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EVALUATION OF PTEN AND PI3K/AKT EXPRESSIONS IN STANOZOLOL-TREATED RAT KIDNEYS

STANOZOLOL UYGULANAN SIÇAN BÖBREĞİNDE PTEN VE PI3K/AKT EKSPRESYONLARININ DEĞERLENDİRİLMESİ

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ABSTRACT

Objective: Stanozolol is an anabolic androgenic steroid (AAS) that is widely used by teenagers and athletes in bodybuilding, sports, and athletics. The potential effects of stanozolol in kidney functions have not been defined. In this study we investigated the expression of tumor suppressor protein phosphatase and tensin homolog (*Pten*) and mRNA levels of phosphatidylinositol 4,5-bisphosphate 3 kinase (*Pi3k*) and the protein kinase B (*Akt1*) signaling pathway in rat kidneys treated by stanozolol.

Materials and Methods: Rats were randomized to 5 groups as control, solvent control, steroid (stanozolol), solvent-exercise, and steroid-exercise. Subcutaneous injection of stanozolol (5 mg/kg) was applied for 28 days and swimming exercise was performed 20 min/day, 5 days/week in exercise groups. Expression of PTEN was evaluated by immunohistochemistry. Also, *Pten*, *Pi3k*, and *Akt1* mRNA expressions were analyzed via RT-PCR.

Results: Mesangial cells and renal tubules in the control, solvent control, and solvent exercise groups showed strong (+++) PTEN reactivity against weak PTEN reactivity in the steroid group. Moderate PTEN reactivity was detected in cells of the steroid exercise group. *Pten* mRNA expression was significantly decreased, and *Pi3k* and *Akt1* mRNA expression were significantly increased in the steroid group versus other groups ($p<0.001$). *Pten* expression showed increase while *Pi3k* and *Akt1* expression showed decrease with exercise treatment ($p<0.05$).

Conclusion: Our findings suggest that AAS usage may inhibit PTEN expression in kidneys, which can be associated with increased *Pi3k* and *Akt1* mRNA levels. Exercise performed with AAS usage can be protective on stanozolol-exposed kidneys due to increased levels of PTEN expression and decreased levels of *Pi3k* and *Akt1*.

Keywords: Stanozolol, kidney, *PTEN, PI3K, Akt1*

ÖZET

Amaç: Stanozolol, gençler ve sporcular tarafından vücut geliştirme, spor ve atletizmde yaygın kullanımı olan bir anabolik androjenik steroiddir (AAS). Stanozololün böbrek fonksiyonlarındaki potansiyel etkileri tanımlanmamıştır. Bu çalışmada, stanozolol ile tedavi edilen sıçan böbreklerinde tümör baskılayıcı protein fosfotaz tensin homolog (*Pten*), Phosphatidylinositol-3-kinase (*Pi3k*) ve *protein kinaz B* (*Akt1*)'nin mRNA düzeylerinin ekspresyonunu araştırdık.

Gereç ve Yöntem: Sıçanlar, kontrol, çözücü kontrol, steroid, çözücü kontrol egzersiz, steroid egzersiz olarak 5 gruba ayrıldı. Yirmi sekiz gün boyunca subkutan stanozolol enjeksiyonu (5 mg/kg) uygulandı ve egzersiz gruplarındaki hayvanlara 20 dk/gün, 5 gün/hafta yüzme egzersizleri yaptırıldı. PTEN ekspresyonu immünohistokimya ile değerlendirildi. Ayrıca *Pten*, *Pi3k* ve *Akt1* mRNA ekspresyonları RT-PCR yoluyla analiz edildi.

Bulgular: Kontrol, çözücü kontrol ve çözücü kontrol egzersiz gruplarındaki mezanjiyal hücreler ve böbrek tübülleri, steroid grubunda saptanan zayıf PTEN reaktivitesine karşı güçlü (+++) PTEN reaktivitesi gösterdi. Steroid egzersiz grubunun mezanjiyal ve tübül hücrelerinde orta düzeyde PTEN reaktivitesi saptandı. *Pten* mRNA ekspresyonu, steroid grubunda kontrol ve diğer gruplara göre anlamlı düşüş gösterdi, *Pi3k* ve *Akt1* mRNA ekspresyonu anlamlı olarak arttı ($p<0.001$). Egzersiz tedavisi ile *Pten* ekspresyonu artış, *Pi3k* ve *Akt1* ekspresyonu azalma gösterdi ($p<0.05$).

