# Sub-chronic testosterone treatment increases the levels of epithelial sodium channel (ENaC)-α, β and γ in the kidney of orchidectomized adult male Sprague-Dawley rats

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Testosterone has been reported to cause the blood pressure to increase. However mechanisms that underlie the effect of this hormone on this physiological parameter are currently not well understood. The aims of this study were to investigate effects of testosterone on expression of α, β and γ- epithelial sodium channel (ENaC) proteins and messenger RNAs (mRNAs) in kidneys, the channel known to be involved in $Na^+$ reabsorption, which subsequently can affect the blood pressure. **Methods**: Adult male Sprague-Dawley (SD) rats were orchidectomized fourteen days prior to receiving seven days treatment with testosterone propionate ( 125µg/kg/day or 250µg/kg/day) with or without flutamide (androgen receptor blocker) or finasteride (5α-reductase inhibitor). Following sacrifice, kidneys were removed and were subjected for α, β and γ -ENaC protein and mRNA expression analyses by Western blotting and Real-time PCR (qPCR) respectively. The distribution of α, β and γ -ENaC proteins in kidneys were observed by immunofluorescence. **Results**: α, β and γ-ENaC protein and mRNA levels in kidneys were enhanced in rats which received testosterone-only treatment. In these rats, α, β and γ-ENaC proteins were distributed in the distal tubules and collecting ducts of the nephrons. Co-treatment with flutamide or finasteride resulted in the levels of α, β and γ- ENaC proteins and mRNAs in kidneys to decrease . In conclusions, increase in α, β and γ-ENaC protein and mRNA levels in kidneys mainly in the distal tubules and collecting ducts under testosterone influence might lead to enhance $Na^+$ reabsorption which subsequently might cause an increase in blood pressure .

# Sub-chronic testosterone treatment increases the levels of epithelial sodium channel (ENaC)-α, β and γ in the kidney of orchidectomized adult male Sprague-Dawley rats

Su Yi Loh, Nelli Giribabu and Naguib Salleh*

Department of Physiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603 Malaysia

*Corresponding author

Tel: 00603 7967 7532

Fax: 00603 7967 4775

E-mail: naguib.salleh@yahoo.com.my

**Abstract**

Testosterone has been reported to cause the blood pressure to increase. However mechanisms that underlie the effect of this hormone on this physiological parameter are currently not well understood. The aims of this study were to investigate effects of testosterone on expression of α, β and γ- epithelial sodium channel (ENaC) proteins and messenger RNAs (mRNAs) in kidneys, the channel known to be involved in $Na^+$ reabsorption, which subsequently can affect the blood pressure. **Methods**: Adult male Sprague-Dawley (SD) rats were orchidectomized fourteen days prior to receiving seven days treatment with testosterone propionate (125μg/kg/day or 250μg/kg/day) with or without flutamide (androgen receptor blocker) or finasteride (5α-reductase inhibitor). Following sacrifice, kidneys were removed and were subjected for α, β and γ-ENaC protein and mRNA expression analyses by Western blotting and Real-time PCR (qPCR) respectively. The distribution of α, β and γ-ENaC proteins in kidneys were observed by immunofluorescence. **Results**: α, β and γ-ENaC protein and mRNA levels in kidneys were enhanced in rats which received testosterone-only treatment. In these rats, α, β and γ-ENaC proteins were distributed in the distal tubules and collecting ducts of the nephrons. Co-treatment with flutamide or finasteride resulted in the levels of α, β and γ-ENaC proteins and mRNAs in kidneys to decrease. In conclusions, increase in α, β and γ-ENaC protein and mRNA levels in kidneys mainly in the distal tubules and collecting ducts under testosterone influence might lead to enhance $Na^+$ reabsorption which subsequently might cause an increase in blood pressure.

## 1. Introduction

Epithelial sodium channel (ENaC), which consists of three homologous subunits (α, β and γ) plays an important role in $Na^+$ reabsorption in kidneys [1]. The α-subunit is required for $Na^+$ conductance while β- and γ-subunits are needed to enhance the channel activity [1]. Expression of ENaC in kidneys was under aldosterone influence [2]. Besides aldosterone, other hormones that could influence kidney ENaC expression include insulin and vasopressin [3, 4]. Mutation of ENaC gene could lead to hypotension, while its prolonged activation could lead to severe hypertension [5]. Expression of ENaC in kidneys could also be affected by sex hormones i.e. estrogen and progesterone [6]. There were evidences which suggest the involvement of testosterone in regulating kidney ENaC expression. Quan and colleagues [7] reported that dihydrotestosterone (DHT) injection to adult male Sprague-Dawley rats could increase $Na^+$ reabsorption in kidney proximal tubules, suggesting that this could be mediated via ENaC. Meanwhile, administration of testosterone in spontaneous hypertensive (SHR) rats was found to decrease the pressure-induced natriuresis, again pointing towards the involvement of ENaC [8].

To date, the information with regard to effect of testosterone on ENaC expression in kidneys were far from complete. Quinkler et al, [9] reported that sub-chronic (14 days) treatment of adult male Wistar rats with testosterone resulted in elevated *α-Enac* mRNA levels in kidney homogenates. Meanwhile, administration of dihydrotestosterone (DHT) causing the same effects, but was lesser than testosterone. Additionally, their study also showed that incubation of human kidney proximal tubule cell line with testosterone but not DHT caused increase in *α-ENaC* mRNA level with testosterone effect was being antagonized by flutamide. However, no expression for β and γ-ENaC was detected in these cells. In the meantime , testosterone and DHT were found to decrease expression of α, β and γ-ENaC subunits in

ovariectomised female Wistar rat kidney [10]. Based on these findings, we hypothesized that testosterone affects expression of all ENaC subunits i.e. α, β and γ in the kidneys, in which their co-existence will lead to a fully functioning ENaC channel, thus would augment $Na^+$ reabsorption which might result in the rise in blood pressure under testosterone influence [11]. Therefore, the aims of this study were to investigate testosterone effect on α, β and γ ENaC protein and mRNA expression levels in kidneys. In addition, the possible involvement of androgen receptor and DHT in mediating testosterone effects were also investigated.

## 2. Materials and methods

### 2.1 Animal preparation and hormonal treatment

Eight weeks old male Sprague-Dawley (SD) rats were housed in a clean and well ventilated environment under light-dark cycle (12/12h). The animals were fed with standard rat diet (Harlan, Germany) and tap water *ad libitum*. All procedures were approved by Institutional Animal Care and Use Committee (IACUC), University of Malaya (ethics number: 2014-05-07/physio/R/NS). Sham operation and orchidectomies were performed under ketamine/xylazine anesthesia. Two (2) weeks following recovery, hormonal treatments were initiated, which coincide with the age of the rats at 10 weeks old. Rats were divided into eight (8) treatment groups with eight (8) rats per group and were given the following treatment for seven (7) consecutive days.

