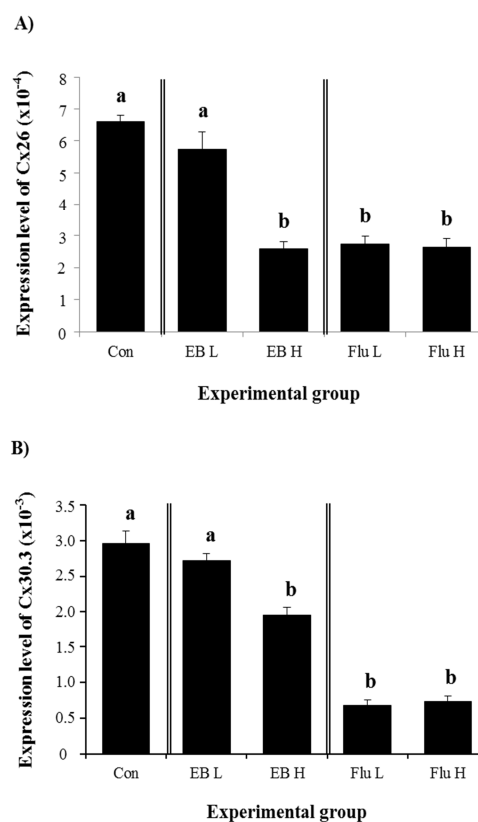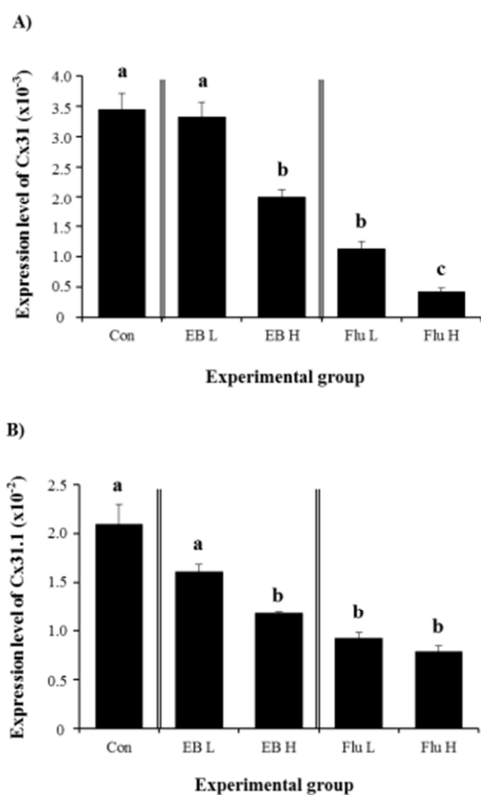


**Fig. 1. Expression patterns of *Cx26* and *Cx30.3* in the corpus epididymis of adult rat.** Seven-days old of male rats were treated with either estradiol benzoate at low dose (EB L, 0.015 μg/kg BW) or high dose (EB H, 1.5 μg/kg BW) or flutamide at low dose (Flu L, 500 μg/kg BW) or high (Flu H, 50 mg/kg BW) dose. Statistical differences among experimental groups on transcript levels of *Cx26* (A) or *Cx30.3* (B) at $p < 0.05$ level are shown in different letters

level in the adult corpus epididymis was detected by high-dose EB treatment at 7 days of postnatal age (Fig. 1B). Treatment of Flu at low- or high-dose at seven-day of old resulted in significant decrease of *Cx30.3* expression in the adult corpus epididymis (Fig. 1B).

### 2. Expressional modification of *Cx31* and *Cx31.1* in the corpus epididymis exposed to EB or Flu at 7 days of postnatal age

