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Nandrolone administration with or without strenuous exercise promotes overexpression of nephrin and podocin genes and induces structural and functional alterations in the kidneys of rats

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ABSTRACT

Among the various adverse effects of nandrolone administration with or without strenuous exercise, kidney abnormalities, where there are associations between nandrolone decanoate consumption, have not been well defined yet. The aim of this study was to investigate the effect of nandrolone decanoate intake with or without strenuous exercise on nephrin and podocin gene expressions, cystatin C, oxidative DNA damage, and histological changes in the kidneys of rats. Thirty-two male wistar rats were assigned into four groups, namely control, nandrolone, nandrolone with strenuous exercise, and strenuous exercise groups. After six weeks of treatment, the results revealed a significant increase in the nephrin and podocin gene expression, plasma cystatin C, and the amount of 8-OHdG in the kidney tissue; as well as a decrease in creatinine clearance in nandrolone and nandrolone with strenuous exercise groups compared to the control group. Moreover, compared to the control group, the nandrolone and the nandrolone with strenuous exercise groups, showed histological changes such as fibrosis and kidney tissue cells proliferation. These findings indicate that nandrolone induces kidney abnormalities, which may in part be associated with overexpression of nephrin and podocin genes mediated by oxidative stress, which was manifested in increased 8-OHdG in kidney tissue.

1. Introduction

Anabolic-androgenic steroids (AAS) as a synthetic form of testosterone are produced for therapeutic purposes due to their prolonged biological activities, less androgenic and more anabolic activities compared to the parent molecule (Evans, 2004). Apart from therapeutic aims, athletes often use these substances to improve their performance, form a greater muscle mass and enhance cosmetic effects (Kindlundh et al., 1998; Nasr and Ahmad, 2009). However, excessive use and supra-physiological doses of AAS can have acute and chronic toxic effects on the reproductive system and different tissues such as the brain, heart, and kidney (Maravelias et al., 2005; Vasilaki et al., 2016; Wallin-Miller et al., 2016). Although we do not have much information on the potential effects of AAS on renal function, some previous studies have demonstrated that nandrolone exposure leads to some functional and structural abnormities in the renal system. From the functional aspect, it has been reported that AASs exposure led to the elevation of serum creatinine, blood urine nitrogen, and uric acid (Juhn, 2003; Mochizuki and Richter, 1988). Moreover, acute renal failure, as a complication of rhabdomyolysis, has been reported among body builders using AAS by previous studies (Hageloch et al., 1988). From a structural perspective, some previous studies have reported that after nandrolone exposure, membrano-proliferative glomerulonephritis increased the volume of renal cortex and decreased the density of α_{1B}-adrenoceptors in the kidney (Hoseini et al., 2009; Lindblom et al., 2005; Revai et al., 2003). However, severeal studies have proposed that AAS may directly induce harmful effects on glomerular cells, leading to mesangial matrix accumulation and podocyte depletion, independent of structural and functional alterations (Herlitz et al., 2010). Although previous studies have identified some features of kidney structural and functional abnormalities following AASs administration, the precise mediating steps between exposure of kidney to nandrolone and initiation of the cascade of responses leading to kidney abnormality have not yet been completely clarified. Therefore, the output remains poorly understood. However,

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studies have often postulated that oxidative stress or inflammation mechanisms are involved and research has not provided sufficient information on this matter (Riezzo et al., 2014; Summers et al., 2012). Among dozens of molecular mediators related to the kidney function, nephrin and podocin are very important constituents of the glomerular filtration barrier. Moreover, alterations of their gene expression are associated with severe nephropathy (Aaltonen et al., 2001; Chen et al., 2015). The podocyte proteins including nephrin and podocin have major roles in the slit diaphragm of podocyte and are essential elements for maintaining the selective permeability of the glomerular filtration barrier (Piscione and Licht, 2011; Welsh and Saleem, 2010). Previous studies have shown that alterations in the expression of nephrin and podocin genes result in worsening of proteinuria, glomerulosclerosis, and renal function in diabetic nephropathy (Wang et al., 2007). Hence, in the current study, we evaluated the possible adverse effects of nandrolone decanoate on kidney tissue of male rats, regarding both the histological and molecular levels during sedentary and sever training status. Thus, proliferating cell nuclear antibody (PCNA) and masson trichrome staining were applied in evaluation of the histopathological alterations. In addition, creatinine clearance, cystatin C, and cystatin C/plasma creatinine ratios were measured for evaluation of functional alterations of kidneys caused by exposure to nandrolone. Due to the fundamental role of nephrin and podocin in both physiological and pathological status of the kidney, we also proposed that nandrolone consumption resulted in kidney abnormalities, which are mediated in part via overexpression of nephrin and podocin related genes in the kidney tissue. Through determination of Ox-DNA damage, it was found that Oxidative stress was induced in rats' kidneys by administration of nandrolone.

