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ARAŞTIRMA / RESEARCH

Redox modulatory effects of testosterone administration in liver tissue of aging rats

Yaşlanan karaciğer sıçan dokusunda testosteron uygulamasının redoks modulator etkisi

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Abstract

Purpose: The aim of this sudy was to evaluate if testosterone administration possesses any ameliorative effects on age-related redox imbalance in liver tissue of male rats.
Materials and Methods: Our study consists of three groups of male rats (n=24). Group 1: Young controls and Group 2: Aged rats that were administrated peanut oil intramuscularly andGroup 3: Aged rats that were administered a single dose of testosterone enanthate (25 mg/kg body weight) in peanut oil intramuscularly as a vehicle. To determine oxidative damage and also antioxidative capacity, we analyzed the levels of protein carbonyl groups, advanced oxidation end products, dityrosine, kynurenine, lipid hydroperoxides, advanced glycation end products, total thiol fractions, and Cu, Zn-superoxide dismutase activities.
Results: The current study results indicate that redox balance is severely impaired in aged liver tissue via higher levels of oxidative protein damage, increased rate of lipid peroxidation, and also reduced antioxidant defense capacity. the present study showed that, testosterone administrationpartially ameliorated impaired redox balance.
Conclusion: Testosterone administration partially improved impaired redox status by alleviating macromolecular oxidative damage and increase antioxidant defense capacity but these ameliorative effects not sufficient as young controls.
Keywords:. Aging, liver, oxidative stress, redox balance, testosterone

Öz

Amaç: Bu çalışmanın amacı, testosteron uygulamasının erkek sıçanların karaciğer dokusunda yaşlanmaya bağlı bozulan redoks homeostasisi üzerine iyileştirici bir etkisinin olup olmadığını değerlendirmektir.
Gereç ve Yöntem: Çalışmamız üç gruptan oluşmaktadır (n=24). Grup 1: Kontrol grubu erkek sıçanlara ve Grup 2: Yaşlı erkek sıçanlara yerfıstığı yağı taşıyıcısı içinde intramusküler olarak verilmiştir. Grup 3'ü oluşturan yaşlı erkek sıçanlara ise tek doz testosteron enanthate (25 mg/kg -) yerfıstığı yağı taşıyıcısı içinde intramuskuler olarak uygulanmıştır. Karaciğer dokusunda makromoleküler hasarı ve antioksidan kapasiteyi belirlemek üzere protein karbonil grupları, ileri oksidasyon protein ürünleri, ditriozin, kinürenin, lipit hidroperoksit, ileri glikasyon son ürünleri, total tiyol fraksiyonları ve Cu, Zn-süperoksit dismutaz aktiviteleri analiz edilmiştir.
Bulgular: Çalışma bulgularımız, yaşlı sıçanların karaciğer dokularında redoks dengesinde ileri düzeyde bir bozulmanın olduğunu artan protein, lipit peroksidasyonu biyobelirteçleri ile ve kısmen azalan antioksidan savunma kapasiteleriyle göstermektedir. Bu çalışmada, testosteron uygulamasının bozulmus redoks dengesinde kısmen iyileştirici yönde etki ettiği saptanmıştır.
Sonuç: Testosteron uygulamasının makromoleküler oksidatif hasarı hafifletmek ve antioksidan kapasiteyi kısmen arttırarak bozulmuş redoks homeostasisini kısmen iyileştirdiği ancak bu etkinin genç kontrollerinki kadar etkili olmadığı sonucuna varılmıştır.
Anahtar kelimeler: Yaşlanma, karaciğer, oksidatif stres, redoks dengesi, testosteron

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INTRODUCTION

Aging is a gradually developing degenerative biological phenomenon. It is characterized as a progressive, universal, multifactorial process with irreversible deterioration of macromolecular structures and functions[1]. Transition to settled life, developments in the field of health sciences and technologies, educational levels have led to an increase in life expectancy from past to present [2-4]. Increasing life expectancy leads to the dawn of aging science.