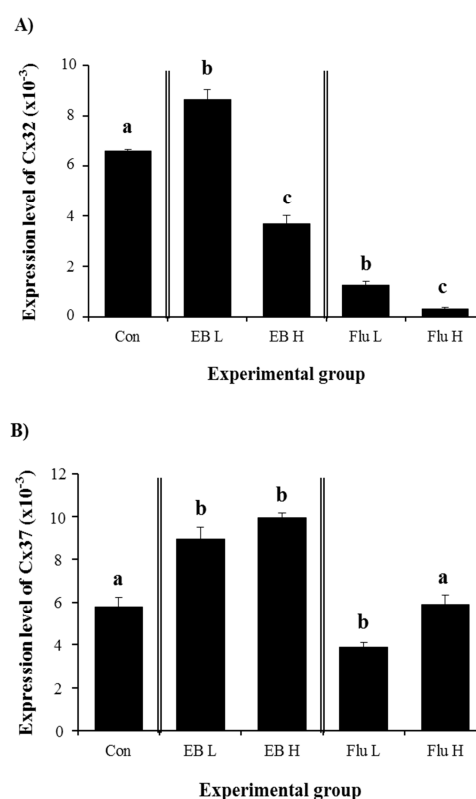Treatment of low-dose of EB at 7 days of age didn't

A)



B)



**Fig. 2. Expression patterns of *Cx31* and *Cx31.1* in the corpus epididymis of adult rat.** Seven-days old of male rats were treated with either estradiol benzoate at low dose (EB L, 0.015 μg/kg BW) or high dose (EB H, 1.5 μg/kg BW) or flutamide at low dose (Flu L, 500 μg/kg BW) or high (Flu H, 50 mg/kg BW) dose. Statistical differences among experimental groups on transcript levels of *Cx31* (A) or *Cx31.1* (B) at $p < 0.05$ level are shown in different letters

give an influence on transcription of *Cx31* in the corpus epididymis at the adult (Fig. 2A). However, a decrease of *Cx31* transcript level was detected by high-dose EB treatment in the corpus epididymis (Fig. 2A). Exposure to low-dose Flu at 7-days old of age resulted in a significant decrease of *Cx31* expression in the corpus epididymis (Fig. 2A). A further decrease of *Cx31* expression was found in a treatment of high-dose Flu (Fig. 2A). Administration of EB at a low-dose at 7 days of age didn't cause aberrant expression of *Cx31.1* in the adult corpus epididymis, even though treatment of high-dose EB led to a significant de-

crease of *Cx31.1* expression (Fig. 2B). Treatments of Flu at two doses resulted in significant decreases of *Cx31.1* transcript levels, compared with that of control group (Fig. 2B)

## 3. Expressional modification of *Cx32* and *Cx37* in the corpus epididymis exposed to EB or Flu at 7 days of postnatal age

A significant increase of *Cx32* expression was found in the corpus epididymis treated with low-dose of EB at 7 days of age (Fig. 3A). However, treatment of high-dose
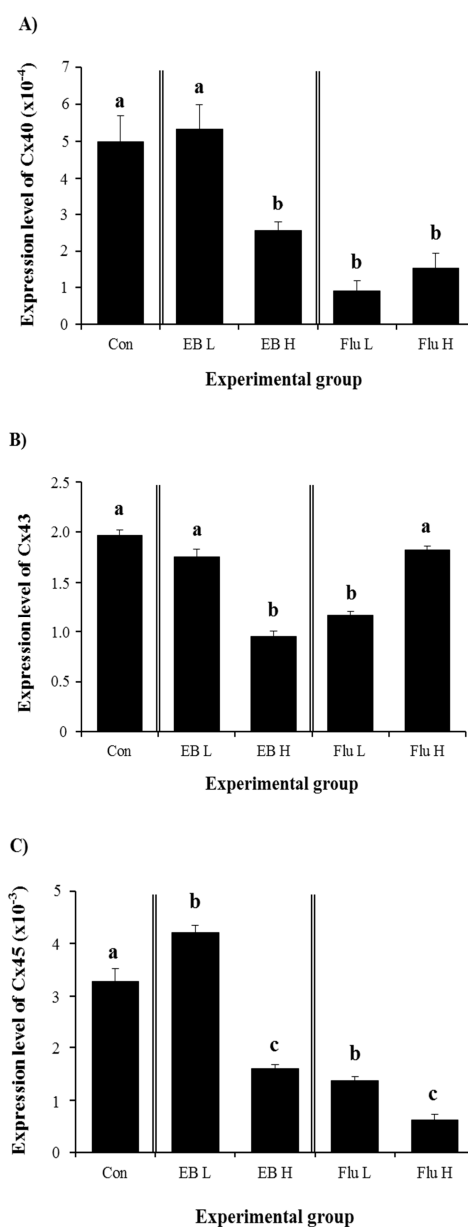
A)