Sonuç: Bulgularımız, AAS kullanımının, artan *Pi3k* ve *Akt1* mRNA seviyeleri ile ilişkili olabilecek böbreklerde PTEN ekspresyonunu inhibe edebileceğini düşündürmektedir. AAS kullanımı ile yapılan egzersiz, PTEN ekspresyon seviyelerinin artması ve *Pi3k* ve *Akt1* seviyelerinin düşmesi nedeniyle stanozolole maruz kalan böbrekler üzerinde koruyucu olabilir.

Anahtar Kelimeler: Stanozolol, böbrek, *PTEN, PI3K, Akt1*

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INTRODUCTION

Anabolic androgenic steroids (AAS), which are synthetic testosterone derivatives, exert their androgenic effects via enhancing anabolic activity. These effects cause increases in muscle growth and strength, improving athletic performance. AASs are prescribed for hypogonadism. They prevent muscle mass loss during medical use, however, these types of chemicals are used by non-expert athletes due to their anabolic effects (1-3). An abusive dose of AAS is 10–100 times higher than its pharmacological dose. These higher doses cause several adverse effects that include hepatotoxicity, reproductive system problems, cardiovascular problems, psychiatric and behavioral disorders, and cancer (4). It has been documented that AASs have different types of adverse effects, such as acute kidney injury, chronic kidney diseases, glomerular toxicity, etc., in kidneys (5). One of the adverse effects of AAS misuse is renal failure in bodybuilders and athletes (6, 7). It has been reported that an increase in athletes' serum creatinine levels may be due to AAS abuse. Serum creatinine, blood urine nitrogen (BUN), and uric acid levels can increase through steroid use (8). In some cases, Wilm's tumor development was reported in athletes (9). It has been reported that administration of stanozolol can cause renal failure in some cases. Stanozolol is a C17α-alkylated derivative of testosterone and is used as an AAS (3, 4, 10-13).

The *PTEN*/*PI3K*/*AKT1* pathway is an important modulator of cell proliferation and cell death (14). The *PTEN/PI3K/AKT1* signaling inhibition suppresses cellular adhesion and induces apoptosis (15). Tumor suppressor *PTEN* (the phosphatase and tensin homolog gene) dysfunction has an important role in different types of cancer. Mutations, deletions, and protein folding disruptions may cause *PTEN* function loss. *PTEN* dysfunction negatively affects the phosphoinositide 3-kinase (*PI3K*) signaling pathway that causes phosphatidylinositol (3-5)-triphosphate accumulation. These accumulations result in inhibition of the *PI3K/AKT1* pathway (16). In different studies and case reports it has been reported that AASs cause kidney failure, however, the potential effects of stanozolol in kidney functions have not been evaluated. It has been known that exercise has benefits in the whole-body system and regular exercise activity has a preventive role against kidney cancer (17, 18).

In this study, we aimed to clarify the effects of stanozolol on the expression of the tumor suppressor protein PTEN and the levels of *Pten*, *Pi3k*, and *Akt1* mRNA in rat kidneys, and also if there is a potential protective effect of exercise on kidneys.

MATERIALS AND METHODS

Animal study

Study procedures were approved by the Istanbul University Institutional Animal Care and Use Committee (protocol number of Animals Ethics approval: HADYEK; 2013-100). Thirty-four 8 month-old, 230 gr. Sprague Dawley rats were used in this study. The animals were divided into 5 groups: group I - control group (n=5), group II - solvent (propylene glycol) control (n=5), group III - steroid (stanozolol 5 mg/kg) (n=8), group IV - solvent exercise (n=8), group V - steroid exercise (n=8). Subcutaneous injections of stanozolol (5 mg/kg) and vehicle propylene glycol (1 ml/kg) were applied for 28 days, once a day. Through the experiments, swimming exercise was applied to the exercise groups for 20 minute a day, 5 days/week. After animal scarification under anesthesia, one of the kidneys was fixed with 10% neutral buffered formalin and the other was stored at -80°C after being frozen with liquid nitrogen.