According to the free radical theory of aging, increased free radical production and impaired enzymatic and non-enzymatic antioxidant defense system activity lead to oxidative damage in cellular macromolecules [6]. The type and levels of oxidative protein modifications can be assessed by conventional oxidative protein biomarkers such as protein carbonyl groups (PCO), advanced oxidation protein products (AOPP), and also specific oxidative protein biomarkers such as dityrosine (DT), kynurenine (KYN). Furthermore, the grade of lipid peroxidation can be assessed by early stage markers such as lipid hydroperoxides (LHP), and late stage markers such as malondialdehyde (MDA), 4-hydroxynonenal (4-HNE)[7]. On the other hand, antioxidant systems include enzymatic and non-enzymatic biomarkers. Variations in the levels of non-enzymatic biomarkers are mainly assessed by levels of thiol (SH) fractions, and also activity of enzymatic system alterations is evaluated by the activity of Cu, Zn-superoxide dismutase (Cu, Zn-SOD) and catalase (CAT)[8,9]. Liver tissue constitutes a vital field of aging research due to its central role in metabolism.

Testosterone is a primary male sex hormone synthesized by interstitial Leydig cells via the stimulation of luteinizing hormone secreted from the anterior pituitary gland. Testosterone, which is an anabolic-androgenic hormone with steroid structure, is effective on bones, liver, kidney, brain, cardiovascular systems, muscles, prostate, Leydig cells, and hematopoiesis[10-27]. According to the experimental and clinical evidence of testosterone deficiency and replacement therapies have controversial effects on metabolism, coagulation, inflammatory process, body composition, cognitive function and behaviour, and prostatic effects[10].

The concentration of circulating testosterone falls with aging[11]. This study is novel because it evaluates redox modulatory effects of testosterone administration on wide variety of macromelecular redox homeostasis biomarkers in the liver tissue of aging rats. The effects of testosterone on the extend of macromolecular redox homeostasis biomarkers has not been previously studied. So this study may promote to the current literature how testosterone administration affects impaired macromolecular redox homeostasis in liver tissue of aging ratsThe current study was aimed to evaluate the redox modulatory effects of testosterone administration in liver tissue of elderly male rats. The serum testosterone concentration return to normal levels by 14 day[12]. Thus, rats wre sacrificed after 14 days to testosterone administration.

MATERIALS AND METHODS

Animal model

The current study was performed with 6 month-old (n = 8) (equal to an 18-year-old human) and 24 month-old male Sprague-Dawley rats (n=16) (equal to a 60-year-old human)[28]. All of the experimental studies were conducted following the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978). The ethical protocol of the current research was approved by the Ethics Committee of Istanbul University, Istanbul, Turkey (number 2012/67, date 25/05/2012). Participants who take part in ethical application form approved to this paper.

Experimental animals were maintained in ambient temperature (20–22 °C) and 55–60% humidity with the normal 12-h light-dark cycles. All of the rats were fed a standard rodent chow and given water *ad libitum*. The rats were separated into three groups (n=8 in each group). Group 1(YC): Young control rats (5 months old) and Group 2 (AC): Aging control rats (24 months old) without testosterone administration that were given a single dose of peanut oil intramuscularly, Group 3 (ATA): Aging rats (24 months old) with testosterone administration that were administered redox modulatory dose of testosterone enanthate (25 mg/kg body weight) in peanut oil intramuscularly as the vehicle. All animals were sacrificed 14 days after the following testosterone administration[29] and their blood samples were collected to analyze routine function tests and other biochemical parameters. In our previous study,

serum testosterone levels were found significantly increased with testosterone enanthate administration (25 mg/kg).

Chemicals and equipment

All chemicals were purchased from Merck (Darmstadt, Germany) or Sigma-Aldrich (St. Louis, MO). Absorbance values were recorded with a Biotek Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek US, Winooski, VT).

Tissue preparation

The liver samples were washed in cooled serum physiologic saline for remove surface contamination. Then samples placed in the tube with beads and add homogenizing buffer (phosphate buffer saline pH 7.4,plus 0.1% digitonin) and a homogenized with bullet blender (Next advance, NY 12180, USA)

Conventional protein oxidation biomarkers

Protein carbonyl groups (PCO)

PCO concentrations were assayed as formerly described colorimetric method by Reznick and Packer[30]. In the acidic condition, protein samples react with 10 mM 2,4-dinitrophenylhydrazine (DNPH) reagent (1:4 ratio). After the precipitation process with %20 and %10 cold TCA, respectively the pellets undergo further processing involving extensive washing with an organic solvent (ethanol: ethyl acetate 1:1 ratio) three times. Finally, 6 N Guanidine-HCl solution added on the resulting protein precipitates and the optic density values of the final samples were read at 360 nm after the incubation period at 37°C for 10 minutes.

