Cukurova Medical Journal
ÇUKUROVA ÜNİVERSİTESİ TIP FAKÜLTESİ

Cukurova Med J 2025;50(4):955-963
DOI: 10.17826/cumj.1662549

RESEARCH

Effects of cyproterone acetate treatment interruption on testicular histology: an experimental rat study relevant to transgender females with incomplete sperm cryopreservation

Siproteron asetat tedavisinin kesilmesinin testiküler histoloji üzerindeki etkileri: sperm kriyoprezervasyonu tamamlanmamış transseksüel kadınlarla ilişkili deneysel bir sıçan çalışması

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Abstract

Purpose: The aim of this study was to investigate the reversibility of the deterioration of testicles as Cyproterone acetate (CPA) treatment ceased in an experimental rat model, and possible contribution of two angiotensin converting enzyme inhibitors on the process.

Materials and Methods: In the first step, the effects of CPA treatment (14 days) on testicular histology on rats were investigated. In the second step, following the treatment period of 14 days with CPA, the effects of captopril or lisinopril application (for three days or seven days or 14 days) were investigated. Sixty-six rats in eleven groups were investigated. The testicles of the rats were planned to be examined on the following day of the last treatment schedule. Right testicular weight values were recorded. Testicles were histologically evaluated based on light microscopical findings, and also a scoring system was used.

Results: CPA treatment caused weight loss of the testicles, and caused histological scoring value changes. The second step findings revealed that the weight values of the testicles returned to control values in the third day and thereafter. Also, histological scoring values were indifferent compared to control, statistically. Histopathological findings, noted, besides scoring, showed that edema, present in all rats in the seventh day in physiological serum treatment group, was not the case for captopril groups. Also, in captopril groups, no rat with germ cell depletion was observed.

Conclusion: We conclude that the changes in testicular histology were over in the third day in our rat population, and inflammatory reactions or a delay in recovery may be

Öz

Amaç: Bu çalışmada, deneysel bir sıçan modelinde Siproteron asetat (SA) tedavisinin kesilmesiyle testislerdeki bozulmanın tersine dönebilirliğinin ve iki anjiyotensin dönüştürücü enzim inhibitörünün bu sürece olası katkılarının araştırılması amaçlanmıştır..

Gereç ve Yöntem: İlk aşamada, sıçanlarda SA tedavisinin (14 gün) testis histolojisi üzerindeki etkileri araştırılmıştır. İkinci aşamada, SA ile 14 günlük tedavi süresini takiben, kaptopril veya lisinopril uygulamasının (üç gün veya yedi gün veya 14 gün boyunca) etkileri araştırıldı. On bir grupta altmış altı sıçan incelenmiştir. Sıçanların testisleri son tedavi uygulamasının ertesi günü incelendi. Sağ testis ağırlık değerleri kaydedildi. Testisler ışık mikroskopik bulgulara dayanarak histolojik olarak değerlendirildi ve ayrıca bir skorlama sistemi kullanıldı.

Bulgular: SA tedavisi testislerde ağırlık kaybına ve histolojik skorlama değerlerinde değişikliklere neden olmuştur. İkinci aşama bulguları, testislerin ağırlık değerlerinin üçüncü gün ve sonrasında kontrol değerlerine döndüğünü ortaya koymuştur. Aynı zamanda, histolojik skorlama değerleri istatistiksel olarak kontrole kıyasla farksızdı. Skorlamanın yanı sıra kaydedilen histopatolojik bulgular, fizyolojik serum tedavi grubunda yedinci günde tüm sıçanlarda mevcut olan ödemin kaptopril grupları için söz konusu olmadığını gösterdi. Ayrıca, kaptopril gruplarında germ hücresi azalması hiçbir sıçanda gözlenmemiştir.

Sonuç: Testis histolojisindeki değişikliklerin sıçan popülasyonumuzda üçüncü günde sona erdiği ve enflamatuar reaksiyonların veya iyileşmede gecikmenin anjiyotensin dönüştürücü enzim inhibitörlerinin, özellikle

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Received: 21.03.2025 Accepted: 06.09.2025

prevented by use of angiotensin converting enzyme inhibitors, especially a thiol-group containing agent, captopril.
Keywords:. Captopril, cyproterone acetate, Lisinopril, rats, sprague-dawley, testis, transgender persons

de tiyol grubu içeren bir ajan olan kaptoprilin kullanılmasıyla önlenebileceği sonucuna vardık.