2. Materials and methods

All experimental protocols were approved by the Urmia University of Medical Sciences Animal Care Committee in accordance with the guide for the care and use of Laboratory Animal Care published by the National Institutes of Health (NIH publication, no.85–23, revised 1985). Thirty-two male wistar rats aged 4 months with an initial body weight of 220 ± 10 g were randomly divided into four groups (eight in each group) namely control, nandrolone, nandrolone with strenuous exercise, and strenuous exercise groups. Nandrolone group received 10 mg of nandrolone decanoate per body weigh by a single injection in the femoral muscle, three times per week (Saturdays, Mondays, and Wednesdays) for six weeks. The nandrolone with strenuous exercise group received the same amount of nandrolone and was forced to swim with a 20% excess body weight. The strenuous exercise group was only forced to swim with an excess weight of 20%.

Swimming tests were performed in a metal cylinder tank (60 cm hight × 100 cm diameter) filled with clean water having a depth of 40 cm and a temperature of 25 ± 2° C in accordance with our previous protocol (Tofighi et al., 2017). The animals were allowed to swim individually for 20 min three times per week (Saturdays, Mondays, and Wednesdays) for six weeks. A metal piece (20% body weight) was attached to the tails of rats to force them to continue swimming. The rats were anesthetized by 10% chloral hydrate (0.5 ml/kg body weight, IP) after 42 days of therapeutic and exercise programs; the depth of anesthesia was evaluated by pinching a hind paw. To collect urine samples, 24 h before anesthesia, rats were kept in metabolic cages singly, and then urine was collected. Urine samples were immediately centrifuged to remove debris or extra substances before storage at −20 °C. At termination, after weighing the animals, the thoracic cavity was opened and blood sample was taken from the heart of each rat in appropriate glass tubes in order to estimate the concentration of flowing biomarkers: urea, creatinine, and cystatine C. The blood sample was centrifuged at 4000 × *g* for 20 min within 30 min of collection, the plasma was separated and was then stored at −80 °C without repeated freeze-thaw cycles. Next, the abdominal cavities of rats were opened and their kidneys were removed. The left kidney was freed completely from perirenal fat, connective tissue, and blood clots, and was subsequently weighed. The kidney was divided into two parts. For the purpose of histopathological investigations, a part (1/3 mid) of the kidney was fixed in formalin, embedded in paraffin, and sectioned at 5 µm. In order to measure kidneys total antioxidant capacity and 8-OHdG amount, other parts of the kidneys were washed with ice-cold physiological saline and then dried on filter papers. Then, the samples were homogenized using Ultra Turrax (T10B, IKA, and Germany) in ice-cold extraction buffer (10% wt/vol), containing a 50 mM phosphate buffer (pH 7.4). The homogenates were centrifuged at 10,000 × *g* at 4 °C for 20 min. Moreover, the supernatant sample was used for 8-OHdG and total antioxidant assay.

For total RNA isolation, 100 mg of the kidney tissue was immersed in 1 ml RiboxEX (total RNA isolation solution) (GeneALL, Seoul, Korea) and restored at −80 °C, up to the time of RNA isolation.

2.1. Total RNA extraction and quantitative real time −polymerase chain reaction (Real time-PCR)

The total RNA was extracted from 100 mg frozen tissue of kidneys by using a kit (Gene all, South Korea, Cat no 305-101), according to the kit instructions. RNA concentration was verified by spectrophotometric measurement of the absorbance at 260 to 280 nm and determined by mixture of Tris base, acetic acid and EDTA (TAE) agarose gel electrophoresis.

Reverse transcriptase (RT) was carried out using hyperscript™ Reverse Transcriptase (Gene All, South Korea). RT-PCR was performed using an amplification reagent kit (Ampliqon, Denmark) by the XP-Cycler instrument (TCXPD, Bioer, USA) with nephrin and podocin and the rats' glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primers. To amplify the cDNA, the 5′ and 3′ primer sequences (forward and reverse) of the nephrin and podocin genes designed via the Gene Bank (http://blast.ncbi.nlm.gov/Blast.cgi) revealed that the primers were gene specific. Furthermore, Gene Runner software verified all the primers (Table 1). Primers (forward and reverse) were also synthesized to amplify the cDNA encoding GAPDH as a house keeping gene; the sequences of related primers are also provided in Table 1.

Real-time quantification of the target genes was performed by using a Real-Time PCR Master Mix Green kit (Ampliqon, Denmark) in a total volume of 25 µl and in accordance with the manufacturer's instructions. Furthermore, the mentioned genes expressions were analyzed employing an iQ5 real-Time PCR detection system (Bio-Rad, CA, USA). The reactions were then prepared for 10 min at 95 °C in a 96 well optical plate followed by 40 cycles of 20 s each at 59 °C. In order to confirm the specificity of the amplification reactions, a melting curve was recorded. Each sample was replicated three times; the value of the threshold cycle (Ct) was the same as that of the corresponding mean. The relative fold expression of each mRNA was calculated by conducting the $2^{-\Delta\Delta Ct}$ method (-ΔΔCt = ΔCt test sample-ΔCt calibrator sample), with Ct being the threshold cycle. Next, the calculated levels were normalized to GAPDH and were then analyzed for statistical significance performing a one-way analysis of variance.

Table 1
Sequences of primers used to evaluate expression of GAPDH, nephrin and podocin.

