

## Effect of an Anabolic Steroid, Nandrolone Decanoate, on Aquaporin 1 and 9 Gene Expression in the Rat Epididymis

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### ABSTRACT

The epididymis in the male reproductive tract is the site where spermatozoa produced from the testis become mature. The epididymis is divided into 4 different segments, initial segment and caput, corpus, and caudal epididymis, depending upon functional and morphological features. Aquaporins (Aqps) are water channel molecules, which are present in the epididymis and play a major role in removal of epididymal water, resulting in creation of microenvironment for sperm maturation and concentration of sperms. Nandrolone decanoate (ND) is a synthetic anabolic-androgenic steroid, which is used to treat clinical diseases and improve physical ability and appearance. Even though it is well determined that the ND causes the male infertility by affecting the testis, little is known the effect of the ND on the epididymis. The present study was focused to examine the effect of ND at different treatment doses and periods on expression of Aqp1 and Aqp9 genes in the epididymis of pubertal rats. Results showed that mRNA expression of Aqp1 and Aqp9 genes among the parts of the epididymis was differentially regulated by ND treatment doses. In addition, treatment periods of ND caused differential expression of Aqp1 and Aqp9 mRNAs among segments of the epididymis. Therefore, it is believed that male infertility induced by ND could be resulted not only from malfunction of the testis but also from aberrant gene expression of Aqp1 and Aqp9 in the epididymis.

(Key words : Epididymis, Aquaporin, Nandrolone decanoate, Anabolic steroid, Male reproduction)

### INTRODUCTION

The male reproduction is regulated and maintained by the action of a complex of various factors, including sex steroid and peptide hormones. Sperms produced from the testis acquire fertilizing capacity throughout travelling excurrent ducts of the male reproductive tract, including efferent ductules and epididymis (Cornwall, 2009). The epididymis has a coiled tubular structure, divided into 4 morphologically and histologically distinct regions: initial segment (IS), caput epididymis, corpus epididymis, and caudal epididymis (Cosentino and Cockett, 1986). Epithelial cells in the epididymis create the micro-environment for proper maturation of spermatozoa. The fluid in each region of the epididymis harbors a distinct micro-milieu which is established by functions of numerous molecules (Cosentino and Cockett, 1986; Cornwall, 2009).

An important function of the epididymis is fluid reabsorption of the testicular fluid (Cornwall, 2009). Aquaporins (AQPs) are a family of small proteins which are integrated in the plasma membrane and mainly involve in water transport across the membrane by osmotic gradients (Ishibashi *et al.*, 2008). Up to date, 13 AQPs have been identified in mammalian (Da Silva *et al.*, 2006). The physiological function and distribution of AQPs in human and animal tissues have been extensively examined from a number of researches (Da Silva *et al.*, 2006; Huang *et al.*, 2006; Kruse *et al.*, 2006). It is believed that AQPs involve in fluid reabsorption in the epididymis, resulting not only in concentration of spermatozoa but also in maintenance of micro-environment for proper sperm maturation (Huang *et al.*, 2006). The presence of multiple AQPs has been recognized in the male reproductive tract, including testis, excurrent ducts, and accessory glands (Badran and Hermo, 2002; Domeniconi *et al.*, 2008;

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Hermo *et al.*, 2004). Recent studies have found that at least 8 AQPs (AQP 0, 1, 2, 3, 5, 8, 9, and 10) have been detected in differential patterns of segmental and cellular localization in the epididymis (Da Silva *et al.*, 2009; Hermo *et al.*, 2004; Huang *et al.*, 2006). Others have demonstrated that expression of AQPs, especially AQP1 and AQP9, in the epididymis is regulated by androgen and testicular factor(s) (Badran and Hermo, 2002; Oliveira *et al.*, 2005; Pastor-Soler *et al.*, 2002; Shayu *et al.*, 2005).