Anahtar kelimeler: Kaptopril, lizinopril, siproteron asetat, sprague-dawley sıçanlar, testis, transseksüel kadınlar

INTRODUCTION

Cyproterone acetate (CPA) is a steroidal antiandrogen, a synthetic progestin, with potent peripheral androgen receptor competitive antagonist properties and with the capability of activating progesteron receptors which in turn causing negative feedback leading to suppression of GnRH secretion[1-4]. Use of CPA as an antiandrogen in addition to the main drug estradiol (E2) to establish the feminine characteristics while lowering serum testosterone levels in transgender women with a functioning gonad was dating back to 1980s[2, 5, 6]. Today, in available countries, CPA is the most frequently prescribed antiandrogen which can lower serum testosterone levels to female reference ranges[3,4]. The relation between lowered serum testosterone levels and complete suppression of spermatogenesis were widely studied[2,3,5,6]. In transgender women, related guidelines advise to cryopreserve sperm before a gender affirming surgery[6-11]. Also, it is clearly advised to cryopreserve sperm before initiating any gender affirming hormonal treatment (GAHT)[7, 8, 11, 12]. However, a large group of transgender women is found to be under GAHT in their admission to healthcare to cryopreserve sperm[8, 11]. To cryopreserve sperm, the cessation of GAHT may take place before any intervention. In the literature, the use of CPA in transgender women was commonly investigated for adverse effects or adverse outcomes, and CPA-related deterioration in histology was proven before[2,3,7,8,11,13-16].

Renin-Angiotensin System (RAS) was shown to affect inflammatory and oxidant processes (also, inhibition of RAS had contributions concerning fertility, inflammatory responses, recovery from injury) on male reproductive organs[17-26]. The effects of CPA treatment on rat testicles were documented in a study where CPA treatment caused germ cell layer depletion and sloughing of spermatogenic cells into the lumen which are indicators of a toxicity[16]. On the other hand, inhibition of RAS by angiotensin converting enzyme inhibitors (ACE-i) were studied in several studies; Al-Maghrebi and colleagues presented that an injury related to ischemia and reperfusion could be decreased by inhibition of RAS pathways, and Gianzo and colleagues emphasized that components of RAS were synthesized in testes and in other male genital tissues, locally, which have contributions to the complex biological systems orchestrating sperm functions[17,24]. Two molecules from ACE-i family were chosen for the study, one of which contains a thiol group in the molecule, namely, captopril.

Here, we tried to investigate the reversibility of histological deterioration in testicles due to CPA use. Also, besides the time interval for a probable recovery, any probable contribution by ACE-i molecules were aimed to be investigated in a rat model. The results of the study were thought to shed light to reproductive potential issues in transgender women with a functioning gonad. In case a recovery could be demonstrated, studies to focus on fertility of transgender women may be of concern.

MATERIALS AND METHODS

Procedure

The study was approved by "Karadeniz Teknik University Animal Care and Ethics Committee" (2014-June-10, file 2014/38). For the study, sixty-six adult male Sprague-Dawley rats, weighing 250-350 grams, were divided into eleven groups (6 rats in each group). The study was performed in Karadeniz Teknik University Animal Experiments Laboratory under the supervision of a group assigned by "Karadeniz Teknik University Animal Care and Ethics Committee" where all surgical and anesthesiological steps were monitored.

The groups were named according to the following convention: X-YYnn, where X is for the step of the study (A for first step studies, B for the second step studies), where YY is for the drug or solution given (PS for physiological serum, CA for cyproterone acetate, CP for captopril, LP for lisinopril), where nn is for the treatment duration (03 for a treatment duration of 3 days, 07 for a treatment duration of 7 days, 14 for a treatment duration of 14 days).

Step-1 studies

The rats in A-PS14 group were given oral physiological serum and solvent-ethanol treatment for 14 days (solvent and physiological serum; 1/3 to 2/3 – once daily).