Target Gene	Primer sequence	Product size
Podocin forward	GGCGAGTGGACAAGAGTAAT	201
Podocin reverse	TGAATGATGAGACGACCCAC	
Nephrin forward	ATCCACTTTAGGGGGTCATTA	231
Nephrin reverse	CTTGTGCTTCTCCTCTCTCAG	
GADPH forward	AGA CAG CCG CAT CTT CTT GT	207
GADPH reverse	CTT GCC GTG GGT AGA GTC AT	

2.2. Biochemical assay

The 8-OHdG amount in the kidney tissue was assayed by adopting the quantitative sandwich enzyme immunoassay method and using a commercial rat 8-hydroxy-desoxyguanosine Elisa kit (Cusabio, China), in accordance with manufacturer's instruction. Measuring the amount of total antioxidant capacity in rat's kidney tissue was carried out by using an antioxidant assay kit (Cayman chemical, USA). Aqueous and lipid soluble antioxidants were not separated in this protocol, thus the combined antioxidant activities of all its constituents including vitamins, proteins, lipids, glutathions, uric acid, etc. were assessed. The assay relies on the ability of antioxidants in the sample to inhibit the oxidation of ABTS (2,2′-Azino-di-[3-ethylbenzthiazoline sulphonate]) to ABTS•+ by metmyoglobin and measures all aqueous- and lipid-soluble antioxidants including vitamins, proteins, lipids, glutathione, uric acid etc. The capacity of the antioxidants in the sample, in terms of prevention of ABTS oxidation, was compared with that of Trolox, a water-soluble tocopherol analogue, and was quantified as millimolar Trolox equivalent. Detection range of the kit was 0.044–0.330.

The amount of plasma Cystatin C was measured carrying out the quantitative sandwich enzyme immunoassay method and using a commercial rat cystatin C Elisa kit (Cusabio, China) following the protocol provided by the manufacturer. Furthermore, plasma and urine creatinine and urea levels were measured using urea and a creatinine commercial kit (Pars Azemoon, Karaj, IRAN). Glomerular filtration rate (GFR), as the best overall index of kidneys function, was estimated by calculating creatinine clearance (GFR = [UCr x V]/PCr) and using plasma (PCr) and urine creatinine (UCr) concentrations and the urine flow rate or volume (V).

2.3. Histopathological examinations

To perform histopathological staining, 5-μm thick histological sections from paraffin-embedded kidney tissue were used. Proliferating cells were implemented, in accordance with our published protocol and by performing immunohistochemistry using an antibody against the proliferation cell nuclear antigen (PCNA) (Shirpoor et al., 2016). In brief, after taking tissue processing steps, such as deparaffinization, rehydration, and gradual ethanol passage, sections from the kidney tissue with a thickness of 5-μm were stained using the Monoclonal anti-PCNA antibody (Dako Denmark A/S, Denmark). Optimal results were achieved with the EnVision™ visualization system (Dako Denmark A/S, Denmark). Furthermore, Hematoxylin was used as a counterstain. The assessment included proper negative controls. Moreover, all the slides were inspected by two expert pathologists, independently. PCNA-positive indices were considered as indicators of kidney cells proliferation. In order to assess the percentage of PCNA-positive indices, four non-overlapping fields of view per section from two to three sections per animal were analyzed. The number of positively stained cells and the total number of cells were counted for each field of view. In addition, for each animal, the number of positively stained cells was then presented as a percentage of the total number of counted cells. The criteria applied in scoring the quality of PCNA-positive indices were as follows: normal (i.e. PCNA-positive indices are present in less than 5% of the kidney cells), mild (i.e. PCNA-positive indices are present in less than 25% of the kidney cells), mild to moderate (i.e. PCNA-positive indices are present in 25–50% of the kidney cells), moderate to severe (i.e. PCNA-positive indices are present in 50–75% of the kidney cells), and severe (i.e. PCNA-positive indices are present in 75–100% of the kidney cells) (Shirpoor, et al., 2016). In order to evaluate the kidney tissue fibrosis, 5 μm kidney tissue sections were stained using Masson Trichrome, in accordance with the manufacturer's instructions (Asiapajohesh, Amol, Iran). The severity of tissue fibrosis was estimated maintaining a semi-quantitative method explained by Ashcroft et al. and our published protocol (Ashcroft et al., 1988; Shirpoor, et al., 2016). A score ranging from zero (normal kidney) to eight (total fibrosis) was set. The criteria appointed in scoring the kidney fibrosis were as follows: grade 0 = normal kidney; grade 1 = minimal fibrosis thickening of kidney tissue, grade 2 and 3 = moderate thickening of kidney tissue without obvious damage to the structure of kidney tissue; grade 4 and 5 = increased fibrosis with definite damage to the architecture of the kidney and formation of fibrosis bands or small fibrosis masses; grade 6 and 7 = severe distortion of the structure and large fibrosis areas; and finally grade 8 = total fibrotic obliteration (Ashcroft, et al., 1988).

All histological measurements were carried out by two independent examiners in a blinded manner and expressed in comparison to the controls.

2.4. Statistical analyses

Normal distribution of data within each group was verified by carrying out a Kolmogorov-Smirnov test. The statistical differences between the groups were tested by conducting a one-way ANOVA and then the Tukey's post hoc test. The data obtained from each test are presented as the mean ± S.E., and $p < 0.05$ is considered as statistically significant.