Advanced oxidation protein products (AOPP)

AOPP were estimated with the colorimetric method described by Hanasand et al [31]. Liver homogenates (10 µL) and citric acid solution (200 µL) were added in a UV microplate. The resulting optic density values were recorded at 340 nm within 2 min after 10 µL potassium iodide pipetting.

Specific oxidative protein biomarkers

Dityrosine (DT)

DT levels were estimated with the previously described by Sadowska-Bartosz et al [32]. Fluorescence values of the final samples were determined at the 330/ 415 nm

Kynurenine (KYN)

KYN levels were estimated according to the spectrofluorometric method as previously described by Sadowska-Bartosz et al.[32]. Fluorescence values were recorded at the excitation and emission wavelengths of 480 nm and 365nm, respectively.

Lipid peroxidation and glycoxidation biomarkers

Lipid hydroperoxides (LHPs)

LHPs levels were measured colorimetrically as previously described by Wolff SP (Wolff 1994)[33]. LHPs formed ferric ions in acidic solutions. Xylenol orange binds ferric ions and forms a blue-purple colored complex. Samples (10 µL) and Ferrous Oxidation with Xylenol orange, version 2 reagent (FOX2) (950 µL) were added to the microcentrifuge tubes. After 30 min incubation time with FOX2 reagent at darkroom optic density values of supernatants were recorded at 560 nm wavelength.

Advanced glycation end products (AGEs)

AGE levels were analyzed according to the spectrofluorometric method as previously described by Sadowska-Bartosz et al.[32] Fluorescence value was determined at the excitation and emission wavelengths of 415 and 330 nm respectively.

Non-enzymatic and enzymatic antioxidant defense system biomarkers

Total thiol fractions (T-SH) (Non enzymatic antioxidant defense system biomarker)

Total thiol (T-SH) levels of the liver tissue homogenates were assayed spectrophotometrically as previously described by Sedlak and Lindsay[34]. Twenty µL of supernatant, 200 µL of Tris buffer at pH 8.2 and 10 µL of 5,5-dithiobis 2- nitrobenzoic acid (DTNB) were mixed to determine T-SH. The absorbance values were recorded at 412 nm wavelength.

Cu, Zn-superoxide dismutase (Cu, Zn-SOD) activity (enzymatic antioxidant defense system biomarker)

Cu, Zn-SOD (EC 1.15.1.1) activities of the liver tissue samples were measured using the method of Sun et al. in an alkaline medium pH10.2[35]. The main technical principle of this method is the inhibition of nitrobluetetrazolium (NBT) reduction with xanthine-

xanthine oxidase. Superoxide anion produced by xanthine oxidase. SOD catalyzes the dismutation reaction of superoxide anion into hydrogen peroxide and O_2 . The final concentration of xanthine oxidase was then 167 U/L[36].The production of water soluble formazon was determined spectrophotometrically at 560 nm.

Total protein concentrations:

Total protein concentrations were analyzed fluorometrically with Qubit analyzer[29].

Statistical analysis

The statistical analyses were performed by IBM SPSS software version 25 (Chicago, IL, USA). Data normality was verified by Shapiro Wilk test. Data were expressed as the mean ± standard deviation and median. Parametric One-Way ANOVA was used with normally distributed data and the nonparametric Kruskal-Wallis test was used to with not normally distributted data. Post hoc tests were managed by the Bonferroni–Dunn test or Man Whitney U test according to normally or not normally distrubeted data, respectively. p values < 0.05 were considered as statistically significant for all tests. Parametric One-Way ANOVA was used for DT, KYN, AGE, TSH and Cu, Zn-SOD parameters. Mean and standard deviations were given and marked statistical significances were marked in mean ±standard deviation values. Non parametric Kruskall-Wallis test was used for PCO, AOPP, LHP. Due to Kruskall-wallis test is a ranking statistic, median values were given and statistical significances were marked in median values.

RESULTS

PCO and AOPP levels are given in Table 1. PCO and AOPP levels were significantly higher in AC rats than YC and ATA (***p<0.001 for both groups and both parameters) rats. On the other hand, PCO and AOPP levels were significantly decreased in ATA when compared to AC (***p<0.001 for both parameters). Also, no differences were found in the median values of PCO and AOPP between ATA and YC.