Anabolic-androgenic steroids (AASs) are structurally testosterone-derived synthetic hormones, which possess a strong anabolic effect, as well as androgenic effect (Chrousos, 2006). The AASs are frequently employed for treatment of various clinical diseases. Of commercially available AASs, nandrolone decanoate (ND) is commonly used to treat a number of diseases, such as anemia, kidney failure, breast cancer, growth deficiency, and HIV-associated muscle loss (Basaria *et al.*, 2001; Gurnfeld *et al.*, 2006; Mulligan and Schambelan, 2002; Ranke and Bierich, 1986). In addition, the ND is often used for a testosterone replacement therapy in the old (Dohle *et al.*, 2003), due to its very low conversion rate to dihydrotestosterone (DHT), a potent androgen (Basaria *et al.*, 2001). A recent study shows an increased rate of ND misuse among the public and athletes to improve athletic performance and physical appearance by increasing muscle content (Sjöqvist *et al.*, 2008). Many researchers have revealed that inappropriate use of ND results in a number of side effects, such as increases of thyroid gland and liver weights (Fortunato *et al.*, 2006; Vieira *et al.*, 2008) and a decrease of male fertility (Karbalay-Doust and Noorafshan, 2006; Takahashi *et al.*, 2004). A prolonged use with ND causes atrophy of Leydig cells, degeneration of seminiferous tubules and a decrease of sperm numbers in the testis, and eventually leads to male infertility (Karbalay-Doust and Noorafshan, 2006; Koeva *et al.*, 2003; Takahashi *et al.*, 2004). Even though the effect of ND on the testis of the male reproductive tract has been well documented, little information is available for the effect of ND on the epididymis.

In the present study, we attempted to determine the effect of ND on the epididymis, in aspect of expression of AQP1 and AQP9 genes. In addition, we examined if different periods and doses of ND treatment influence expression of AQP1 and AQP9 genes in the epididymis.

## MATERIALS AND METHODS

### Animals and Treatment

Male Sprague Dawley rats at 23 days of age were obtained from Samtako (O San, S. Korea) and individually housed under controlled conditions and given *ad libitum* food and water for whole experimental period.

Rats were randomly divided into 2 experimental groups, short treatment (2 weeks) and long treatment (12 weeks) of nandrolone decanoate (ND). These experimental animals were further divided into 3 subgroups for each treatment period, control (olive oil), low ND (2 mg ND/kg body weight/week), and high ND (10 mg ND/kg body weight/week). Thus, the present study had 6 experimental groups, having 5–6 rats for each group. Subcutaneous injection of ND was began at 30 days of age and continued for proper treatment periods, 2 or 12 weeks. Physical condition and movement of experimental animals were monitored every day. The 2 doses of ND used for the present study were lower than the therapeutic doses, usually 25–100 mg given for treatments of debilitating illness and postmenopausal breast carcinoma and 50 mg given for treatment of postmenopausal osteoporosis (Karbalay-Doust S, Noorafshan A, 2006).

### Collection of Tissues and RNA Isolation

At the end of ND injection, rats were anesthetized by CO<sub>2</sub> stunning and male reproductive tracts were isolated. The epididymis was separated from the rest of male reproductive tract and further dissected into 4 parts, initial segment (IS, head part of caput epididymis), caput (head), corpus (body), and caudal (tail) epididymis. Tissues were briefly washed in cold PBS buffer, quickly frozen in liquid nitrogen, and stored in –80°C. Total RNAs were isolated from tissues using easy-Blue total RNA extraction solution (iNtRON Biotech, Sungnam, S. Korea) and a polytron homogenizer (Fisher Scientific, Pittsburgh, USA). The RNA pellets were dissolved in RNA storage buffer (Ambion, Austin, USA) and kept in –80°C freezer until used for reverse-transcription (RT) reaction. The purity and yield or the qualities of total RNAs were determined by an UV spectrophotometer (Eppendorf, New York, USA) or gel electrophoresis, respectively.