Table 2
Effect of nandrolone, nandrolone with strenuous exercise, and strenuous exercise on changes of kidney tissue 8-OHdG amount and Total antioxidant capacity, Gene expression of nephrin,podocin, urine and plasma levels of creatinine, urea, creatinine clearance and cys-c/creatinine ratio after days treatment.

	Control	Nandrolone	Nan-exe	Exer
Cystatin C(ng/ml)	7.42 ± 0.13	8.32 ± 0.18 *	8.55 ± 0.14 *	7.47 ± 0.13 †, ±
Creat.blood(mg/dl)	0.45 ± 0.019	0.46 ± 0.05	0.42 ± 0.01	0.42 ± 0.02
Creat.Urine(mg/dl)	6.37 ± 0.28	2.4 ± 0.23 *	0.6 ± 0.28 †,*	2.4 ± 0.14 *, ±
Urea Blood(mg/dl)	32.8 ± 2.2	44.7 ± 2.9 *	44.6 ± 4.4 *	34 ± 5.3 †, ±
Urea.Urine(mg/dl)	329 ± 22	201 ± 25 *	100 ± 17 *,†	200 ± 23 *, ±
Creat Clearance(ml/min)	1.12 ± 0.22	0.44 ± 0.07 *	0.13 ± 0.03 *,†	0.48 ± 0.13 *, ±
Cys.Creat(ng/ml/ mg/dl)	16.49 ± 0.35	17.6 ± 0.61 *	20.31 ± 0.89 *,†	17.56 ± 0.84 ±
Nephrin(Fold)	26.86 ± 0.66	30.6 ± 0.64 *	29.75 ± 0.11 *	28.68 ± 0.42 *,†
Podocyn(Fold)	27.71 ± 0.9	35.38 ± 0.54 *	33.82 ± 0.71 *	34.77 ± 0.59 *
8-OHdG(ng/ml)	1.2 ± 0.04	1.72 ± 0.02 *	1.82 ± 0.08 *	1.22 ± 0.02 †, ±
T.A.C(ng/ml)	0.46 ± 0.025	0.52 ± 0.01	0.68 ± 0.02 *,†	0.62 ± 0.008 *,†
Kidweig.Bodyweig(mg/gr)	6.33 ± 0.43	8.15 ± 0.93 *	8.58 ± 0.1 *	6.76 ± 0.86 †, ±

Values are mean ± SE for 8 rats per group.
Nan-exe: nandrolone-exercise, Cys.Creat: Cystatin C/creatinine, T.A.C: total antioxidant capacity.
* Denotes significant difference ($p < 0.05$) compared to the control.
† Denotes significant difference ($p < 0.05$) compared to the nandrolone group.
± Denotes significant difference ($p < 0.05$) compared to the nandrolone with strenuous exercise.

3. Results

Several biochemical markers were monitored, the results of which are summarized in Table 2. Nandrolone and nandrolone with strenuous exercise elevated plasma cystatin C amount among the two groups compared to the control group ($p < 0.01$). Plasma cystatin C amount showed no significant difference between nandrolone and nandrolone with strenuous exercise group. Strenuous exercise alone was not able to induce elevation of plasma cystatin C among the group compared to the control group. Plasma creatinine level showed no significant difference among different groups. Plasma urea levels showed significant elevation in the nandrolone and nandrolone with strenuous exercise groups compared to the control group ($p < 0.05$), but there were no significant differences between the strenuous exercise and the control group. There were no significant differences in urea levels in the plasma between the nandrolone and the nandrolone with strenuous exercise groups. In the nandrolone, nandrolone with strenuous exercise, and strenuous exercise groups urine creatinine and urine urea levels were significantly lower than that in the control group ($p < 0.05$), and this decline was more severe in nandrolone with strenuous exercise group compared to the nandrolone alone and exercise groups ($p < 0.05$). Urine creatinine and urine urea levels showed no significant difference between nandrolone and exercise groups. In all the treated groups, the creatinine clearance, as an indicator of glomerular filtration rate, was significantly lower than that in the control group ($p < 0.05$). Among the treated groups, creatinine clearance in the nandrolone-exercise group was significantly lower than nandrolone and exercise alone groups ($p < 0.050$). The ratio of serum cystatin C to serum creatinine increased significantly in the nandrolone and nandrolone with strenuous exercise groups compared to the control group ($p < 0.03$). There were no significant differences found between the exercise groups and the control group in terms of cystatin C/creatinine ratio. The ratio of serum cystatin C to serum creatinine increased significantly in the nandrolone with strenuous exercise group compared to that in the nandrolone group ($p < 0.05$). The results of nephrin and podocin genes expression in the kidneys of animals treated with nandrolone, nandrolone with strenuous exercise, and severe exercise groups were increased significantly compared to that of the control group ($p < 0.05$). There were no significant differences in nephrin and podocin genes expression in the kidney between the nandrolone and the nandrolone with strenuous exercise groups. The 8-OHdG amount in the kidneys of the animals treated with nandrolone and nandrolone with strenuous exercise were increased significantly in comparison to that in the control group ($p < 0.05$), but there were no significant differences between the exercise and control group as well as between nandrolone and the nandrolone with strenuous exercise groups in term of the 8-OHdG amount. Slight increases in the total antioxidant capacities were found in the nandrolone, nandrolone with strenuous exercise and exercise groups compared to the control group ($p < 0.05$). Total antioxidant capacities in the nandrolone with strenuous exercise and exercise groups were significantly higher than that in the nandrolone group ($p < 0.05$). There was no significant difference in terms of the total antioxidant capacity between the nandrolone and the control group. The ratio of kidney weight to body weight increased significantly in the nandrolone and nandrolone with strenuous exercise groups compared to that in the control group ($p < 0.03$). There was no significant difference found between the exercise and control group in terms of kidney weight to body weight ratio.