The rats in A-CA14 group were given oral cyproterone acetate (calculated as 25 mg/kg solved in the solvent-ethanol plus physiological serum; 1/3 to 2/3 - once daily) for 14 days. The rats in A-PS14 and A-CA-14 groups were sacrificed at the fifteenth day.

Step-2 studies

The rats in B-PS03, B-PS07, B-PS14, B-CP-03, B-CP07, B-CP14, B-LP03, B-LP07 and B-LP14 groups were treated for 14 days with cyproterone acetate (calculated as 25 mg/kg solved in the solvent-ethanol plus physiological serum; 1/3 to 2/3 - once daily) and later they were given physiological serum or captopril or lisinopril as follows. The rats in B-PS03 group were given orally the physiological serum treatment for three days following the cyproterone acetate treatment and were sacrificed one day following the last physiological serum treatment. The rats in B-PS07 group were given orally the physiological serum treatment for seven days following the cyproterone acetate treatment and were sacrificed one day following the last physiological serum treatment.

The rats in B-PS14 group were given orally the physiological serum treatment for fourteen days following the cyproterone acetate treatment and were sacrificed one day following the last physiological serum treatment. The rats in B-CP03 group were given orally captopril (30 mg/kg/day) treatment for three days following the cyproterone acetate treatment and were sacrificed one day following the last captopril treatment. The rats in B-CP07 group were given orally captopril (30 mg/kg/day) treatment for seven days following the cyproterone acetate treatment and were sacrificed one day following the last captopril treatment. The rats in B-CP14 group were given orally captopril (30 mg/kg/day) treatment for fourteen days following the cyproterone acetate treatment and were sacrificed one day following the last captopril treatment. The rats in B-LP03 group were given orally lisinopril (5 mg/kg/day) treatment for three days following the cyproterone acetate treatment and were sacrificed one day following the last lisinopril treatment. The rats in B-LP07 group were given orally lisinopril (5 mg/kg/day) treatment for seven days following the cyproterone acetate treatment and were sacrificed one day following the last lisinopril treatment. The rats in B-LP14 group were given orally lisinopril (5 mg/kg/day) treatment for fourteen days following the cyproterone acetate treatment and were sacrificed one day following the last lisinopril treatment.

The rats were kept in room temperature 21$\pm$2°C. Twelve hours dark and twelve hours light cycles were applied, and the rats were fed ad libitum. Each rat was kept in its own cage. All steps of the study were monitored and supervised by a group assigned by "Karadeniz Teknik University Animal Care and Ethics Committee". The rats were given ketamine hydrochloride (60mg/kg) for anesthesia. The testicles were exposed and made ready to be excised. Then a lethal dose of ketamine hydrochloride was administered. Excisions of both testicles were performed on all rats when sacrificed.

Histopathological evaluation

The right testicles were weighed and both testicles were put into Bouin solution for histopathological evaluation.

Histopathological preparation steps started with dehydration of the fixed samples, followed by the embedding of the samples into paraffin. Serial sections, 5 micrometers in thickness, were obtained using a microtome. Sections were transferred to slides where a deparaffinization took place. Then, the slides were stained (Hematoxylin and Eosin), and were made ready for the evaluation. All histopathological slides were evaluated by only one expert pathologist using a light microscope (Nikon E 200).

Histopathological evaluation was based on light microscopical principles; and a scoring system was used (as defined by D.M. Creasy) [27].

Score-0: Normal

Score-1: Spermatid retention emerging with chemicals or hormonal disturbance

Score-2: Missing germ cell layers in seminiferous tubules

Score-3: The presence of multinucleate giant cells formed from cell cytoskeletal disintegration and cytoplasm fusion due to a slow degenerative process

Score-4: Impairment of the spermatogenic cycle due to the slow degenerative process and sloughing of spermatogenic cells into the lumen

Score-5: Increased interstitial space volume associated with seminiferous tubule cell loss.

Statistical analysis

For Step-1 study groups, statistical analyses for the weight values and histological scoring values were done using Mann-Whitney U test (comparison of A-PS14 and A-CA14 groups). For Step-2 study groups, statistical analyses for the weight values for variance were done by One-Way-ANOVA test, and in case a difference was found, two-sample tests were done using Mann-Whitney U tests with Bonferroni correction.