### cDNA Synthesis and Real-Time Polymerase Chain Reaction (Real-Time PCR)

The RT reaction was carried out according to the instruction in ImProm-II<sup>TM</sup> reverse transcription system (Promega, Madison, USA). Briefly, 1 µg of total RNA was used for RT reaction in total volume of 20 µl with oligo-dT primer. RT reactions were carried out at 25°C for 5 min, 42°C for 1 hr, and 70°C for 15 min. The real-time PCR was performed in a mixture of 1 µl of cDNA, 0.75U of GoTaq polymerase (Promega, Madison, USA), 5 µl of 5X buffer, 0.2 mM of dNTPs (Promega, Madison, USA), 2.5 µl of 3000X SYBR Green (BMA, Rockland, USA), and 10 pmol of each primer. A total volume of the mixture for real-time PCR was 25 µl. The PCR program employed an initial step of pre-denaturation at 95°C for 5 min, followed by denaturation at 94°C for 30 sec, annealing at T<sub>m</sub> for 30 sec, and extension at 72°C for 30 sec of cycles. The PCR conditions are summarized in

**Table 1. Primer sequences, expected product sizes, and PCR conditions for real-time PCR**

Gene (GenBank access number)	Primer sequence	T <sub>m</sub> (°C)	Expected PCR size (bps)
<i>Aqp1</i> (NM_012778)	F: GTCCACATGGTCTAGCCTTGCTG (983~1017) R: GGGAAGGGTCTGGAGGTAAGTCA (1333~1356)	65	374
<i>Aqp9</i> (AF016406)	F: GAAGCTCGACCCAGACATGAAGG (1055~1078) R: GTAAAAAAGGTCTCTGAGGCTATCCA (1402~1428)	65	374
<i>Ppia</i> (NM_017101)	F: GGCAAATGCTGGACCAAACAC (342~362) R: TTAGAGTTGTCCACAGTCGGAGATG (513~537)	65	196

*Aqp1* : aquaporin 1; *Aqp9* : aquaporin 9; and *Ppia* : cyclophilin A.

Numbers in parentheses of primer sequences indicate the positions of bases in GenBank sequences.

Table 1. Oligonucleotide primers for PCR were prepared by utilizing published information. The final extension at 72°C for 10 min was carried out for each PCR. The size of PCR products were confirmed by fractionation on 1.2% agarose gels, which were photographed under UV using an image documentation system (Vilber Lourmat, Marne-la-Vallée, France). In the PCR assay, we included *Ppia* (cyclophilin A) as an internal PCR control.

#### Data Analysis

The RT reaction and real-time PCR were repeated at least 3~4 times for each experimental group to obtain a mean and a standard deviation. The expression levels of *Aqp1* and *Aqp9* mRNAs were compared with those of *Ppia* and presented as relative expression ratio between *Aqp* mRNA and *Ppia*. Mean differences among 3 experimental groups in a treatment period were compared using one-way ANOVA, followed by a post-hoc test, Tukey's test. In all cases, results were considered significant when  $p < 0.05$ .

## RESULTS

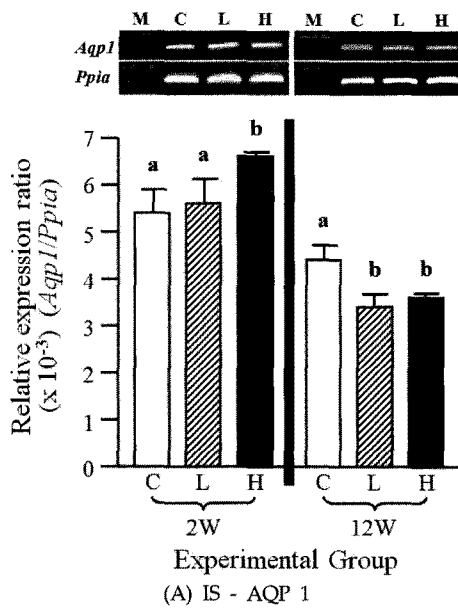
### Expression of *Aqp1* and *Aqp9* in Initial Segment and Caput Epididymis