Total RNA extraction and cDNA synthesis

Twenty mg tissue samples obtained from animals were incubated with RNA Stabilization Reagent (RNAlater; Qiagen), then TissueLyser II (Qiagen) was added. RNA purification was performed with RNeasy Mini Kit (Qiagen) in QIAcube (Qiagen) and the manufacturer's instructions were followed. Reverse transcription to cDNA was performed with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). cDNA quality was assessed with an Epoch Spectrophotometer System and Take3 Plate (BioTek, Winooski, VT, USA).

Relative quantification of the gene expression

StepOnePlus Real-Time PCR System technology (Applied Biosystems) was used to evaluate *Pten*, *Pi3k*, and *Akt1* mRNA expression analyses with TaqMan Probe-based technology (Primer Design Ltd., Southampton, UK). Study results were shown as relative fold increase/decrease compared with the control animals using the $2^{-\Delta\Delta Ct}$ method (Livak, KJ and Schmittgen, TD). The primer sequences are shown in Table 1. All assays were done in triplicate and on three different days. For each sample, 100 ng cDNA was added into the PCR mix with 1 µL of

Table 1: The primer sequences for real time PCR

Gene	Forward (5′–3′)	Reverse (5′–3′)	Assay ID
Pten	TTGGCGGTGTCATAATGTCT	GCAGAAAGACTTGAAGGCGTA	Rn00477208
Pı3k	AACACAGAAGACCAATACTC	TTCGCCATCTACCACTAC	Rn01769524
Akt1	GTGGCAAGATGTGTATGAG	CTGGCTGAGTAGGAGAAC	Rn00583646
β-actin	TGGTGGGTATGGGTCAGAAG	GACAATGCCGTGTTCAATGG	Rn00667869

Primer Perfect Probe and QuantiTect Probe PCR Master mix (Qiagen). Beta-actin was used as a reference. Total reaction mix was 20 µL for each sample. The cycle procedure was heating for 2 min, 95°C for 10 min, then 40 cycles of 15 sec. at 94°C and 60 sec. at 60°C (19).

Immunohistochemistry

PTEN expression was evaluated by the immunohistochemistry method. Kidney tissues were fixed with 10% formaldehyde and embedded in paraffin. Sections from the paraffin block (approximately 2–3 µm) were taken with a microtome and emplaced on charged microscope slides. After overnight incubation at 56°C, sections passed one by one decreasing alcohol series for the rehydration process. Then sections were incubated in 5% hydrogen peroxide (in methanol) for 15 min. for blockage of endogen peroxidase. Sections were washed with PBS. 10% citrate buffer incubation in a microwave was performed 3 times for 5 min for the antigen retrieval process. After cooling of sections, 1/100 diluted PTEN primary antibody (Abbiotec 251264) was added onto sections and incubated overnight at +4°C. Then sections were washed with PBS and subsequently biotin-labeled secondary antibody and DAB chromogen solutions were performed for 10 min each. After washing with distillated water, sections were dyed with Mayer's hematoxylin for 30 seconds. Then sections were washed with tap water, covered with coverglass, and scored under light microscopy. In the immunohistochemistry evaluation, the tissue samples of each animal were examined and the data were evaluated. PTEN antibody staining intensity was scored according to the overall intensity with 3 levels (+ = weak staining; ++ = moderate staining; and +++ = strong staining).

Statistical analysis

We used SPSS version 20 software (IBM Corp., Armonk, NY, USA) for statistical analyses for gene expression evaluation only between all 6 groups. Mean value and standard deviation were calculated. Comparison of means was performed by ANOVA one-way analysis of variance and the Tukey test. Differences were considered significant if $p<0.05$.