*Group 1*: Intact, sham-operated receiving peanut oil only - S

*Group 2*: Orchiectomized, receiving peanut oil only - O

*Group 3*: Orchiectomized, receiving 125µg/kg/day testosterone - T125

*Group 4*: Orchiectomized, receiving 250µg/kg/day testosterone - T250

*Group 5*: Orchiectomized, receiving 125µg/kg/day testosterone plus flutamide (8mg/kg/day), - T125+FU

*Group 6*: Orchiectomized, receiving 250µg/kg/day testosterone plus flutamide (8mg/kg/day), - T250+FU

*Group 7*: Orchiectomized, receiving 125µg/kg/day testosterone plus finasteride (5mg/kg/day)- T125+FN

*Group 8*: Orchiectomized, receiving 250µg/kg/day testosterone plus finasteride (5mg/kg/day)- T250+FN

At the end of the treatment, rats were sacrificed by cervical dislocation and kidneys were removed for molecular biological and histological analyses.

### 2.2 Quantification of *Enac* mRNA levels in kidneys by high throughput qPCR (Fluidigm)

Immediately following removal, the kidneys from n = 4 rats per group were stored in RNAlater solution (Ambion, CA, USA) in order to preserve the RNA integrity. Tissues were then weighted and disrupted in a tissue lysis buffer by using a rotor-stator homogenizer (Heidolph DIAX 600). RNA extraction was performed by using a Macherey-Nagel Nucleo Spin RNA kit (Düren, Germany) according to the manufacturer's guidelines. cDNA synthesis was then carried out by using a Biorad iScript Reverse Transcription Supermix for RT-qPCR (California, USA). A high throughput qPCR-based microfluid dynamic array technology (Fluidigm) was performed to evaluate the changes in gene expression. The converted cDNA was pre-amplified by using Applied Biosystems PreAmp Master Mix, first, one cycle 95˚C for 10 min, then 10 cycles, 95˚C for 15 sec and finally one cycle, 60˚C for 4 min. The pre-amplified cDNA was diluted accordingly and was then used to perform the qPCR in Fluidigm dynamic array chip following the manufacturer's protocol with the aid of applicant specialists. The chip was run in the BioMark Instrument for qPCR at 95˚C for 10 min, followed by 40 cycles at 95˚C for 15 sec and lastly at 60˚C for 1 min. *Gapdh* was selected as a house-keeping gene as its expression were the most stable in kidneys. Taqman primers and probes for *Gapdh, Scnn1a (Enac-α), Scnn1b (Enac-β)* and *Scnn1g (Enac-γ)* were purchased from Applied Biosystems, CA, USA. All experiments were carried out in triplicates and the data were analyzed by using a Fluidigm Real-Time PCR analysis software.

131 **2.3 Quantification of ENaC protein levels in kidneys by Western blotting**

132

133 The frozen kidneys, obtained from n = 4 rats per group were weighted, cut and the tissues submerged in
134 RIPA buffer solution (BioVision, CA, USA) containing a protease inhibitor (BioVision, CA, USA), then
135 disrupted using a rotor-stator homogenizer (Heidolph DIAX 600). The total proteins were obtained by
136 centrifugation at 14,000×g for 15 min at 4˚C and protein concentration was quantified by a Thermo
137 Scientific Micro BCA Protein Assay Kit (Rockford, USA) following the manufacturer guidelines. Equal
138 amount of proteins were added into a loading dye, boiled for 5 min and loaded onto a 10% SDS-PAGE
139 gel. Following separations, the proteins were transferred onto a polyvinylidene fluoride (PVDF)
140 membrane (BIORAD, US), then blocked in 5% bovine serum albumin (BSA) (Sigma-Aldrich, USA) for
141 60 min at room temperature. The membranes were then probed with primary antibodies against α-ENaC,
142 β-ENaC, γ-ENaC (sc-22239, sc-25354, sc-22245, Santa Cruz Biotechnology, CA, USA respectively) for
143 90 min at room temperature. This was followed by incubation in horseradish peroxidase (HRP)
144 conjugated secondary antibody (Santa Cruz Biotechnology, CA, USA) for 60 min at room temperature.
145 The blots were then developed by using a Thermo Scientific Super Signal West Pico Chemiluminescent
146 Substrate (Rockford, USA) according to the manufacturer's manual. Chemiluminescent signals were
147 captured by using a highly sensitive CCD camera-based imager (BioSpectrum Imaging System). The
148 band intensity of each target was analyzed by using Image J software. GAPDH (Santa Cruz
149 Biotechnology) was used as endogenous control. The experiment was performed in triplicate and the
150 average ratios of target protein/GAPDH band intensity were then determined.

151

152 **2.4 Whole body perfusion fixation and detection of ENaC distribution in kidneys by**
153 **immunofluorescence**

154

155 Another set of animals (n = 4 rats per group), which were duplicate of those used for Western blotting and
156 qPCR analyses were anaesthetized with ketamine/xylazine anesthesia and a transcardial perfusion was

157 performed with injection of 4% paraformaldehyde (PFA) in phosphate buffer saline (PBS). The animals
158 were then killed and the kidneys were removed and fixed in 30% sucrose for 3 days. The tissues were
159 then processed and embedded in a paraffin wax. The kidneys were sectioned coronally into 5µm by using
160 a microtome. Sections were then mounted onto poly-L-lysine coated glass slides. Sections were then de-
161 parafinized and antigens were retrieved by boiling the sections in 1mM EDTA (BioVision InC.), pH 8.0
162 containing 0.05% (v/v) Tween20 (Sigma Aldrich). Sections were then blocked with appropriate 10%
163 (v/v) normal serum (Santa Cruz Biotechnology, CA, USA) in PBS for 1 h at room temperature and were
164 then incubated with target primary antibodies (as above) at the ratio of 1:100 at 4ºC, overnight. After
165 three times of washing with PBS, sections were then incubated in appropriate fluorophore conjugated
166 secondary antibodies at a dilution of 1:200, for 1 h at room temperature and then sealed with UltraCruz
167 mounting medium (Santa Crus Biotechnology, CA, USA). Secondary antibodies used in this experiment
168 were donkey anti-goat lgG (H+L) conjugated with DyLight 488 (SA5-10086) and goat anti-rabbit lgG
169 (H+L) conjugated with DyLight 550 (SA5-10033) (Thermo Scientific, Rockford, USA). The images were
170 viewed and captured by using a confocal laser scanning microscope (Leica TCS SP5 II) at a fixed
171 exposure time. All experiments were carried out in four (4) replicates and the representative images were
172 selected. All images were taken at 63x magnification and the slides without tissue were used as white
173 balancer.
174
175 **2.5. Measurement of serum testosterone level by enzyme-linked immunoassay (ELISA)**
176
177 Immediately following sacrifice and prior to transcardial perfusion, the blood from n = 8 rats per group
178 was collected via heart puncture and the serum was separated by centrifugation at 3500× g, 25°C for 10
179 min. Levels of testosterone in serum was analysed by enzyme-linked immunosorbent assay (ELISA) kit
180 (BioSource International, Inc., Camarillo, CA, USA), according to the manufacturer guideline.
181
182 **2.6 Statistical Analysis**

All data were analyzed by SPSS software with mean ± standard error of mean (S.E.M) obtained. Statistical significant between groups was evaluated by Student t-test followed by one-way analysis of variance (ANOVA). Statistically significant difference were denoted as $p<0.05$. Tukey's post-hoc test was used to examine adequacy of the samples and all values were > 0.08 which indicate adequate sample size.