Statistical analyses for the histological findings for variance were done using Kruskal-Wallis test, and in case a difference was found, two-sample tests were done using Mann-Whitney U tests with Bonferroni correction. For statistical analyses, IBM SPSS version 23 (Chicago, IL, U.S.A.) software was used.

RESULTS

For the weight values of right testicles of rats from A-PS14 and A-CA14 groups, a significant weight decrease was observed in cyproterone acetate treated rats (members of A-CA14 group). The mean values of weight with standard deviation were 1617$\pm$120 mg and 1440$\pm$21 mg for A-PS14 and A-CA14 groups, respectively. The p value obtained using Mann Whitney U test was smaller than 0.001.

For the histological scoring values of rats from A-PS14 and A-CA14 groups, a significant score difference was observed where all rats from A-PS14 group presented "score-0", while 1 rat from A-CA14 group presented "score-2" and the remaining 5 rats presented "score-4". Data were presented in Table 1.

The p value obtained using Mann Whitney U test was smaller than 0.001. Histological view of a testis of a rat from A-PS14 group showing "normal testicular histology" was presented as Figure 1, and from A-CA14 group showing "sloughing of spermatogenic cells into the lumen" was presented as Figure 2.

When B-PS03, B-PS07, B-PS14 groups were compared to A-PS14 group, for the weight values of right testicles of rats, a statistically significant difference could not be demonstrated (a p value of 0.036 was found for One-Way-ANOVA test, but the tests between groups were found insignificant for a difference). When B-CP03, B-CP07, B-CP14 groups were compared to A-PS14 group, for the weight values of right testicles of rats, a statistically significant difference could not be demonstrated (the p value was 0.827).

Table 1. Histological scoring values of testicles of rats from A-PS14 and A-CA14 groups.

	rat-1	**rat-2**	**rat-3**	**rat-4**	**rat-5**	**rat-6**
A-PS14 group	0	0	0	0	0	0
A-CA14 group	2	4	4	4	4	4

$p < 0.001$; Mann-Whitney U test; Step-2 studies:

When B-LP03, B-LP07, B-LP14 groups were compared to A-PS14 group, for the weight values of right testicles of rats, a statistically significant difference could not be demonstrated (the p value was 0.260). The weight values (mean and standard deviation) of right testicles of rats for B-groups were presented in Table 2.

In comparison of B-PS03, B-PS07, B-PS14 groups to A-PS14 group for histological scoring values, the results were not found to be different (the p value obtained from Kruskal-Wallis test was 0.554). In comparison of B-CP03, B-CP07, B-CP14 groups to A-PS14 group for histological scoring values, the results were not found to be different (the p value obtained from Kruskal-Wallis test was 0.778). In comparison of B-LP03, B-LP07, B-LP14 groups to A-PS14 group for histological scoring values, the p value obtained from Kruskal-Wallis test was 0.020. However, the tests between groups were found insignificant for a difference.

Table 2. Weight values of right testicles of rats from Step-2 study groups (B-PS03, B-PS07, B-PS14, B-CP03, B-CP07, B-CP14, B-LP03, B-LP07, B-LP14).

	"03" groups	**"07" groups**	**"14" groups**
B-PS (physiological serum)	1588 ± 111 mg	1784 ± 123 mg	1613 ± 120 mg
B-CP (captopril)	1647 ± 089 mg	1591 ± 228 mg	1677 ± 195 mg
B-LP (lisinopril)	1666 ± 178 mg	1582 ± 315 mg	1812 ± 158 mg

Data are presented as mean ± standard deviation.
No statistically significant difference; One-Way-ANOVA.

Rows for the drugs applied, columns for the duration of treatment; together to define the study group. The histological scoring values of testicles of rats for B-groups were presented in Table 3.

Besides scoring values, the histopathological findings, presented by the pathologist, revealed that edema was prominent in all rats from B-PS07 group, while edema was observed in one rat from B-LP14 group; it was also demonstrated that germ cell depletion was present in one rat from B-PS07 group, and in one rat from B-LP14 group. In "captopril groups" (B-CP03, B-CP07, B-CP14) no testicles were found to have edema or germ cell depletion. The findings related to edema or germ cell depletion were presented in Table 4. Histological views of testes from "Step 2 studies" (nine slides) were given as Figure 3.