RESULTS

Gene expression

The gene expression levels of all groups are summarized in Table 2. In our study, upon completion of the treatment, the expression of *Pten, Pi3k,* and *Akt1* mRNA was measured using RT-PCR. *Pten* mRNA expression downregulated significantly in the steroid group ($p<0.05$) compared to other experimental groups. In the steroid exercise group, *Pten* upregulated significantly compared to the steroid group ($p<0.001$) (Figure 1). A significant in-

Table 2: Gene expression levels of treated animal groups

	PTEN		PI3K		AKT	
	Mean	SD	Mean	SD	Mean	SD
Groups						
Control	2.02	±0.28	1.37	±0.13	1.29	±0.17
Solvent control	1.78	±0.21	1.41	±0.26	1.58	±0.22
Steroid	0.57	±0.03	3.04	±0.48	3.21	±0.39
Solvent exercise	2.17	±0.31	1.46	±0. 23	1.19	±0.16
Steroid exercise	1.3	±0.18	2.05	±0.3	2.26	±0.24

PTEN: phosphatase and tensin homolog, PI3K: phosphoinositide 3-kinase, AKT: protein kinaz B, SD: Standard deviation

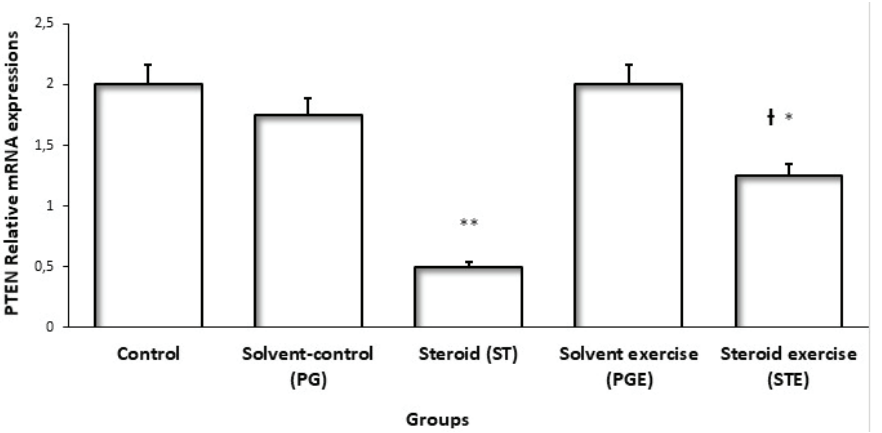

Figure 1: Compares the *Pten* mRNA expression levels. Significant changes steroid versus other groups (**$p<0.05$), and steroid exercise versus steroid group (*$p<0.001$), steroid exercise versus other groups (ŧ$p<0.001$).

crease in *Pi3k* mRNA expression was seen in the steroid group compared to other groups (p<0.05). A significant increase in *Pi3k* mRNA expression was also seen in the steroid exercise group compared with the control, solvent control, and solvent exercise groups (p<0.001), but decrease was detected compared with the steroid group (p<0.001) (Figure 2). The steroid treatment group led to a significant increase in *Akt1* mRNA expression compared with the other groups (p<0.05). The steroid exercise treatment group led to a significant increase in *Akt1* mRNA expression compared with the control, solvent control, and solvent exercise groups (p<0.001) but decreased compared with the steroid group (p<0.001) (Figure 3).

Immunohistochemistry

The results of the PTEN immunoreactivity are summarized in Table 3. Similar reactivity was detected in light

Table 3: PTEN immunohistochemistry reactivity scoring

Pten reactivity	Control	Solvent control	Steroid	Solvent exercise	Steroid exercise
Proximal tubules	-	-	-	-	-
Distal tubules	+++	+++	+	++	++
Mesangial cells	+++	+++	-	+++	-
Medullar tubules	++	++	+	++	++