Expressions of *Aqp1* and *Aqp9* mRNAs were detected in IS and caput epididymis at all experimental groups (Fig. 1 and 2). In the IS, 2 week treatment with a high dose of ND caused a significant increase of *Aqp1* mRNA expression level (Fig. 1A), whereas 12 week treatment of ND resulted in significant decreases of *Aqp1* mRNA levels, regardless doses of ND treated in the present study (Fig. 1A). Unlike *Aqp1*, a significant decrease of *Aqp9* mRNA level was observed from a high dose treatment of ND for 2 weeks (Fig. 1B). However, expression levels of *Aqp9* mRNAs were significantly decreased with ND treatment for 12 weeks (Fig. 1B). In the

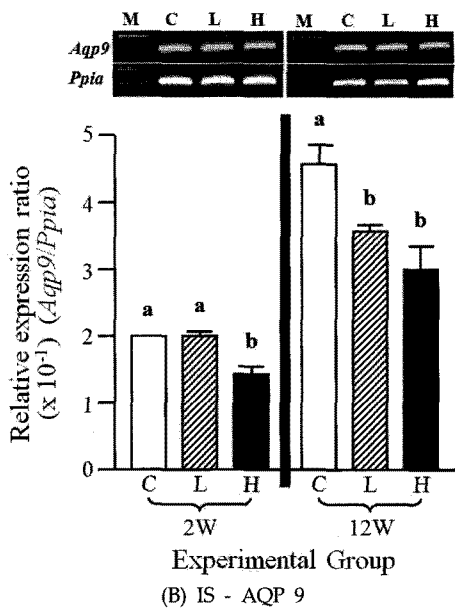
caput epididymis, a low dose ND treatment for 2 weeks led to a significant decrease of *Aqp1* mRNA levels, but a significant increase of *Aqp1* mRNA expression was found with a high dose ND treatment for same period (Fig. 2A). Interestingly, the expression level of *Aqp1* mRNA was not changed by ND treatment for 12 weeks (Fig. 2A). Expression level of *Aqp9* mRNA in the caput epididymis was significantly decreased at a low dose, but not a high dose, ND treatment for 2 weeks (Fig. 2B). Twelve-week treatment of ND caused a significant increase of *Aqp9* mRNA level at a high dose only (Fig. 2B).

### Expression of *Aqp1* and *Aqp9* in the Corpus and Caudal Epididymis

Significant increases of *Aqp1* mRNA levels in the corpus epididymis were found by ND treatment at low and high doses for 2 weeks (Fig. 3A). However, no *Aqp1* mRNA expression at all experimental groups was detected in the corpus epididymis with 12 week ND treatment (Fig. 3A). Expression of *Aqp9* mRNA in the corpus epididymis was significantly reduced by ND treatment for 2 weeks (Fig. 3B). Like *Aqp1* mRNA expression, there was no detectable *Aqp9* mRNA expression with 12-week ND treatment (Fig. 3B). *Aqp1* mRNA expression in the caudal epididymis was significantly decreased by a low dose ND treatment for 2 weeks, and further significant decrease of *Aqp1* mRNA level was found with a high ND dose at same treatment period (Fig. 4A). Treatment with ND for 12 weeks resulted in significant decreases of *Aqp1* mRNA expression levels at both low and high doses (Fig. 4A). *Aqp9* mRNA level was significantly increased with a high dose ND treatment for 2 weeks (Fig. 4B). However, a significant decrease of *Aqp9* mRNA level was detected in the caudal epididymis by a low dose ND treatment for 12 weeks (Fig. 4B). Furthermore, *Aqp9* mRNA level in the caudal epididymis was significantly decreased with a high dose ND treatment, compared with its with a low dose ND treatment (Fig. 4B).