Table 3. Histological scoring values of testicles of rats from Step-2 study groups (B-PS03, B-PS07, B-PS14, B-CP03, B-CP07, B-CP14, B-LP03, B-LP07, B-LP14).

	"03" groups	**"07" groups**	**"14" groups**
B-PS (physiological serum)	0, 0, 0, 0, 0, 4	0, 0, 0, 0, 0, 0	0, 0, 4, 0, 0, 0
B-CP (captopril)	4, 0, 0, 0, 0, 0	0, 4, 0, 0, 0, 0	4, 0, 0, 0, 0, 0
B-LP (lisinopril)	0, 0, 0, 0, 0, 0	0, 4, 4, 0, 4, 0	0, 0, 0, 0, 0, 0

No statistically significant difference; Kruskal Wallis test.
Rows for the drugs applied, columns for the duration of treatment; together to define the study group.

Table 4. Histopathological findings of testicles of rats besides scoring values from Step-2 study groups (B-PS03, B-PS07, B-PS14, B-CP03, B-CP07, B-CP14, B-LP03, B-LP07, B-LP14).

	"03" groups	**"07" groups**	**"14" groups**
B-PS (physiological serum)	none	edema: 6 rats germ cell depletion: 1 rat	none
B-CP (captopril)	none	none	none
B-LP (lisinopril)	none	none	edema: 1 rat germ cell depletion: 1 rat

Rows for the drugs applied, columns for the duration of treatment; together to define the study group

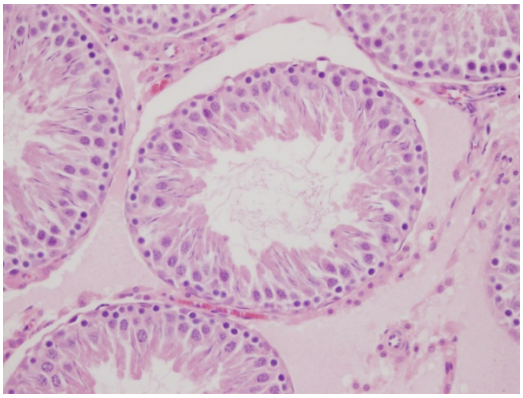

Figure 1. Histological view (400x) of a testis of a rat from A-PS14 group showing "normal testicular histology" (Score-0).

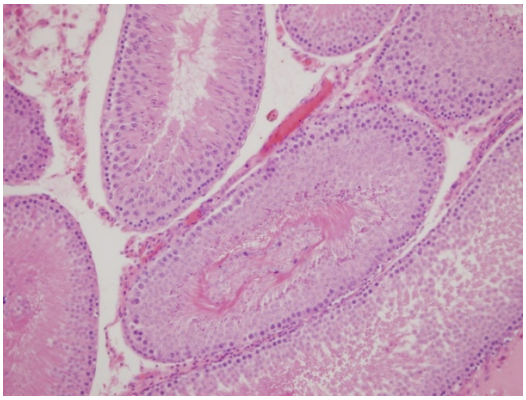

Figure 2. Histological view (200x) of a testis of a rat from A-CA14 group showing "sloughing of spermatogenic cells into the lumen" (Score-4).