Fig. 1 shows microscopic fibrosis scores in different parts of nephron tubules in different groups. There were no lesion score in the glomerulus, proximal tubules, and distal tubules in the control group (grade 0). The microscopic lesion score in the glomerulus, proximal tubules, and distal tubules of exercise group was 1, which is an indication of minimal fibrosis thickening of the kidney tissue. The microscopic lesion scores in the glomerulus, proximal tubules, and distal tubules of the nandrolone, and nandrolone with strenuous exercise groups were 4 to 5, which is an indication of increased fibrosis with definite damage to the kidneys architecture and formation of fibrosis bands or small fibrosis masses. The ratio of proliferated cells (PCNA-positive indices) in different parts of nephron is shown in Fig. 2 and Table 3. The PCNA-positive indices were dramatically increased in the nandrolone, and nandrolone with strenuous exercise treated groups compared to the control group ($p < 0.05$). There were no significant differences between the exercise and the control group. In the nandrolone with strenuous exercise group, PCNA-positive indices were significantly higher than that in the nandrolone group ($p < 0.05$). In the proximal section of nephron tubules, the ratio of PCNA-positive indices showed a significant increase in the nandrolone, and nandrolone with strenuous exercise treated groups compared to the control group ($p < 0.05$). PCNA-positive indices did not differ significantly between the exercise group and the control group. In the distal portion of nephron tubules, the percentage of PCNA-positive indices of nandrolone, and nandrolone with strenuous exercise treated groups were significantly higher than that in the control group ($p < 0.05$). There were no significant differences between the exercise group (1.5%) and the control group in terms of PCNA-positive indices percentage.

4. Discussion

Among the various adverse effects of administering extra doses of AAS in different organs, the impact of supra-physiological doses of AAS on kidneys function has received little attention (Riezzo, et al., 2014). The results of the current study provide strong evidence that long-term administration of supra-physiological doses of nandrolone decanoate with or without severe exercise could lead to alterations in the kidney function and structure along overexpression of nephrin and podocin genes in the experimental rats' kidneys. Among dozens of molecular mediators and genes expression related to kidney function, mutations in expression of genes encoding nephrin and podocin were shown to play a major role in the alteration of glomerular permeability and early onset of nephritic syndrome in humans (Boute et al., 2000). Mechanistically, nephrin interacts with various other transmembran proteins such as podocin, CD2AP, as well as other proteins, which still need to be identified, to build the filtration complex at the slit diaphragm in the glomerular podocyte. Regarding the special structural and signaling functions of every protein in filtration complex, the stoichiometric ratio between filtration complex proteins is essential for a normal functioning of ultrafiltration (Benzing, 2004). Alteration in genes expression associated with each element in the filtration complex lead to a dysfunction of the barrier, flattening of foot process, and uncontrolled permeability of the glomerular filtration barrier (Schaefer et al., 2004). The underlying molecular mechanism through which the nandrolone exerts its over-expressive effects on nephrin and podocin genes expression still is not very well understood, but the action mechanism of all AASs is similar to all other steroid hormones (Tsitsimpikou et al., 2016). In general, all steroid hormones had receptors at cellular level and their effects were exerted by binding to receptors, which were translocated to binding sites on chromatin, then promoted gene transcription and stimulated the production of mRNA, and finally increased protein synthesis (Maravelias, et al., 2005). In agreement with results of our study, it has been previously reported that androgens induce the transcription of a number of genes in the mouse kidneys (Asadi et al., 1994). In addition, previous studies have indicated a parallel association between overexpression of nephrin mRNA and renal failure demonstrated by an increased protein concentration in the urine (Schaefer, et al., 2004). To the best of our knowledge, this is the first *in vivo* study showing that nandrolone exposure with or without severe exercise influences the kidney function by overexpression of genes related to nephrin and podocin as two key proteins of slit pores.