## 3 Results

### 3.1 Testosterone increases α-ENaC protein and mRNA levels in kidneys

In Fig. 1A, the levels of *α-Enac* mRNA were highest following testosterone-only treatment. Co-treatment with flutamide or finasteride caused *α-Enac* mRNA levels to decrease ($p<0.05$). The effect of flutamide was greater in rats which received 125 mg/kg/day testosterone when compared to 250 mg/kg/day testosterone. However, the effect of finasteride in rats treated with 125mg/kg/day testosterone- was not significantly different when compared to its effect in rats treated with 250mg/kg/day testosterone.

In Fig. 1B and IC, expression levels of α-ENaC protein was highest in rats which received 250 mg/kg/day testosterone. In these rats, co-administration of flutamide or finasteride caused α-ENaC protein expression level to decrease ($p<0.05$). Flutamide effect was greater in rats treated with 125 mg/kg/day testosterone than in rat treated with 250 mg/kg/day testosterone. However, following co-administration of finasteride, no significant difference in α-ENaC protein expression level was observed between rats which received 125 mg/kg/day testosterone and rats which received 250 mg/kg/day testosterone.

### 3.2 Testosterone increases β-ENaC protein and mRNA levels in kidneys

In Fig. 2A, the levels of *β-Enac* mRNA were highest in rats which received testosterone-only treatment. In these rats, co-administration of flutamide or finasteride resulted in *β-Enac* mRNA levels to decrease ($p<0.05$). The effect of flutamide was greater in rats which received 125 mg/kg/day testosterone when compared to rats which received 250 mg/kg/day testosterone. However, co-administration of finasteride did not cause *β-Enac* mRNA levels in rats which received 125mg/kg/day testosterone to be difference from rats which received 250mg/kg/day testosterone.

In Fig. 2B and 2C, the levels of expression of β-ENaC protein were highest in rats which received 250 mg/kg/day testosterone. In these rats, co-administration of flutamide or finasteride resulted in β-ENaC protein expression level to decrease ($p<0.05$). The effects of flutamide were greater in rats which received 125 mg/kg/day testosterone when compared to rats which received 250 mg/kg/day testosterone. However, co-administration of finasteride did not cause β-ENaC protein expression level in rats which received 125 mg/kg/day testosterone to be different from rats which received 250 mg/kg/day testosterone.

### 3.3 Testosterone increases γ-ENaC protein and mRNA levels in kidneys

In Fig. 3A, the levels of *γ-Enac* mRNA were highest in rats which received 250 mg/kg/day testosterone. In these rats, co-administration of flutamide or finasteride resulted in *γ-Enac* mRNA levels to decrease ($p<0.05$). The effect of flutamide was not significantly different between rats which received 125 mg/kg/day testosterone and rats which received 250 mg/kg/day testosterone. Similarly, the effect of finasteride in rats which received 125mg/kg/day testosterone was not significantly different from its effect in rats which received 250mg/kg/day testosterone.

In Fig. 3B and 3C, the levels of γ-ENaC protein was highest in rats which received 250 mg/kg/day testosterone. In these rats, co-administration of flutamide or finasteride resulted in γ-ENaC protein expression level to decrease ($p<0.05$). Co-administration of flutamide with testosterone did not cause γ-

ENaC protein expression level in rats which received 125 mg/kg/day testosterone to be different from rats which received 250 mg/kg/day testosterone. In contrast, co-administration of finasteride with testosterone caused greater decrease in the level of this protein in rats which received 250 mg/kg/day testosterone when compared to rats which received 125 mg/kg/day testosterone.

**3.4 Distribution of α-ENaC protein in nephrons**

In Fig 4, α-ENaC protein was seen to be highly distributed in distal tubules and collecting ducts of rats which received 125 and 250 mg/kg/day testosterone. In these rats, co-administration of flutamide or finasteride resulted in α-ENaC protein distribution level to be relatively lower (Fig 5).

**3.5 Distribution of β-ENaC protein in nephrons**

In Fig. 4, β-ENaC protein was highly distributed in distal tubules and collecting ducts in rats which received 250 mg/kg/day testosterone. However, co-administration of flutamide or finasteride with testosterone resulted in relatively lower β-ENaC protein distribution level when compared to rats which received testosterone-only treatment (Fig 6).

**3.6 Distribution of γ-ENaC protein in nephrons**

In Fig. 4, γ-ENaC protein was highly distributed in distal tubule and collecting ducts in rats which received 125 and 250 mg/kg/day testosterone. However, co-administration of flutamide with testosterone resulted in relatively lower γ-ENaC protein distribution when compared to testosterone-only treatment (Fig 7). Similar effects could be seen in rats following concomitant co-administration of finasteride with testosterone.

### 3.7 Serum testosterone level

In Table 1, administration of 125 mg/kg/day testosterone resulted in serum level of this hormone to be 13.5 fold greater than its level in non-testosterone-treated orchiectomised rats. Treatment with 250 mg/kg/day testosterone resulted in serum testosterone level to be 16.5 fold greater than in non-testosterone treated orchiectomized rats.

## 4. Discussion

To the best of our knowledge, this study has for the first time revealed that testosterone was able to up-regulate expression of ENaC subunits' (α, β and γ) proteins and mRNAs in the kidneys of orchidectomised adult male rats *in-vivo*. The dose-dependent increase in serum testosterone levels was observed when testosterone was subcutaneously injected to these rats at increasing doses which suggested that this hormone most likely was not metabolized as it bypassed the first-pass effect. It was found that equal amount of α-ENaC protein was expressed in the orchidectomised male rats' kidneys following administration of 125 and 250mg/kg/day testosterone, however higher expression of β and γ ENaC proteins were found in the kidneys of rats which received 250mg/kg/day testosterone compared to 125 mg/kg/day testosterone. The latter findings were consistent with the findings by Keinitz et al, [12] who reported that administration of testosterone at 500 mg/kg/ in male rats resulted in high level of α-ENaC protein in kidneys. Another study has revealed that the levels of *α-ENaC* mRNA in human kidney cell line was enhanced by testosterone, however this hormone was found to have no significant effect on β and γ-*ENaC* mRNA levels [13]. In contrast, we found that the levels of β and γ- ENaC proteins and mRNAs were highly up-regulated in the orchidectomised male rats' kidneys by testosterone. We postulated that co-expression of all ENaC subunits (α, β and γ) would result in a fully operating channel as their co-existence was required for the maximal ENaC channel function [14].