The levels of DT, and KYN originated from oxidized tyrosine and tryptophan residues are given in Table 2. A decrease in specific protein oxidation parameters was observed in the ATA group when compared to AC. This reduction was statistically significant in KYN, whereas in DT it was found to be insignificant (*p<0.05 YC vs AC for DT, **p<0.01 AC vs ATA). DT concentrations were higher in the AC group when compared to YC (*** p<0.001 YC vs AC)

Table 1: Concentrations of conventional protein oxidation biomarkers.

		YC	AC	ATA
PCO (nmol/mg protein)	Mean+SD	0.78±0.08	1.34±0.08	0.81±0.1
	Median	0.76	1.37a***	0.79b***
AOPP (µmol / L Chloramine- T equivalent)	Mean+SD	81.25±4.30	125.25±10.43	88.75±11.87
	Median	81.5	126.5 a***	87.0b***

PCO; protein carbonyl group, AOPP; advanced oxidation protein products, YC; Young Control, AC; Aging Control; ATA; Aging rats with Testosterone Administration, SD; standard deviation, a; vs YC, b; vs AC, *** p<0.001

Table 2. Concentrations of specific protein oxidation biomarkers

		YC	AC	ATA
DT (FU/mg protein)	Mean+SD	427.13± 28.66	521.13±107.11a*	485.38±44.84
	Median	426.5	540.0	492.0
KYN (FU/mg protein)	Mean+SD	117.5±9.68	152.13±15.46a***	127.88±11.94b**
	Median	118.0	151.0	132.5

DT; Dityrosine, KYN; Kynurenine, FU; fluorescence unit, YC; Young Control, AC; Aging Control; ATA; Aging rats with Testosterone Administration, SD; standard deviation, a; vs YC, b; vs AC, *; p< 0.05, **p<0.01, *** p<0.001

LHP and AGE concentrations and median values are given in Table 3. Testosterone administration represented a detractive effect on age-related lipid peroxidation rate. LHP median values were significantly higher in the AC group when compared to YC (p<0.001). Median values of LHP in ATA groups were higher than AC and lower than YC (p<0.01 for both groups). AGE levels were

significantly lower in the YC group when compared to AC and ATA (*** $p<0.001$ for both groups) but AGE levels in ATA rats were no significantly different from AC. T-SH and Cu, Zn-SOD activities are given in Table 4. It was found that testosterone administration leads to elevated T-SH levels and Cu, Zn-SOD activity compared to AC (* $p<0.05$, and *** $p<0.001$, respectively) although testosterone administration was found to be insufficient to reach the enzymatic antioxidant capacity levels of YC.

Table 3. Lipid peroxidation and glycoxidation biomarkers

		YC	AC	ATA
LHP (µmol/mg protein)	Mean+SD	1.26±0.16	1.55±0.06	1.44±0.05
	Median	1.3	1.55 [a***]	1.44[a**,b**]
AGE (FU/mg protein)	Mean+SD	448.5±23.29	524.0±20.63[a***]	497.38±17.70[a***]
	Median	444.5	532.5	499.0

LHP; Lipid hydroperoxides, AGE; advanced glycation end products, YC; Young Control, AC; Aging Control; ATA; Aging rats with Testosterone Administration, SD; standard deviation, a; vs YC*** b; vs AC, **$p<0.01$, ***$p<0.001$

Table 4. Concentrations and activities of non-enzymatic and enzymatic antioxidant defense system biomarkers

		YC	AC	ATA
T-SH (nmol/mg protein)	Mean+SD	9.01±0.45	7.63±0.53[a***]	8.36±0.53[b*]
	Median	9.0	7.5	8.35
Cu, Zn-SOD (U/mg protein)	Mean+SD	7.4±0.31	5.63±0.44[a***]	6.61±0.38[a***,b***]
	Median	7.45	5.8	6.7

T-SH; total thiol fractions, Cu, Zn-SOD; cupper-zinc superoxide dismutase, U;unit YC; Young Control, AC; Aging Control; ATA; Aging rats with Testosterone Administration, SD; standard deviation, a; vs YC, b; vs AC, *;$p< 0.05$, *** $p<0.001$

DISCUSSION

Aging is a degenerative process with a progressive nature in which structural disorders at the organelle, cell, and tissue level are generally accompanied by functional disorders. The liver is an organ with a significant central function in metabolism. The characteristics of advanced liver diseases are similar to hypogonadal men, however, the relationship among them has not been fully explained[13].