Interestingly, the current study results, along with the “overexpression of nephrin and podocin genes” showed a significant increase in plasma cystatin C and creatinine levels, cystatin C/creatinine ratio,

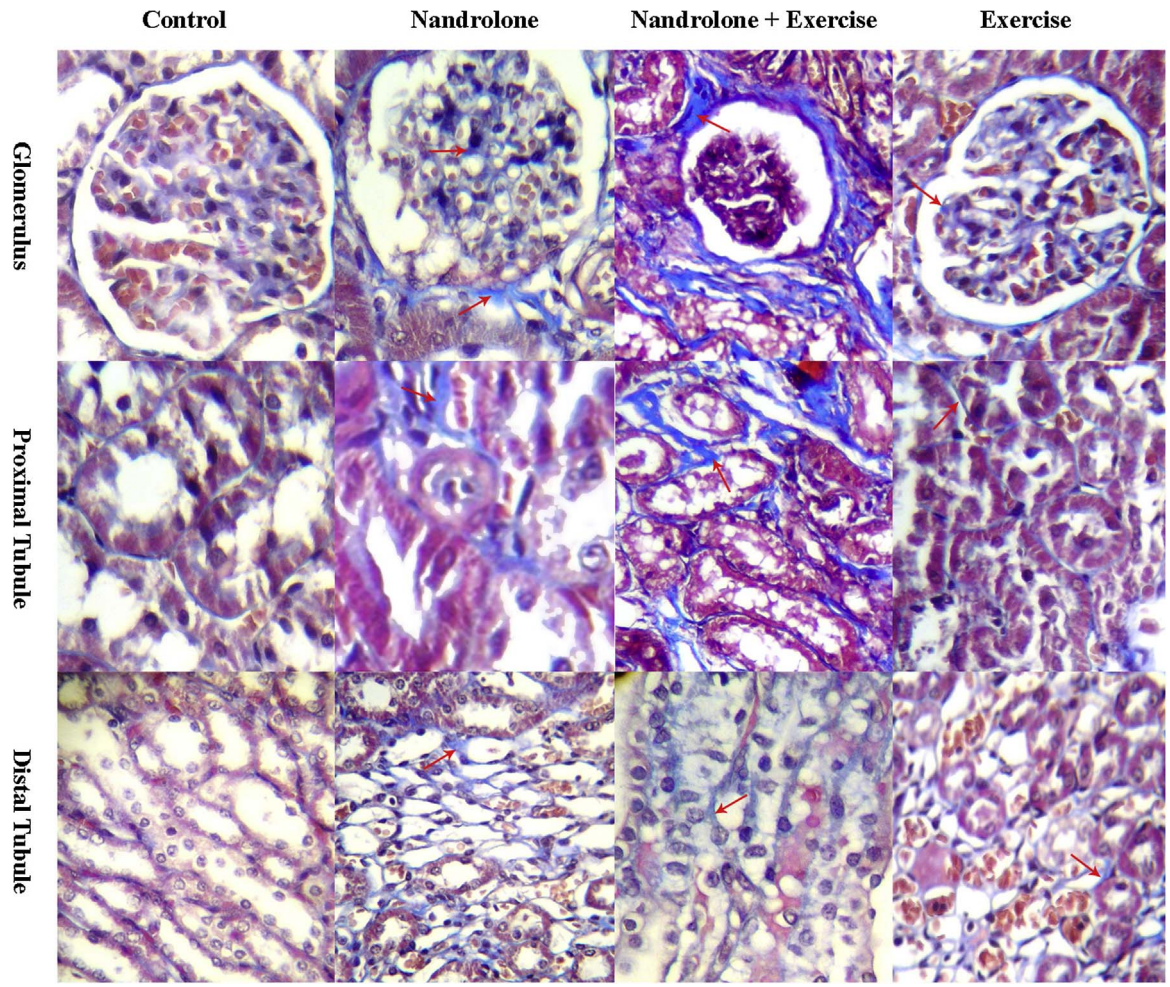

Fig. 1. Photomicrograph of kidney tissue of rats (Masson trichrome staining). In samples obtained from the different groups. (Original magnification ×400). Fibrosis band (⟶).

and a significant decrease in creatinine clearance as an indicator of glomerular filtration rate in the treated groups compared to the control group. All mentioned parameters are indicators of the kidney dysfunction and abnormality. The most commonly used endogenous predictor markers of the kidney function are serum creatinine and cystatin C values. The reduced glomerular filtration rate estimated by calculating creatinine clearance along with the elevated plasma cystatin C levels indicated that nandrolone consumption influenced renal function in rats that received nandrolone with or without severe exercise. Although plasma creatinine and cystatin C are two golden markers for prediction of the kidney function, the Achilles heel of these markers as a predictor of the kidney function is that their amount is affected by some non-renal factors such as body weight, higher white blood cells count, and increased markers of inflammation (Stevens et al., 2009; Tangri et al., 2011). To overcome this issue, Grubb et al. recommended the ratio of cystatin C/plasma creatinine be used as an indicator of alterations in the glomerular filtration quality (Grubb et al., 2015). The ratio of serum syctatin C/creatinine increased due to shrinking of glomerular pores in a condition named 'shrunken pore syndrome'. This incident manipulates the composition of glomerular filtrate as follows: serum concentrations of large molecules such as cystatin C increase when pore size is reduced a little, but more pore shrinkage leads to retention of smaller molecules such as urea and creatinine, which results in their accumulation in the blood (Lund et al., 2003; Shirpoor et al., 2013). When a lower degree of pore shrinkage occurs, cystatin C is retained more than creatinine because its molecule is 100 times larger than creatinine (Hayashi et al., 2008). Therefore, the ratio of cystatin C/creatinine can serve as an indicator of the kidney dysfunction at an earlier stage (Hayashi, et al., 2008). Interestingly, the current study results revealed a significant increase in the cystatin C/creatinine ratio in nandrolone and nandrolone with strenuous exercise groups compared to the control group. In the current study, oxidative damage and inflammatory reaction were also evidenced by oxidative DNA damage (8-OHdG) elevation, kidney cells proliferation, and fibrosis in the kidneys obtained from the treated groups, compared to the control group. The mechanism through which nandrolone induced cell proliferation and fibrosis is not fully understood, but it may have resulted from oxidative stress and inflammatory reactions. Our recent work and others have indicated that exposure of rats to nandrolone leads to enhancement of pro-inflammatory and oxidative stress markers such as Il-1β, HSP90, Ox-LDL, NADPH oxidase, 8-OHdG, as well as heart tissue fibrosis and proliferation (Riezzo, et al., 2014; Tofighi, et al., 2017). In addition, the key role of reactive oxygen species and inflammatory cytokines in promoting the kidney tubular proliferation and kidney fibrosis has been evidenced by previous studies (Shirpoor, et al., 2016). Regarding the total antioxidant capacity level in the kidney tissue, no significant differences between the nandrolone and control group were observed. Total antioxidant capacity was significantly higher in kidneys obtained from nandrolone with strenuous exercise, and strenuous exercise groups compared to the control and nandrolone groups. Similar to our study, previous studies have reported that nandrolone had no marked effect on the antioxidant protection system or the total antioxidant capacity in the kidneys and heart tissues of the animal (Riezzo, et al., 2014; Tsitsimpikou, et al., 2016). Oxidative and inflammatory nature of nandrolone documented by previous studies, on the one hand, and oxidative DNA damage along with tissue