In this study, we have found that the level of expression of α, β and γ-ENaC proteins and mRNAs in the kidney was significantly decreased following co-administration of flutamide with testosterone. The effect of flutamide on α and β-ENaC but not γ-ENaC was found greater in rats which received 125mg/kg/day when compared to 250mg/kg/day testosterone. The reason behind this effect was unknown, however we postulated that following high dose testosterone treatment i.e. at 250mg/kg/day, some of this hormone might be aromatized to estradiol in the adipose tissue, penis and brain [15] while some bind to the tissue androgen receptor. The aromatized form of testosterone i.e. estradiol could cause increased in α-ENaC levels in the kidney [6]. This could perhaps explain the reason why lesser inhibition was seen following high dose testosterone treatment. In the meantime, at high dose, there was a possibility that testosterone could exert a non-genomic effect which was not inhibited by flutamide [16]. Finasteride was found to inhibit testosterone effect in causing increase in ENaC subunits expression in the kidneys, which suggested that DHT was involved. In the absence of DHT, levels of ENaC subunits in kidneys would be markedly decreased. Besides DHT, other hormone which was also known to cause expression level of α, β and γ-ENaC subunits in the kidneys to increase is aldosterone, which was reported to induce redistribution of all ENaC subunits to the apical membrane of kidneys' distal tubule and collecting ducts [17, 18]. Therefore, redistribution of all ENaC subunits to the apical membrane of distal tubule and collecting duct under testosterone influence might produce similar effect to that reported under aldosterone, in which this would result in fully functioning channels [18]. The resultant effect would lead to increase in $Na^+$ reabsorption. We have found that ENaC subunits' protein were also found to be expressed in the cytoplasm under testosterone influence, in which these distributions could represent the channels at various stages of processing.

The effect of testosterone on ENaC subunit expression as observed in our study could help to explain the mechanisms underlying this hormone effects on kidneys' $Na^+$ handling as have been previously reported [8]. Based on what has been known, we have provided additional information in which in kidneys of male rat model, testosterone was also found to enhance β and γ-ENaC subunit expression in addition to the

already known increase in α-ENaC expression [9]. These testosterone effects would likely result in a fully functioning channel. However, more works need to be done in order to confirm the optimal ENaC channel function at the apical membrane of the distal tubule and collecting duct epithelia for example by using a patch clamp in which kidney epithelial cells obtained from animals treated with testosterone will be exposed to a specific ENaC inhibitor such as amiloride [19]. If in the case where ENaC channels are functioning, inhibition by amiloride will result in reduced $Na^+$ conductance. Additionally, the involvement of DHT can also be confirmed by administering this compound directly to the rats and the involvement of genomic pathway in mediating testosterone effect on ENaC expression can be confirmed by using androgen receptor knock-out animal model.

Our results might be clinically relevance. The role of testosterone in causing development of hypertension has long been debated. So far there has been no studies implicating direct involvement of this hormone in the development of this disease in males. Furthermore, molecular mechanisms underlying hypertension development that were linked to testosterone remain largely unknown. Several evidences showed that testosterone has direct link to blood pressure. Reckelhoff et al, [8] reported that in intact spontaneous male hypertensive (SHR) rats, elevated blood pressure was linked to high serum testosterone levels and this was further confirmed from the observations that ovariectomized female SHR rats that was given testosterone had increase in blood pressure [8]. Furthermore, studies have shown that natriuresis were markedly reduced in intact male and testosterone-treated ovariectomised female SHR rats which indicated that higher amount of $Na^+$ was reabsorbed in the kidney under the influence of this hormone [8]. Similar findings were reported in humans whereby serum testosterone levels correlate with the blood pressure [20].

In conclusions, our findings have provided evidences which might support the role of testosterone in causing a relatively higher blood pressure in males than females. Increased in kidney $Na^+$ reabsorption which might occur secondary to testosterone-induced up-regulation of ENaC might predispose the males

to hypertension and could perhaps be the reason why males have higher incidence of this disease compared to age-matched females before menopause. Finally, the differential effect of testosterone on α, β and γ –EnaC subunits expression in the kidney might contribute towards gender differences in the blood pressure regulation [21, 22].

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# Table 1(on next page)

Serum level of testosterone in different experimental groups

Values were expressed as mean ± S.E.M of eight (8) different observations. $^{\&}p<0.05$ compared to sham-operated rats, *p<0.05 compared to orchidectomized control rats, Sham = sham-operated, ORX = orchidectomized control, T125=125mg/kg/day testosterone, T250=250mg/kg/day testosterone

1

| Groups | Serum testosterone levels (ng/ml) |
|---|---|
| Sham | 2.89 ± 0.38 |
| ORX | 0.25[&] ± 0.20 |
| T125 | 3.37[*] ± 0.40 |
| T250 | 4.12[*] ± 0.55 |