(A) IS - AQP 1

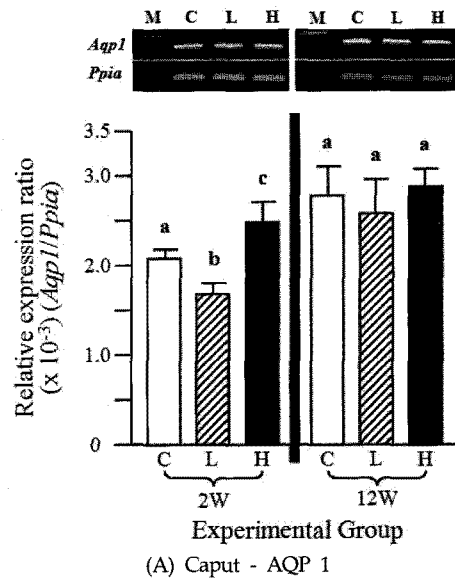


(B) IS - AQP 9

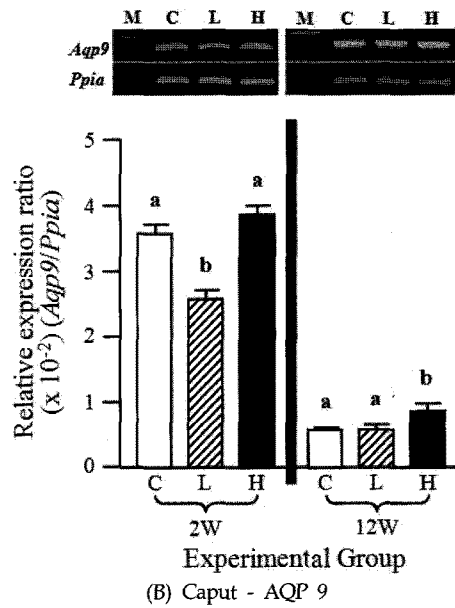
**Fig. 1. Expression of AQP 1 and 9 in the initial segment after nandrolone decanoate treatment.** Expression of *Aqp1* (A) and *Aqp9* (B) mRNA in the IS. C: control, L: low dose of ND treatment (2 mg/kg BW/week), H: high dose of ND treatment (10 mg/kg BW/week), M: 100 bp marker. Cyclophilin A (*Ppia*) was used as an internal control of real-time PCR. Different letters indicate statistically significant at  $p < 0.05$ .

### DISCUSSION

The beneficial effects of ND for treatment of clinical diseases are commonly spoiled by a number of side effects, including impairment of male fertility (Boyadjiev et al., 2000; Monon, 2003). In the testis, the ND affects acti-