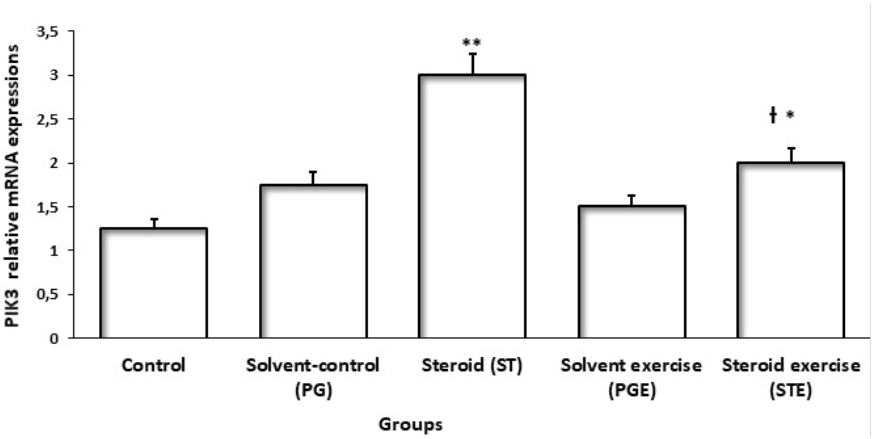

Figure 2: Compares the *Pi3k* mRNA expression levels. Significant changes steroid versus other groups (**p<0.05), and steroid exercise versus steroid group (*p<0.001), steroid exercise versus other groups (†p<0.001).

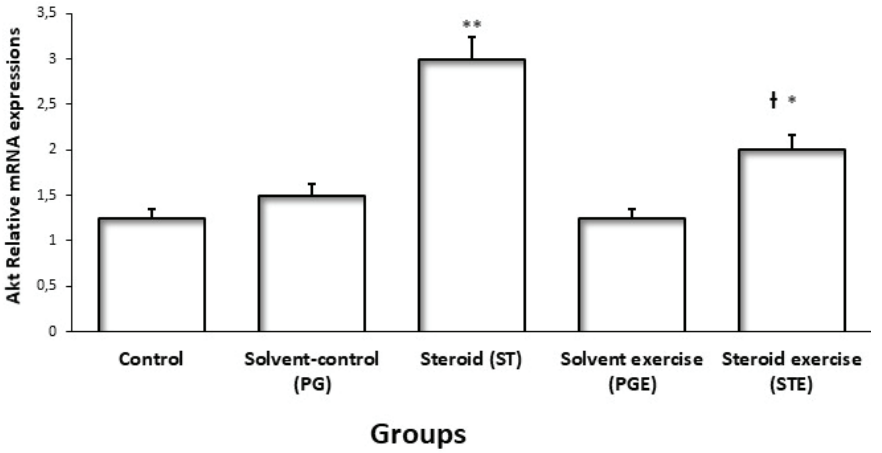

Figure 3: Compares the *Akt1* mRNA expression levels. Significant changes steroid versus other groups (**p<0.05), and steroid exercise versus steroid group (*p<0.001), steroid exercise versus other groups (†p<0.001).

microscopic evaluation of the control, solvent control, and solvent exercise groups. In these groups, glomerular mesangial cells had a strong (+++) PTEN reaction. Cells of distal tubules had strong (+++) immunoreactivity but proximal tubules did not show any reaction. Tubules of medulla showed a moderate (++) reaction. In the steroid-treated group, mesangial cells and proximal tubule cells did not show PTEN immunoreactivity. A few distal tubules showed weak (+) reaction. Medullary tubules also had weak (+) immunoreactivity. In the steroid exercise group, no immunoreactivity was detected in mesangial cells or proximal tubules. But moderate (++) reaction in distal tubules and medullary tubules was detected (Figure 4).

DISCUSSION

Synthetic AAS Stanozolol is a substance that has functions like testosterone. This chemical is used to enhance athletic performance and improve aesthetics (20). Higher doses of AAS can cause several types of adverse effects on the cardiovascular system, reproductive system, urinary system, and hepatic system, and mental health problems (21). AAS mimics testosterone's physiological effect by inducing an altered expression on DNA sequences. Reports showed a close relationship between AASs and cancer formation, progression, and metastases. The metabolites of AASs are inducers of cell proliferation. Like testosterone, AASs are metabolized to 17 beta-estradiol and play an important role in estrogen-dependent can-