B)



**Fig. 3. Expression patterns of *Cx32* and *Cx37* in the corpus epididymis of adult rat.** Seven-days old of male rats were treated with either estradiol benzoate at low dose (EB L, 0.015 μg/kg BW) or high dose (EB H, 1.5 μg/kg BW) or flutamide at low dose (Flu L, 500 μg/kg BW) or high (Flu H, 50 mg/kg BW) dose. Statistical differences among experimental groups on transcript levels of *Cx32* (A) or *Cx37* (B) at $p < 0.05$ level are shown in different letters

EB caused a transient decrease of *Cx32* transcript level in the adult corpus epididymis (Fig. 3A). A great decrease of *Cx32* expression was detected with low-dose Flu treatment (Fig. 3A). Expression of *Cx32* in the adult corpus epididymis was barely detected with a treatment of high-dose Flu (Fig. 3A). Interestingly, treatments of EB at 7 days of age caused expressional increases of *Cx37* in the adult corpus epididymis (Fig. 3B). However, an administration of low-dose Flu resulted in a significant decrease of *Cx37* expression, while there was no change of *Cx37* transcript level with high-dose Flu treatment (Fig. 3B).

### 4. Expressional modification of *Cx40*, *Cx43*, and *Cx45* in the corpus epididymis exposed to EB or Flu at 7 days of postnatal age

Expressional change of *Cx40* in the adult corpus epididymis after exposure to EB at 7 days of age was only detected with high-dose EB treatment (Fig. 4A). Treatment of Flu at low- or high-dose resulted in significant decrease of *Cx40* transcript level, compared with that of control (Fig. 4A). A significant decrease of *Cx43* transcript level was observed with high-dose EB treatment, while no change of *Cx43* expression was found in a treatment of low-dose EB treatment (Fig. 4B). Treatment of low-dose Flu at 7 days of age resulted in a significant decrease of *Cx43* expression in the adult corpus epididymis (Fig. 4B). But, expression of *Cx43* in the corpus epididymis was not influenced by high-dose Flu treatment (Fig. 4B). Expression of *Cx45* in the adult corpus epididymis was significantly increased by low-dose EB treatment but was significantly decreased by high-dose EB treatment (Fig. 4C). Even though Flu treatments caused significant decreases of *Cx45* expression in the corpus epididymis, a further significant decrease of *Cx45* transcript level was detected with high-dose treatment of Flu (Fig. 4C).



**Fig. 4. Expression patterns of *Cx40*, *Cx43*, and *Cx45* in the corpus epididymis of adult rat.** Seven-days old of male rats were treated with either estradiol benzoate at low dose (EB L, 0.015 µg/kg BW) or high dose (EB H, 1.5 µg/kg BW) or flutamide at low dose (Flu L, 500 µg/kg BW) or high (Flu H, 50 mg/kg BW) dose. Statistical differences among experimental groups on transcript levels of *Cx40* (A), *Cx43* (B), or *Cx45* (C) at $p < 0.05$ level are shown in different letters

# DISCUSSION

Disruption of hormonal homeostasis by exogenous admini-stration of EB or Flu to male commonly develops abnormal expression of a number of genes related with functions of the epididymis (Gorowska et al., 2014; Kaushik et al., 2010). In addition, neonatal exposure of steroidal compounds often results in permanent changes of male reproductive function at the adult (Goyal et al., 2003). The present study was designed to determine the effect of EB or Flu treatment at neonatal age on expression of *Cx* isoforms in the corpus epididymis of adult rats.