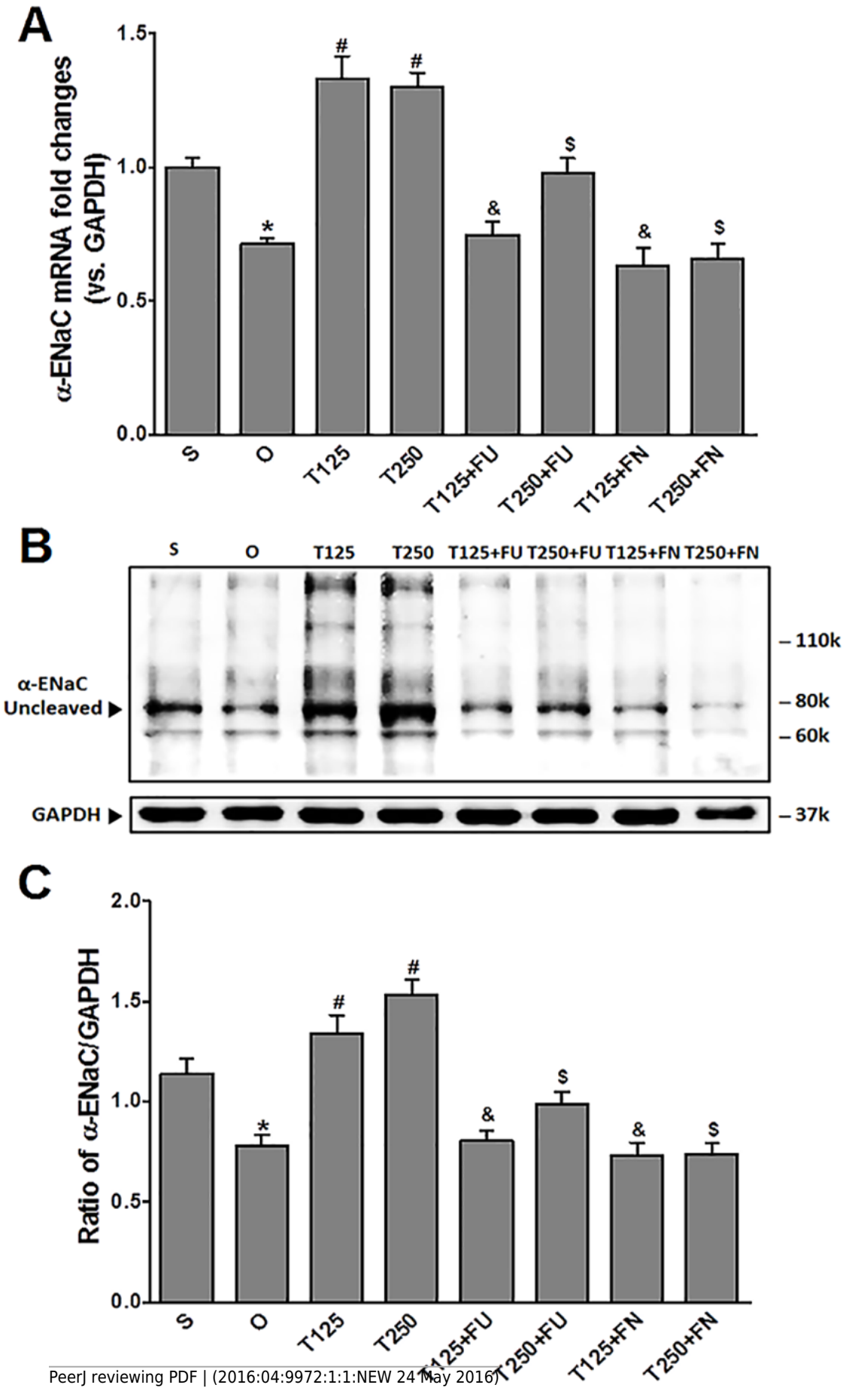
2

3

# 1

(A) *α -Enac* mRNA level (B) Whole membrane image of α -ENaC protein band and (C) Ratio of α -ENaC/GAPDH protein band intensity in kidneys

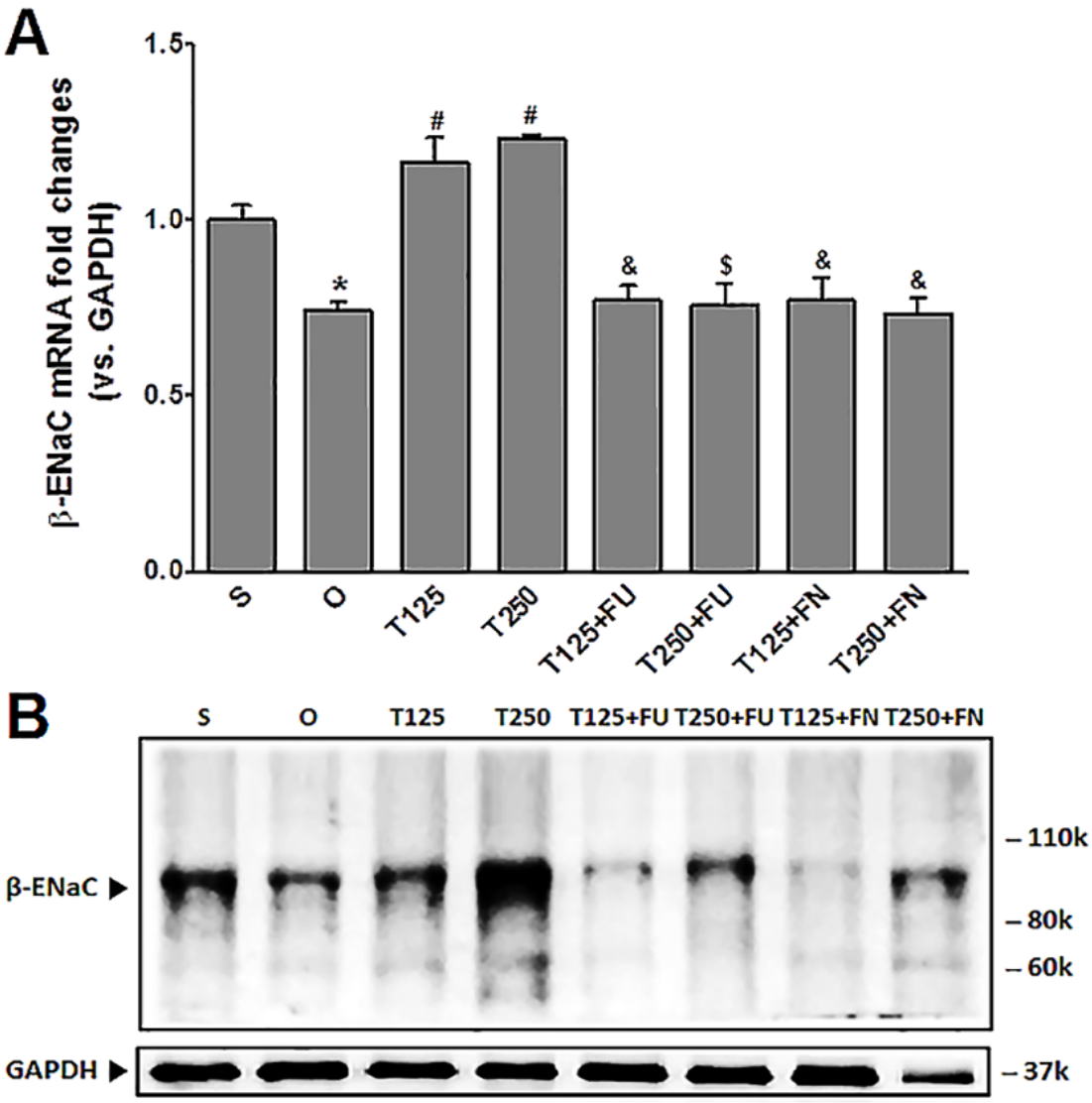
Values represent mean ± S.E.M of four rats. *p<0.05 compared to sham-operated rats, #p<0.05 compared to orcidectomized control rats, $^{\&}$ p <0.05 compared to T125. $^{\$}$p<0.05 compared to T250. S= sham operated, O=orchiectomized non-treated, T125 = 125mg/kg/day testosterone-treated, T250 = 250mg/kg/day testosterone-treated rats, FU= flutamide, FN= finasteride. Molecular weight of α -ENaC protein = 78kDa (cleaved)


A
α-ENaC mRNA fold changes (vs. GAPDH)
1.5
1.0
0.5
0.0
S
O
T125
T250
T125+FU
T250+FU
T125+FN
T250+FN
B
S
O
T125
T250
T125+FU
T250+FU
T125+FN
T250+FN
α-ENaC Uncleaved
GAPDH
110k
80k
60k
37k
C
Ratio of α-ENaC/GAPDH
2.0
1.5
1.0
0.5
0.0

# 2

(A) *β -Enac* mRNA level (B) Whole membrane image of β -ENaC protein band and (C) Ratio of β -ENaC/GAPDH protein band intensity in kidneys.