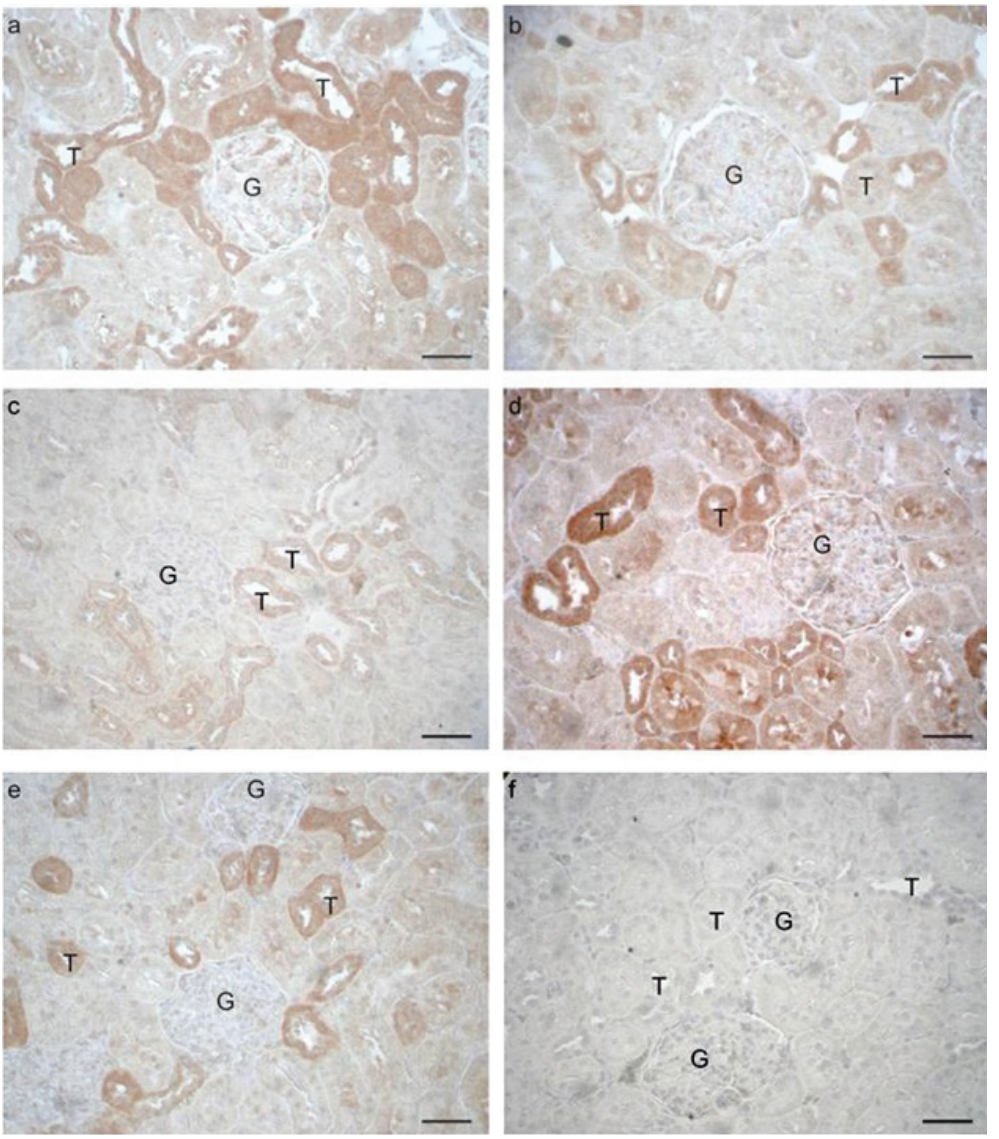

Figure 4: PTEN immunoreactivity, a) Control group, b) solvent control group, c) steroid group, d) solvent exercise group, e) steroid exercise group, G: Glomerulus, T: tubules

cer mechanisms. Additionally, reactive oxygen species (ROS) are increased during AASs catabolism. This can cause genotoxicity and the formation of adenomas (22-25). The misuse of AASs can cause kidney toxicity and increased kidney cancer formation risk (12, 26, 27). Chronic AAS usage can promote apoptosis via oxidative stress in kidney tissue. It has also been reported that AASs induce genetic damage in kidney tissue (27-29). Pak et al. reported that the kidney carcinogenesis mechanism could be triggered by AAS usage through the STAT5 pathway (26). However, AASs' adverse effects on kidney tissue have not been clarified yet. The adverse effects of stanozolol on kidneys have been evaluated in different studies. An excessive dose of stanozolol increased the serum creatinine levels, and induced oxidative stress (4, 10).