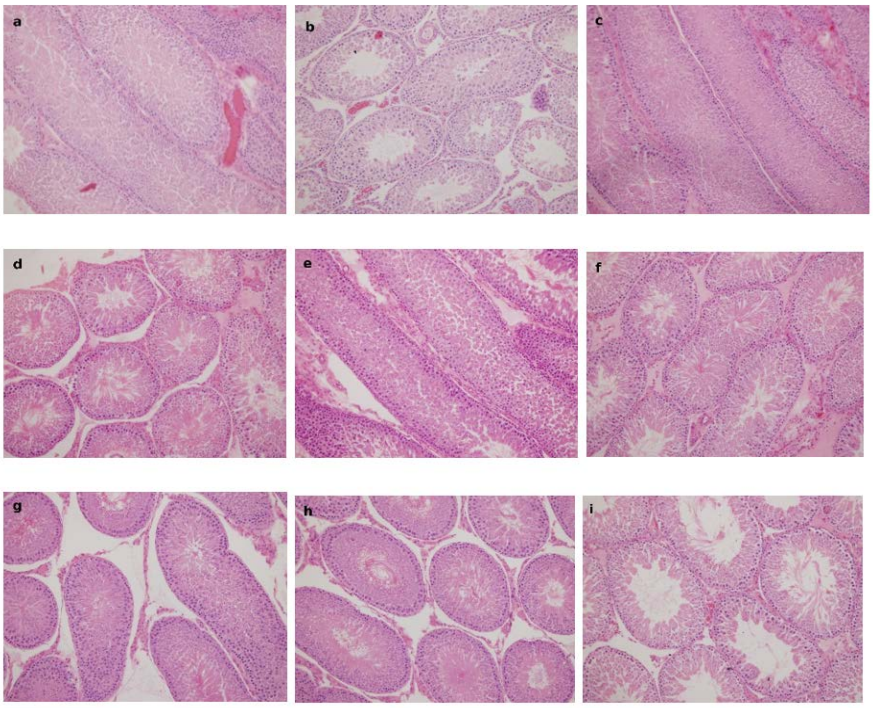

Figure 3. Histological view (200x) of testes from "Step 2 studies"
a. B-PS03, b. B-PS07, c. B-PS14,
d. B-CP03, e. B-CP07, f. B-CP14,
g. B-LP03, h. B-LP07, i. B-LP14
(in Figure 3 b, edema is prominent and germ cell depletion is visible)
(in Figure 3 i, edema is present throughout the slide).

DISCUSSION

CPA, with its progestogenic properties, suppresses GnRH secretion, and results in decreased testosterone production in the testicles leading to lower testosterone levels in the testicles which result in diminished reproductive function and leading to lower serum testosterone levels[1,2,4]. CPA, not available in some countries, is one of two main drugs for transgender women under GAHT before gender affirming surgery to establish feminine characteristics and female range serum testosterone levels [1, 3-5]. The antiandrogens to be chosen are CPA or spironolacton, which were given in conjunction with the main drug estradiol, and were extensively studied[3-5]. CPA was found to be superior to spironolacton in many aspects, especially in lowering serum testosterone levels to female range levels and absence of erections[3, 4]. Lowered serum testosterone level was the result of decreased testicular testosterone production which is crucial for spermatogenesis[2-4]. In other words, CPA treatment is accepted to deteriorate testicular functions, both testosterone production and spermatogenesis [2, 16]. On the other hand, CPA treatment related adverse effects are also of concern (lipid profile abnormalities, serum prolactin level changes, increased observation of meningioma in CPA treated individuals)[2-4, 10, 13, 15]. To find a way to decrease the adverse effect profile of CPA, medical professionals tried to find the lowest possible dose of CPA[2, 3]. The results of the studies confirmed that lower doses of CPA (as 10 mg daily) can be affective in reaching the treatment goals[2, 3]. However, lowest doses of CPA are also devastating for testicles. The possible effects of CPA on testicles were investigated in a retrospective study conducted on transgender women [8]. In the study, orchidectomy specimens of 173 transgender women undergoing sex reassignment surgery were evaluated, and the authors presented that feminizing hormonal treatment resulted in spermatogenesis abnormalities and loss of reproductive function, and the authors concluded that cryopreservation of sperm should be offered and discussed before the initiation of hormonal treatments[8]. Transgender women were advised to cryopreserve sperm for fertility preservation for future family building before initiating any treatment[7, 8, 10-12]. However, in their admission to healthcare for sperm cryopreservation, many of them (at least 86-92%) were found to be under GAHT[11]. In such a scenario, the cessation of GAHT takes place and then attempts to cryopreserve sperm were tried [8]. Data about the negative effects of GAHT on testicular functions and on finding healthy sperm for cryopreservation were boring for transgender women and for the healthcare provider.