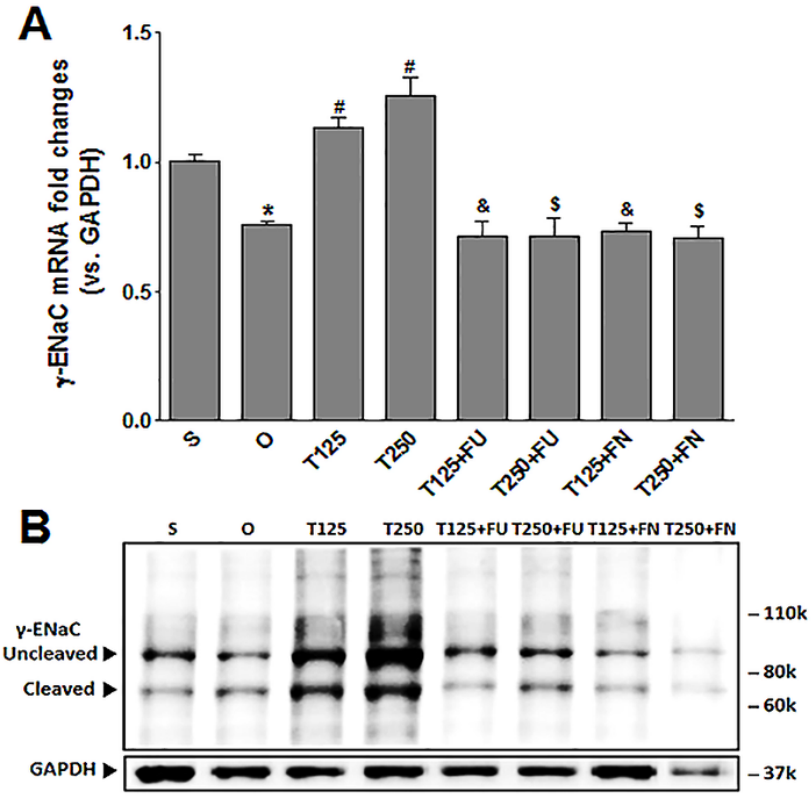
Values represent mean ± S.E.M of four rats. *p<0.05 compared to sham-operated rats, #p<0.05 compared to orcidectomized control rats, $^{\&}$ p <0.05 compared to T125. $^{\$}$p<0.05 compared to T250. S= sham operated, O=orchiectomized non-treated, T125 = 125mg/kg/day testosterone-treated, T250 = 250mg/kg/day testosterone-treated rats, FU= flutamide, FN= finasteride. Molecular weight of β-ENaC protein = 99 kDa.


A
β-ENaC mRNA fold changes (vs. GAPDH)
1.5
1.0
0.5
0.0
S
O
T125
T250
T125+FU
T250+FU
T125+FN
T250+FN
B
S
O
T125
T250
T125+FU
T250+FU
T125+FN
T250+FN
β-ENaC
GAPDH
110k
80k
60k
37k
C
Ratio of β-ENaC/GAPDH
2.0
1.5
1.0
0.5
0.0

# 3

(A) *γ -Enac* mRNA level (B) Whole membrane image of γ -ENaC protein band and (C) Ratio of γ -ENaC/GAPDH protein band intensity in kidneys.

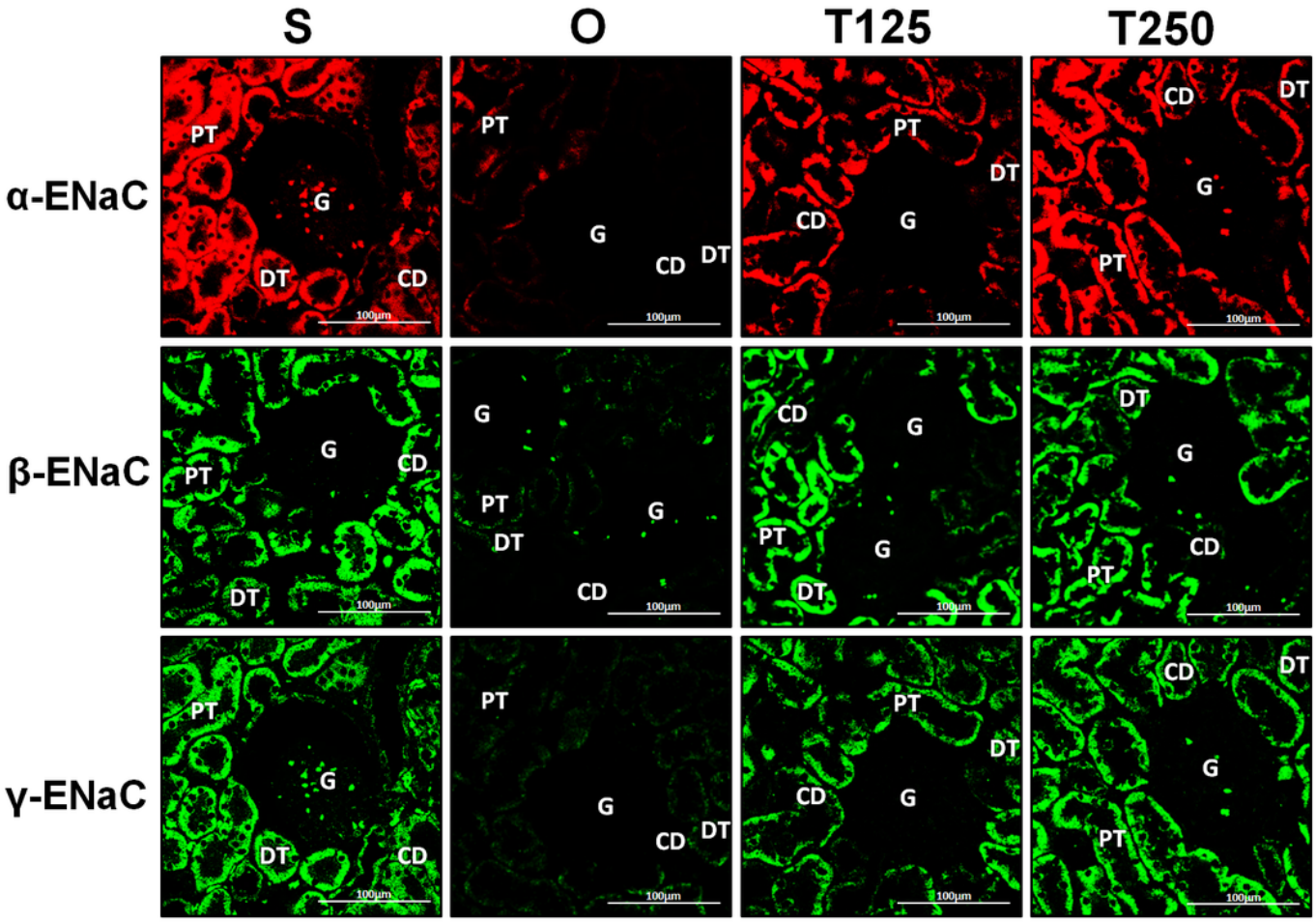
γ -ENaC possesses a cleaved and an un-cleaved forms. Values represent mean ± S.E.M of four rats. *p<0.05 compared to sham-operated rats, #p<0.05 compared to orcidectomized control rats, $^{\&}$ p <0.05 compared to T125. $^{\$}$p<0.05 compared to T250. S= sham operated, O=orchiectomized non-treated, T125 = 125mg/kg/day testosterone-treated, T250 = 250mg/kg/day testosterone-treated rats, FU= flutamide, FN= finasteride. Molecular weight of γ -ENaC= 75kDa (cleaved/unglycosylated).