One of the major regulating molecular mechanisms of cell proliferation and survival is the *PTEN/PI3K/AKT1* pathway. *AKT1* is an anti-apoptotic factor and its overexpression causes cell cycle arrest and inhibition of cellular death. *AKT1* regulates the apoptosis mechanism via inactivating the pro-apoptotic proteins. Alterations in this pathway are associated with several diseases, including cancer. It has been shown that *PI3K/AKT1* signaling is disrupted in many cancer types, such as breast cancer, colon cancer, ovarian cancer, pancreatic cancer, and prostate cancer. *PTEN* inactivation is associated with *AKT1* activation, which causes tumor cell proliferation. *PTEN* function loss might be the cause of mutation, deletion, or epigenetic modulations at high frequency in many primary and metastatic human cancers (30, 31).

Mutations of the *PTEN* gene and disrupted expression of PTEN were reported in several studies. Nassif et al., reported the reduced or absent immunohistochemical reaction of PTEN in primary sporadic colorectal cancer (32). Also, immunohistochemical and RT-PCR analysis has shown a complete loss of *PTEN* mRNA expression in anaplastic thyroid cancers (33). Wang et al. reported mutations in *PTEN* genes in late-stage bladder carcinoma, and Wu et al. also showed loss of *PTEN* expression in melanoma (34, 35). Kanamori et al. reported a negative correlation between *PTEN* and *AKT1* expression in endometrial carcinoma cells (36). Breuksch et al. demonstrated that *PTEN* function loss can play a role in kidney tumor progression (37). In our study, we detected loss of PTEN expression in mesangial cells and cells of kidney tubules due to stanozolol treatment. Our results are consistent with the referenced literature. Several studies reported that androgens and AASs can cause cell proliferation and survival due to the activation of the PI3K/AKT pathway. Sirianni et al. evaluated the effects of nandrolone and stanozolol on breast cancer cell proliferation and detected increased *AKT1* phosphorylation and stimulation of the *PI3K/AKT1* and PLC/PKC pathways (38). It has been reported that androgens stimulate ovarian cancer cell survival via telomerase activity and the *PI3K/AKT1* pathway (39). In our study, the steroid-treated group showed increased mRNA expression of *Pi3k* and *Akt1*. This increased activation of *Pi3k/Akt1* expression is associated with decreased *Pten* expression.

Also, in the steroid exercise group, we detected increased expression of *Pten* with the decreased expression of *Pi3k* and *Akt1* against the steroid group. Physical inactivity is related to cancer development. Recent studies showed that physical activity is effective in reducing the risk of various cancers like pancreatic, colon, prostate, lung, ovarian, breast, and endometrial (40-42). Yu et al. have reported that exercise increased the *Pten* expression levels of mice skin cells compared with a sedentary control group and prevented the risk of skin cancer development. Also, benefits of regular exercise on the development of hepatocellular carcinoma were reported (43). There is no reported article about the protective effects of exercise on kidneys. Our findings suggest that exercise may have a protective effect on kidneys exposed to stanozolol.

As a result of our findings, we suggest that stanozolol usage can cause possible renal cancer development via decreased *Pten* expression and the increased *Pi3k/Akt1* pathway. According to our results, the *Pten* gene expression profile was in accordance with PTEN immunohistochemistry results. Daily exercise during AAS treatment may be beneficial to kidney health and can decrease the risk of cancer development. In this study, the inability to evaluate the parameters associated with kidney dysfunction in the blood in animals and the inability to examine the expression levels of genes and proteins belonging to more molecular pathways in the kidney are limiting factors. In conclusion, in more detailed and long-term chronic studies, the correlation between the *Pten* gene and protein expression levels will be investigated and even more molecular pathway elements can be evaluated.

Informed Consent: Written consent was obtained from the participants.

Ethics Committee Approval: Ethics committee approval was received for this study from the Istanbul University Animal Experiments Local Ethics Committee (Date: 30.09.2013, No: 100).

Peer Review: Externally peer-reviewed.

Author Contributions: Conception/Design of Study-Ç.S., M.K.; Data Acquisition- T.K., Ç.S., M.K.; Data Analysis/Interpretation-T.K., Ç.S., M.K.; Drafting Manuscript- T.K., Ç.S., M.K.; Critical Revision of Manuscript-T.K.,M.K.; Approval and Accountability-T.K., Ç.S., M.K.

Conflict of Interest: Authors declared no conflict of interest

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