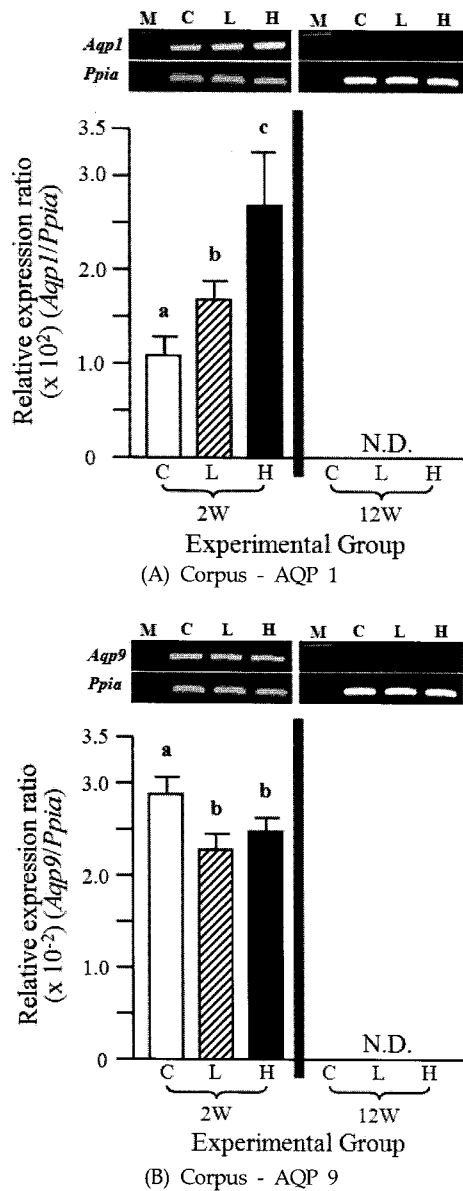
(A) Caput - AQP 1



(B) Caput - AQP 9

**Fig. 2. Gene expression of AQP 1 and 9 in the caput epididymis after nandrolone decanoate treatment.** Expression of *Aqp1* (A) and *Aqp9* (B) mRNA in the caput epididymis. C: control, L: low dose of ND treatment (2 mg/kg BW/week), H: high dose of ND treatment (10 mg/kg BW/week), M: 100 bp marker. Cyclophilin A (*Ppia*) was used as an internal control of real-time PCR. Different letters indicate statistically significant at  $p < 0.05$ .

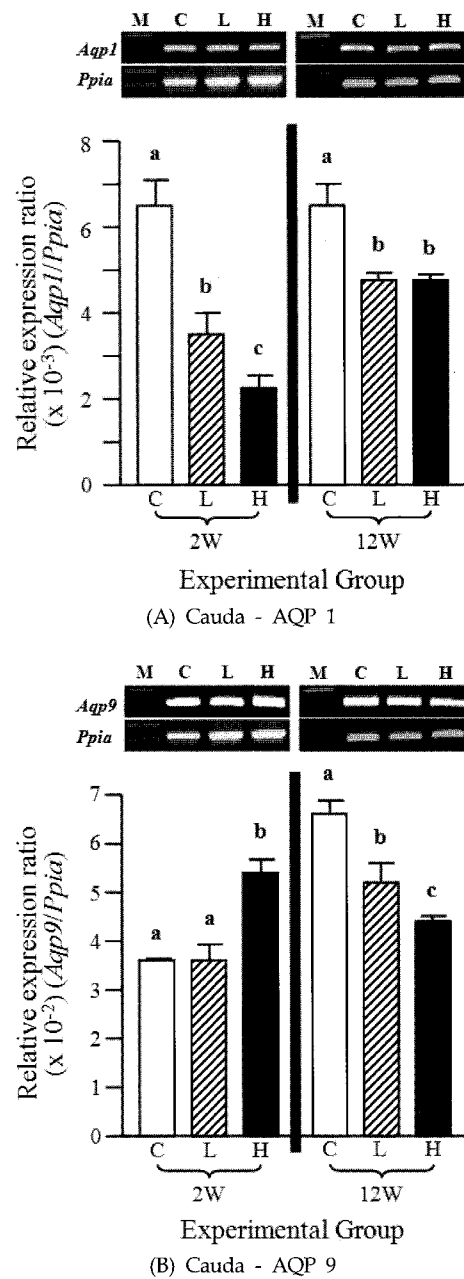
vity of 3 $\beta$ -hydroxysteroid dehydrogenase in Leydig cells (Koeva et al., 2003). In addition, the ND causes the arrest of spermatogenesis and destruction of the seminiferous tubules and a depletion of Leydig cells (Nagata et al., 1999; Noorafshan et al., 2005; Takahashi et al., 2004). Even though a number of researches have demonstrated the effect of ND on the testis, little is known for an influence of ND on the epididymal function. The present study showed that ND differentially regulates mRNA expres-



**Fig. 3. Expression patterns of AQP 1 and 9 genes in the corpus epididymis after nandrolone decanoate treatment.** Expression of *Aqp1* (A) and *Aqp9* (B) mRNA in the corpus epididymis. C: control, L: low dose of ND treatment (2 mg/kg BW/week), H: high dose of ND treatment (10 mg/kg BW/week), M: 100 bp marker. Cyclophilin A (*Ppia*) was used as an internal control of real-time PCR. Different letters indicate statistically significant at  $p < 0.05$ . ND : not detectable.

ssion of *Aqp1* and *Aqp9* genes among segments of the epididymis. In addition, results from the current study imply that expression of *Aqp1* and *Aqp9* genes in particular epididymal regions would be dependent upon doses and periods of ND treatment.