A
γ-ENaC mRNA fold changes (vs. GAPDH)
1.5
1.0
0.5
0.0
S
O
T125
T250
T125+FU
T250+FU
T125+FN
T250+FN
B
S
O
T125
T250
T125+FU
T250+FU
T125+FN
T250+FN
γ-ENaC
Uncleaved
Cleaved
GAPDH
110k
80k
60k
37k
C
Ratio of Uncleaved γ-ENaC/GAPDH
2.0
1.5
1.0
0.5
0.0
D
Ratio of Cleaved γ-ENaC/GAPDH
2.0
1.5
1.0
0.5
0.0

# 4

Immunofluorescenceimages showing distribution of α, β and γ- ENaC proteins in nephrons.

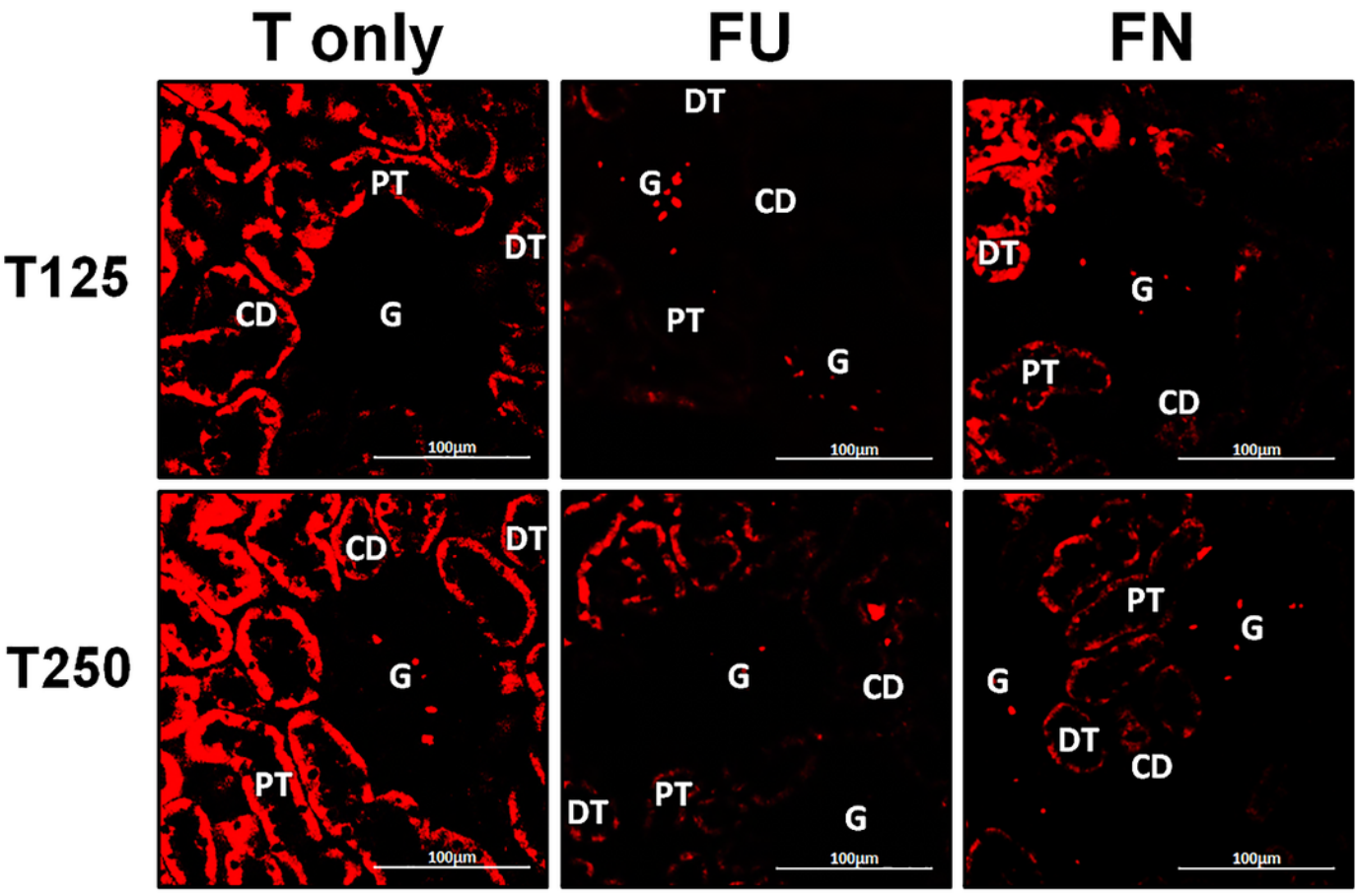
Red or green fluorescence signals indicate the sites where ENaC subunit proteins were expressed. G= glomeruli, PT= prximal tubule, DT= distal tubule, CD= collecting duct. S= sham operated, O=orchiectomized non-treated, T125 = 125mg/kg/day testosterone-treated, T250 = 250mg/kg/day testosterone-treated rats. Scale bar= 100µM



# 5

Effects of flutamide and finasteride on expression of α -ENaC in nephrons.