In transgender women, in an age group from adolescent age to younger ages, effects on testicular functions could not be ignored. To have the testicular functions back in a period for sperm cryopreservation or on the decision of a retransition (to decide to live as a male, as before; sometimes called as detransition) were of great importance. Data about the ratio of transgender women with a decision to retransition cannot be concluded today, but is accepted to be around 3% and studies related to the factors are analyzed [28]. The reproductive potential of retransitioners also is a noteworthy point, then. In other words, for testicles to recover from the deteriorating effects of CPA becomes a crucial question.

Here, our study was conducted to try to answer the question in a rat model. We, once again showed that CPA caused shrunken testicles and histological indicators of diminished reproductive function. In the second phase, we tried to investigate the recovery phase. With the knowledge of RAS presence in testicular tissue, known to be present in many organ systems, and which was shown to have effects on inflammation, testicular blood flow, testicular homeostasis, fertility, responses to ischemia related injury, recovery from injury, we also studied two ACE-i molecules, on this purpose, to show any probable contribution[17-26]. Here, captopril has to be mentioned because of the thiol-group it contains. In ischemia reperfusion studies, especially, the presence of the thiol-group on the molecule was considered to be beneficial in preventing reperfusion injury or in recovery from injury[19,21]. While ACE-i molecules were shown to inhibit inflammatory responses (endotoxin related responses in uveitis patients, ischemia-reperfusion induced responses in experimental models) in presented papers, a thiol-group containing ACE-i was studied comparing to an angiotensin II receptor antagonist in a toxicity research and the authors concluded that testicular injury could be protected by lowering angiotensin II presence, and the protective effects of the inhibition of ACE was discussed to be amplified when the molecule was containing a thiol-group[17-19, 21-23].

The current study revealed that as a cyproterone acetate administration was stopped, the negative changes were found to be reversed (the changes in

weight values and histological scoring values). For the contribution of adjuvant ACE-i treatments, it can be expressed that in the absence of an ACE-i (B-PS groups), observation of edema (in all 6 rats) and germ cell depletion (in one rat) is of interest. To a lesser extent, the observation of edema (in one rat) and germ cell depletion (in one rat) in B-LP groups is of note. We want to emphasize that no rats with edema or germ cell depletion were found in B-CP groups, which were treated with the agent captopril, a thiol group containing ACE-i.

Limitations of the study can be summarized as follows: The result that the presence of recovery from CPA induced testicular injury in rats is promising, and the time necessary for a recovery was found to be short. However, it has to kept in mind that in a human population, the presence of a recovery and time for a recovery from CPA induced testicular injury need investigation. On the other hand, the recovery from injury has to be supported with fertility outcomes, which necessiates further human studies.

Our findings led us to the conclusion that captopril having a thiol-group not found in lisinopril kept testicles away from inflammatory changes that could be observed in the recovery period following the cessation of CPA treatment, in our rat population. We also concluded that the histological injury caused by CPA treatment was reversible in rats. The studies on the fertility potential of human subjects, either cryopreservation outcomes or fertility of retransitioners, are necessary to draw firm conclusions, while our study submits promising findings on the way to advise professionals to tell that the testicular injury related to CPA treatment may be reversible in nature.

Author Contributions: Concept/Design : FÇ, RÖ,NİKÜÇ, MT, ÖK, CB, İOK; Data acquisition: FÇ, RÖ, NİK,ÜÇ, MT, ÖK, CB, İOK; Data analysis and interpretation: FÇ, RÖ, MT; Drafting manuscript: FÇ, RÖ, NİK, ÜÇ, MT, ÖK, CB, İOK; Critical revision of manuscript: FÇ, RÖ, NİK, ÜÇ; Final approval and accountability: FÇ, RÖ, NİK, ÜÇ, MT, ÖK, CB, İOK; Technical or material support: FÇ; Supervision: FÇ, RÖ, MT; Securing funding (if available): n/a.
Ethical Approval: Ethical approval was obtained with the decision number 201/12-4 dated 10.06.2014 of the Karadeniz Technical University Animal Experiments Local Ethics Committee.
Peer-review: Externally peer-reviewed.
Conflict of Interest: Authors declared no conflict of interest.
Financial Disclosure: Authors declared no financial support

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