Expressions of *Aqp1* and *Aqp9* genes in the rodent epididymis have been extensively examined from others (Badran and Hermo, 2002; Lu *et al.*, 2008; Pastor-Soler *et al.*, 2001). It is largely considered that *Aqp9* is a major



**Fig. 4. Expression of AQP 1 and 9 genes in the caudal epididymis after nandrolone decanoate treatment.** Expression of *Aqp1* (A) and *Aqp9* (B) mRNA in the caudal epididymis. C: control, L: low dose of ND treatment (2 mg/kg BW/week), H: high dose of ND treatment (10 mg/kg BW/week), M: 100 bp marker. Cyclophilin A (*Ppia*) was used as an internal control of real-time PCR. Different letters indicate statistically significant at  $p < 0.05$ .

form of AQPs expressing in the epididymal epithelia, especially IS and caudal epididymis (Badran and Hermo, 2002; Da Silva *et al.*, 2006). Our present study also shows that a relative level of *Aqp9* mRNA is higher than those of *Aqp1* mRNA. Expression of *Aqp1* in the epididymis is localized in not the epithelial cells but in adjacent sm-

ooth muscle and endothelial cells of vascular systems (Badran and Hermo, 2002; Brown et al., 1993). However, there is no detailed information available for mRNA expression of *Aqp1* and *Aqp9* genes among segments of the epididymis. In the present study, we found that *Aqp1* and *Aqp9* mRNAs were present in the entire epididymal regions during early pubertal ages. In addition, our current study revealed that there is no expression of *Aqp1* and *Aqp9* mRNA in the corpus epididymis at adult rat, which is in agreement with a previous finding (Badran and Hermo, 2002). Because animals from 2 treatment groups (2 and 12 weeks) in the present study were different ages, such differential expression patterns in the corpus epididymis might be due to age-dependent expression of *Aqp1* and *Aqp9* genes in the epididymal region.

The regulation of *Aqp1* and *Aqp9* expression in the male reproductive tract has been demonstrated from previous studies (Badran and Hermo, 2002; Fisher et al., 1998; Oliveira et al., 2005; Shayu et al., 2005). Pastor-Soler et al. (2002) showed that a treatment with flutamide, an antiandrogen, results in down-regulation of *Aqp9* mRNA expression in the epididymis. The level of *Aqp9* expression is dramatically decreased by ligation of the efferent ductules or orchidectomy, but the *Aqp9* expression is not restored by testosterone replacement (Badran and Hermo, 2002). A long-term treatment with the antioestrogen induces expression of *Aqp1* in the IS (Oliveira et al., 2005). These findings indicate that a complex of various steroid hormones regulates expression of *Aqp1* and *Aqp9* genes in the epididymis. In the present study, *Aqp1* mRNA expression in the epididymis is significantly up-regulated by short term treatment with a high dose of ND, except the caudal epididymis showing a drastic decrease of *Aqp1* mRNA level in a dose-dependent manner. However, regardless of doses of ND, a long term ND treatment led to significant reduction of *Aqp1* mRNA levels in the IS and caudal epididymis. These observations imply that mRNA expression of *Aqp1* gene is differentially regulated among parts of the epididymis by different doses and periods of ND treatment. The current study also demonstrates the segmental and differential regulation of *Aqp9* gene expression in the epididymis by ND treatment. It is known that the ND is capable with androgen receptor in the cell and functions in similar with testosterone (Van Der, 1965). Therefore, it is considered that such segmental and differential expression of *Aqp1* and *Aqp9* gene induced by ND treatment could be due to differences of androgen receptor levels present in each segment of the epididymis.

In summary, the present study demonstrates that the ND treatment influences abnormal mRNA expression of *Aqp1* and *Aqp9* genes in the epididymis. In addition, our current study clearly shows that expression of *Aqp1* and *Aqp9* mRNA among parts of the epididymis is differentially regulated by ND, depending upon treatment peri-

ods and doses. Thus, it is concluded that the ND commonly used for treatment of clinical diseases and for improvement of physical ability and appearance could affect the male fertility by disrupting not only the testicular function but also epididymal function.

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