It is likely that treatment of low-dose EB at neonatal age doesn't greatly affect expression of most *Cx* isoforms in adult corpus epididymis, except *Cx32*, *Cx37*, and *Cx45*, which were significantly increased. These findings were somewhat different from those observed in the initial segment (IS), which treatment of low-dose EB caused significant increases of *Cx30.3*, *Cx31.1*, and *Cx43* expression but no other *Cx* isoforms (Lee, 2014). These results suggest that expression of *Cx* isoform in the IS and corpus epididymis is differentially regulated by low-dose EB. The level of estrogen receptor (ER) α along the epididymis varies with the highest at the corpus than other epididymal regions (Hess et al., 2011). Such differential levels of ERα among the epididymal segments would contribute to differ-rent expressional patterns of *Cx* isoforms induced by low-dose EB treatment. It is also possible that responsiveness to low-dose EB on expression of *Cx* isoform would be different among epididymal regions. At this moment, the current findings couldn't provide firm evidence to answer such differential expression of *Cx* isoforms by low-dose EB treatment between the IS and corpus epididymis. Molecular dissection of promoter regions of *Cx* isoforms would offer a possibility to understand how such differential responsiveness on expression of *Cx* isoforms to low-dose EB occurs within different epididymal regions.

Exposure to high-dose EB at the neonatal age generally led to significant decreases of *Cx* isoforms in adult corpus epididymis, except *Cx37* with a significant increase. These results relatively differ from those detected in the IS, which expression of *Cx* isoform in the adult after neonatal high-dose EB treatment didn't show any specific pattern (Lee, 2014). Aberrant expression of *Cx* isoform by high-dose EB would be partly explained by differential respon-siveness of *Cx* genes at the dose of EB. From the findings from current study, it is generally considered that treatment of EB at 1.5 μg /kg BW at 7 days of age is sufficient to suppress expression of most *Cx* isoforms in adult corpus epididymis, even though an exception is *Cx37*. One might think that the treatment of high-dose EB at neonatal age has a disruptive effect on general hormonal balance and thus causes expressional decreases of most *Cx* isoforms. But, because expression pattern of *Cx37* is not the case, it is more thoroughly considered that expressional regulation of Cx37 by EB would differ from other *Cx* isoforms in the corpus epididymis.

Administration of low-dose Flu at 7 days of age resulted in significant expressional decreases of all *Cx* isoforms in adult corpus epididymis. Even though the same treatment of Flu results in similar results in the IS, expression of some *Cx* isoforms in the IS are not changed or signifi-cantly increased (Lee, 2014). These conflict results observed between the IS and corpus epididymis would be fairly explained by segment-specific expressional regulation of some *Cx* isoforms by low-dose Flu. The level of androgen receptor (AR) in the corpus epididymis is the lowest among epididymal segments (Pujol & Bayard, 1979). As an antiandrogenic compound, the Flu acts on suppression of AR signaling pathway. Thus, it is reasonable to think that expression of *Cx* isoforms in the corpus epididymis is up-regulated by androgen. And, disruption of hormonal homeostasis by exogenous exposure of an antiandrogen at the neonatal age could result in permanent defect on

expression of *Cx* isoforms in the adult. Additional researches are suggested to resolve androgenic regulation on expression of *Cx* isoforms in the corpus epididymis.

Expression of *Cx* isoforms in adult corpus epididymis after high-dose Flu treatment at neonatal age is somewhat unusual. As might expected, expression of most *Cx* isoforms is significantly lower than the control or further decreased than that with low-dose Flu treatment. However, the levels of *Cx37* and *Cx43* do not differ from those of control. These observations are surprisingly and are not simply answered with the findings obtained from present research. A possible suggestion would be addressed from negative dose-dependent expressional regulation of genes. Also, it is considerable that high-dose Flu could influence different signaling pathway at which low-dose Flu does not give an impact. But, it is clear that expressional regulation of *Cx37* and *Cx43* by androgen is more complicate than expected. More detailed examination is required for providing defined data on *Cx37* and *Cx43* expression in the corpus epididymis.

In conclusion, the present research has clearly demonstrated that neonatal exposure to EB or Flu results in aberrant expression of *Cx* isoforms in adult corpus epididymis. The epididymis is a major site for sperm maturation, and *Cx* is particularly important for communicating between different cell types, such as the epididymis. Even though it is not considered at the present research, additional investigations are suggested to examine the effect of EB or Flu treatment at neonatal age on male fertility.

## ACKNOWLEDGEMENT

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