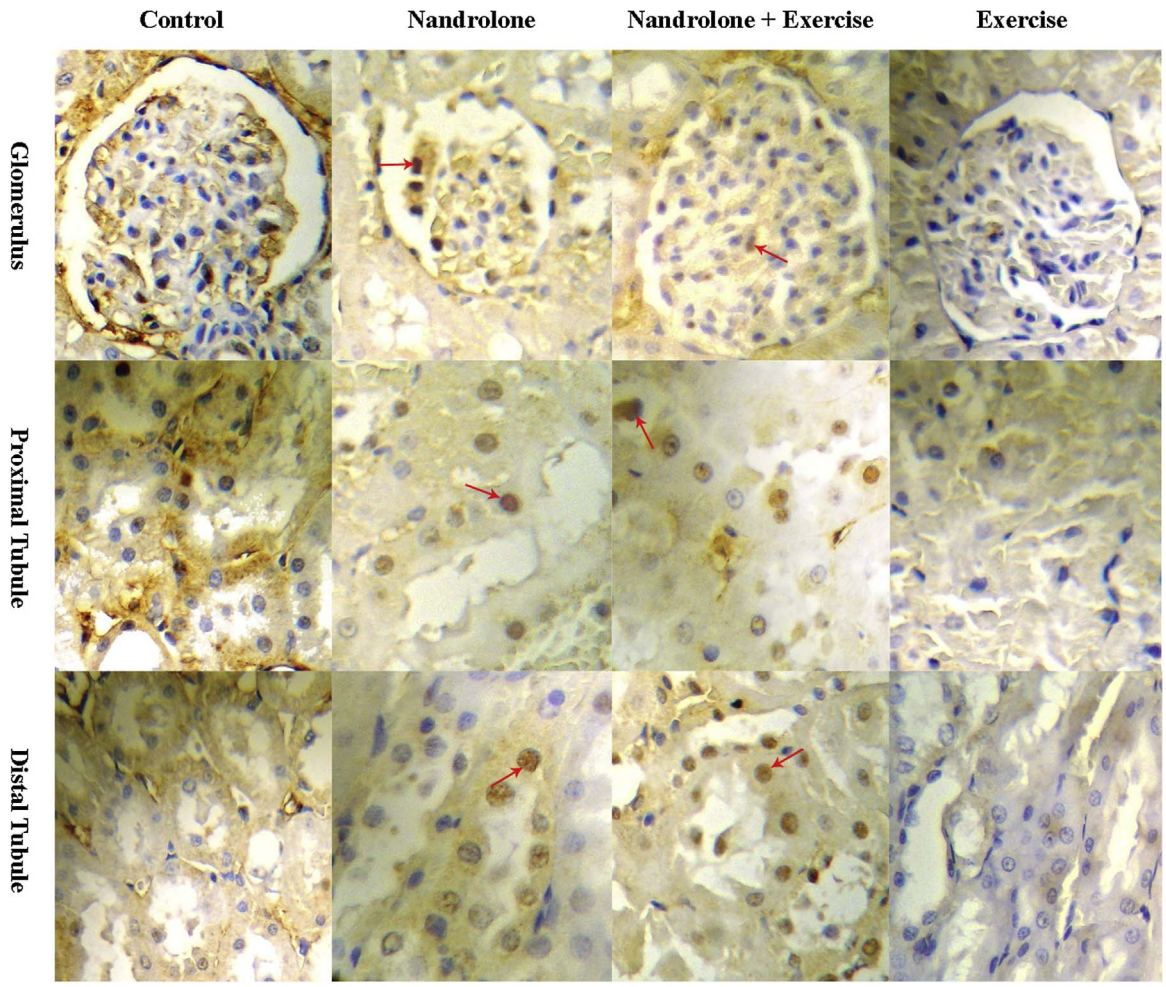

Fig. 2. Immunohistochemical staining of kidney tissue by proliferating cell nuclear antigen(PCNA) anti-body showed mild to moderate kidney cell proliferation, in nandrolone, and nandrolone with strenuous exercise treated groups compared to the control group (Original magnification ×400). PCNA positive indices (⟶).