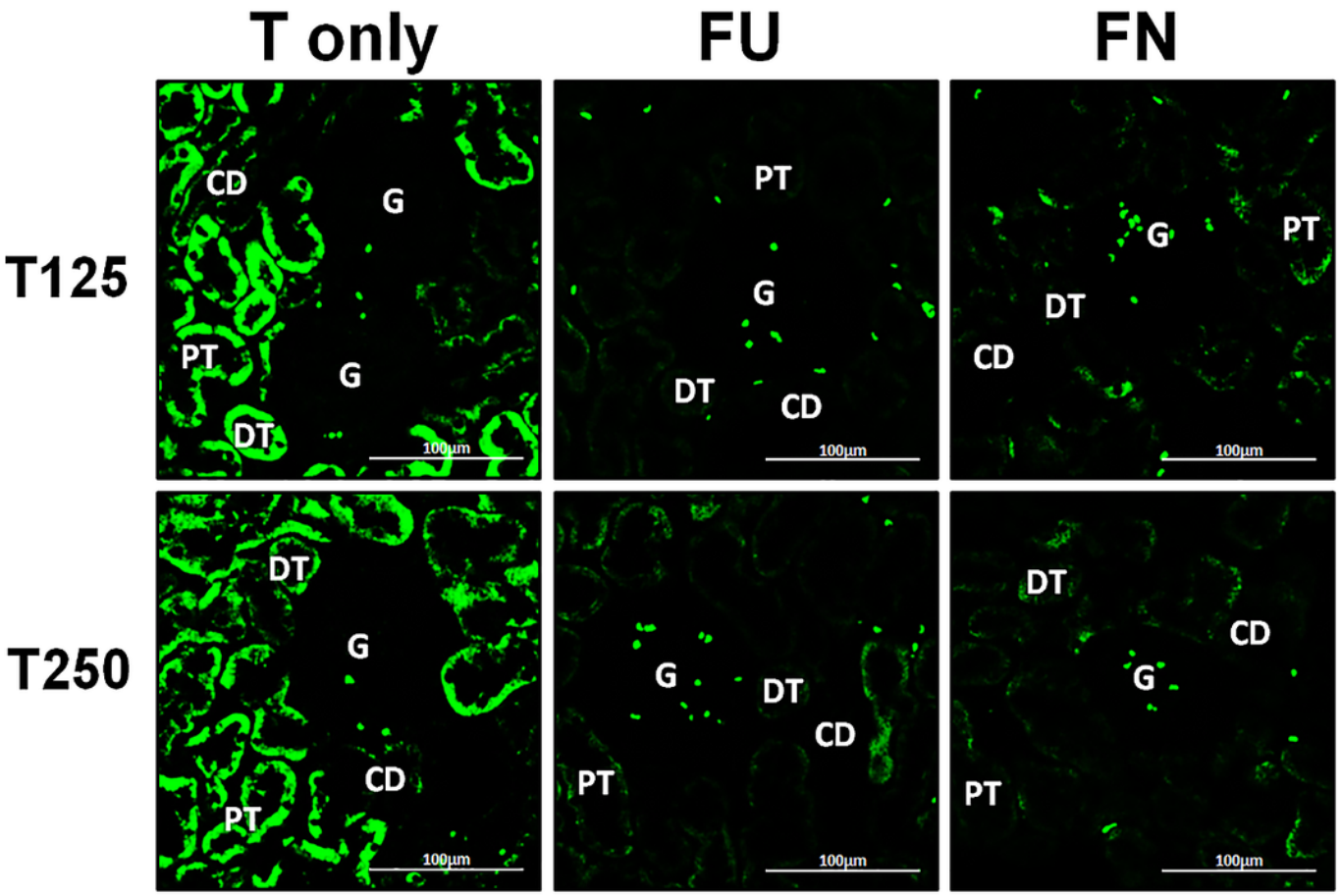
Red fluorescence signals indicate sites where ENaC subunit proteins were expressed. G= glomeruli, PT= prximal tubule, DT= distal tubule, CD= collecting duct. S= sham operated, O=orchiectomized non-treated, T125 = 125mg/kg/day testosterone-treated, T250 = 250mg/kg/day testosterone-treated rats. Scale bar= 100μM



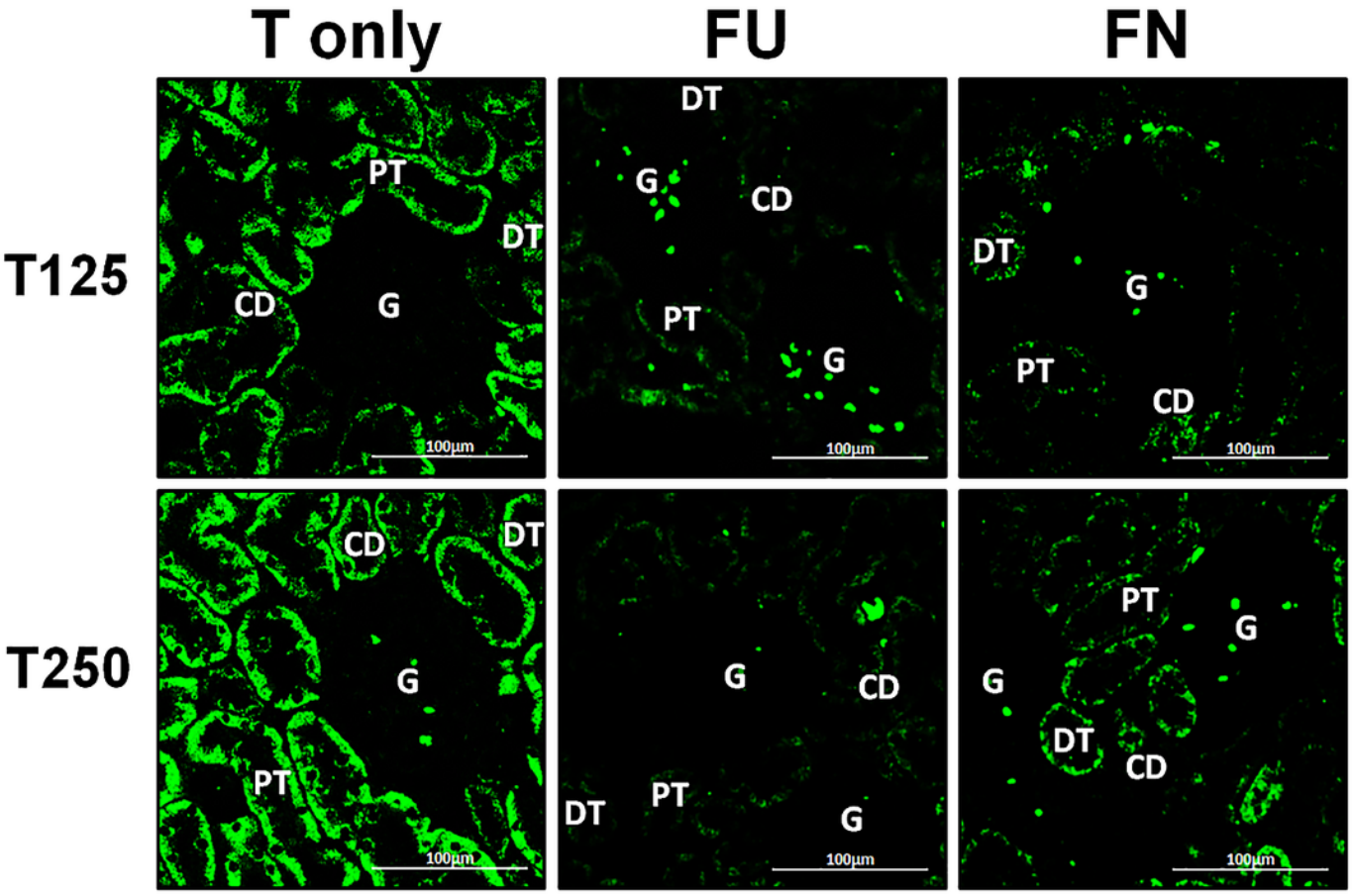
# 6

Effects of flutamide and finasteride on expression of β -ENaC in nephrons.

Green fluorescence signals indicate sites where ENaC subunit proteins were expressed.. G= glomeruli, PT= prximal tubule, DT= distal tubule, CD= collecting duct. S= sham operated, O=orchiectomized non-treated, T125 = 125mg/kg/day testosterone-treated, T250 = 250mg/kg/day testosterone-treated rats. Scale bar= 100μM



# 7

Effectsof flutamide and finasteride on expression of γ -ENaC in nephrons.

Green fluorescence signals indicate sites where ENaC subunit proteins were expressed. G= glomeruli, PT= prximal tubule, DT= distal tubule, CD= collecting duct. S= sham operated, O=orchiectomized non-treated, T125 = 125mg/kg/day testosterone-treated, T250 = 250mg/kg/day testosterone-treated rats. Scale bar= 100μM