In the literature, exogenous administration of testosterone effects are controversial. According to controversial results of these studies, in which molecular pathways and effects of exogenous testosterone replacement depend on gender, dose, duration time and organ similar to aging and free radicals effets[14]. Testosterone enenthianate (20mg/kg) replacement for 6 weeks in 3 months old rats hippocampus lead to prooxidant effects[14]. A testosterone administration (100 mmol/L) has been observed to decrease oxidative stress and cytoprotective effect in Leydig cells but cytotoxic effects were found witha high dosage of testosterone (500mmol/L)[15]. On the other hand, the other study showed that testosterone enanthate administration (500 mg) leads to impaired redox homeostasis [16].

When the literature review is realized in PubMed, it was observed that no study has been investigated the effect of testosterone administration on liver tissue with respect to impaired redox homeostasis. In our study, we aimed to determine whether the redox modulatory dose of testosterone enanthate administration led to an improvement in impaired redox homeostasis in the livers of aging rats. For this purpose, 5 month-old young rats (equal to an 18-year-old human) and 24-month-old aged rats (equal to a 60-year-old human) were included in the study [28].

It is considered that one of the basic mechanisms underlying aging is impaired redox homeostasis. Imbalance occurring in redox homeostasis is one of the reasons that play a major role in the oxidative damage in cellular macromolecules. Oxidative damage in proteins is determined by global biomarkers such as PCO and AOPP and specific amino acid biomarkers such as DT, KYN[7]. The formation and accumulation of these oxidatively modified biomolecules bring along functional losses depending on age [6]. Choobineh et al., reported that testosterone administration (0.1 mg/kg for 7 days and 35 days) did not affect on PCO content in mice[17].

Male rats were selected in the present study due to eliminating effects of estrogen. It is a protective

biomolecule against oxidative damage in female individuals. Decreasing estrogen levels after menopause may be one of the reasons for the increase in oxidative stress. The decrease in the levels of testosterone due to aging and the interruption of its aromatized conversion to estrone may also be active causes. In the study carried out by Tunez et al., it was reported that testosterone administration (0.5mg/kg for 8 days) to ovariectomized rats for a week prevents protein carbonylation and lipid peroxidation rate[18].

In our current study, a significant increase in the levels of general and specific protein oxidation markers of male elderly rats was determined compared to their respective control groups. Our results represent concordance with the previous studies on aging[29,37]. It was observed that testosterone administration alleviated the magnitude of oxidative protein damage and brought it closer to the levels of young controls in kidney[29]. It was observed to have similar effects in the liver and kidney with respect to the rate of lipid peroxidation. Although no significant effect was observed in a previous study on kidney tissue in terms of improving glycoxidative damage, a statistically significant improvement was observed in the liver tissue while it could not approach the controls.

When it was compared in terms of the enzymatic and nonenzymatic antioxidant capacity, it was observed that testosterone administration has started to have an improving effect on decreasing antioxidative capacity in aged tissues, however, it is inadequate to approach it to the antioxidative capacity of the young control group in our current study. Choobieh et al found that testosterone administration results in decreased SOD and glutathione peroxidase activity and also MDA levels in spinal cord[17]. Not only, decreased levels of early lipid peroxidation marker, LHP, but also increased T-SH levels and Cu, Zn-SOD activities were found in ATA rats when compared to AC rats in the current study. These different results may derive from organ differences and also testosterone dosage and duration time.

The current study has some of the limitations. In the present study changes in the level or activities of macromolecular redox status biomarkers were analyzed but the underlying mechanism of endocrinal aging-induced liver redox imbalance is still unclarified. However, our study advocates the possible redox modulatory effects of testosterone administration in aged liver rats.

The results of our study indicate that a single dose of testosterone administration has a significant effect by increasing antioxidative capacity and reducing oxidative damage in aging rats. Further studies should be carried out to clarify the underlying redox regulation mechanism and relation between clinical biomarkers of liver functions and testosterone metabolism. Underlying redox modulatory mechanisms in liver tissue of aging males may provide a potential target for effective testosterone-based therapeutic strategies to prevent age-related liver pathologies.

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