Table 3
ratio of proliferated cells (PCNA-positive indices) in different parts of nephron

		Control	Nandrolone	Nandrolone + Exercise	Exercise
The ratio of proliferated cells (PCNA-positive indices) (%)	Glomerulus	1	30 ± 4*	45 ± 6*,†	1.5 ± 1
	Proximal Tubules	1	58.5 ± 3*	42 ± 4*,†	1.5 ± 1
	Distal tubules	1	47 ± 7*	48 ± 4*	2 ± 1

Values are mean ± SE for 8 rats per group.
* Denotes significant difference ($p < 0.05$) compared to the control.
† Denotes significant difference ($p < 0.05$) compared to the nandrolone group.

fibrosis and cell proliferation, on the other hand, led us to speculate that nandrolone exerts its hazardous effects on kidneys tissue through oxidative stress and inflammatory reaction.

The second issue addressed in the current study was finding an answer to the question that whether exhaustive exercise combined by nandrolone mitigates nandrolone hazardous effects on the kidney tissue through oxidative stress and inflammatory reaction or promotes it. Previous studies have shown that exhaustive exercise alone exerts deleterious effects on different tissues and organs through oxidative stress. Furthermore, it enhanced formation of reactive oxygen (ROS) and nitrogen species (RNS) (Riezzo et al., 2014; Sarmiento et al., 2016) (6,7,8). Sachdev and Davies have recently described mechanisms responsible for exercise-induced free radical formation, includeing an increase in oxygen consumption (and ROS production), autoxidation of catecholamines, activation of inflammatory cells caused by muscle tissue damage and ischemia and/or hypoxia/reoxygenation damage (Sachdev and Davies, 2008)

In the current study, there were moderate differences between the exhaustive exercise group and control group regarding the kidney structure alterations, kidney functional indicators such as creatinine clearance and plasma cystatin C amount, and gene expressions. Despite the kidney function and structure alteration in the exercise group, the total antioxidant capacity showed a significant increase in the exhaustive exercise group compared to the control group, but the 8-OHdG amount showed no significant difference in comparison to the control group. It is possible that the given exercise load or duration in the current study, was not enough to cause overtraining and oxidative stress, but it was sufficient to induce some structural and functional alteration through other ways such as inflammation. In addition, previous studies have shown that severe physical training alone has various effects (increase, decrease, or no change) on oxidative DNA damage or amounts of *-OHdG (Aaltonen, et al., 2001). Moreover, according to the available literature, training has contrary effects on antioxidant enzymes system. For example, vigorous training does not influence heart or liver antioxidant enzymes system as much as it influences the skeletal muscles (Powers et al., 1993). It is possible that different effects of training on various antioxidant enzymes be reflective of the specific cellular locations where ROS are produced. They may also reflect the basal antioxidant capacity in various tissues.

In the current study, some biochemical and histological alterations

such as fibrosis, urine urea and creatinine, as well as creatinine clearance or GFR were more pronounce in the nandrolone with strenuous exercise group compared to the nandrolone treated group, but kidney cell proliferation, genes expression, cystatin C, and 8-OHdG amounts showed no significant differences in the nandrolone with strenuous exercise group compared to the group treated only by nandrolone. According to the current study results, severe exercise training alongside nandrolone exposure may predispose kidney tissue to more damage than when nandrolone is administrated alone. By the way, in our opinion, further research is required in comprehensive details to determine whether strenuous exercise alongside nandrolone consumption augments or does not change renal toxic effects of nandrolone. In conclusion, our work raises three notable points: first, the exposure to chronic nandrolone causes overexpression of nephrin and podocin genes, as two proteins of slit diaphragm in the glomerular podocyte that play a major role in the alteration of glomerular permeability, as well as an increase in the serum cystatine C and a decrease in creatinine clearances. All these processes are known as indicators of renal failure. The second point is that nandrolone-induced genes overexpression and renal failure correlate with oxidative DNA damage and also with the kidney tissue proliferation and fibrosis, and they provide strong evidence for the occurrence of oxidative stress and inflammatory reaction under nandrolone exposure. The third point we established is that exhaustive exercise concurrent with nandrolone promotes some biochemical and histological alterations by nandrolone in the kidneys of rats. Based on our results, we conclude that nandrolone exerts its deleterious effects on the kidney, at least in part, by nephrin and podocin genes overexpression and enhancing the serum cystatine C level mediated by oxidative stress and inflammation. However, further research is still required to elucidate the comprehensive details of the subjects. In addition, whether nandrolone exposure induces renal failure via overexpression of nephrin and podocin genes still needs to be discovered by studies using microarray analysis to clarify underlying molecular mechanism of the subjects.

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Conflict of interest

None.

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