Biogerontology (2010) 11:335–346
DOI 10.1007/s10522-009-9257-8

RESEARCH ARTICLE

Gender-dependent oxidative variations in liver of aged rats

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Received: 3 October 2009 / Accepted: 18 November 2009 / Published online: 28 November 2009
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Abstract A shift from redox regulation to oxidative damage is known to contribute organ dysfunction and aging-related disorders. Exposure to reactive oxygen species throughout the life-span increases the incidence of several liver diseases. A redox basis of the loss of antioxidant capacity of aged livers has not been fully elucidated in both genders. In the current study, we investigated the gender-dependent relations between protein carbonyl (PCO), a commonly used marker of protein oxidation and other protein oxidation parameters such as advanced oxidation protein products (AOPP) and total thiol (T-SH). Our study also covered other oxidative stress markers, such as malondialdehyde (MDA), lipid hydroperoxides (LHP), and glutathione (GSH) in liver tissue of the male and female aged rats. PCO and AOPP levels in old male and female rats were significantly higher than those in the young control groups ($P < 0.001$ and $P < 0.01$, respectively for male rats; $P < 0.001$ for both parameters in female rats). On the other hand, T-SH levels were not found to be different between young and old rat groups. Plasma MDA levels of old male and female rats were significantly higher compared to those of the young control groups ($P < 0.01$ and $P < 0.001$, respectively). LHP levels were only found out to be significantly higher in old female rats when compared to those in young male rats. GSH levels in old male and female rats were significantly lower than in the corresponding young control groups ($P < 0.01$ for male rats; $P < 0.05$ for female rats). Our results demonstrated greater susceptibility to hepatic oxidative damage in females than in males. This appears to contradict the general assumption that females are less susceptible to oxidative injury than males are.

Keywords Aging · Liver · Gender · Protein oxidation · Lipid peroxidation

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Introduction

Gender is a crucial determinant of aging and life span, but little is known about gender differences in free radical homeostasis (Ali et al. 2006). This natural phenomenon can be explained on the basis of the mitochondrial theory of aging (Harman 1972). Superoxide radicals ($O_2^{\cdot -}$) generated adventitiously by the mitochondrial respiratory chain can give rise to much more reactive radicals, resulting in random

oxidation of all classes of cellular macromolecules (de Grey 2006). On the other hand, mitochondria from female rats generate half the amount of $O_2^{\cdot-}$ than those of the males (Balaban et al. 2005; Borrás et al. 2007).

Declining physiological functions are a characteristic of life beyond the reproductive phase in animals and humans. An understanding of the mechanisms cellular and tissue senescence associated with the aging process is important, in terms of minimizing the impact of disease processes in later life and developing strategies for maximizing lifespan (Langley-Evans and Sculley 2005). Although the majority of liver functions seem to be maintained with age, the incidence of several liver diseases such as non-alcoholic fatty liver disease and hepatocellular carcinoma increases with age (Timchenko 2009).

The oxidative damage promoted by reactive oxygen species (ROS) and reactive nitrogen species (RNS) is termed oxidative stress and nitrosative stress, respectively. Efficient regulation of ROS/RNS production and neutralization is essential for avoiding their detrimental effects, and different molecular mechanisms co-operate to preserve this equilibrium termed "redox balance" or redox homeostasis. The gender-dependent factors responsible for such redox-dependent variations in the level of oxidized macromolecules are largely unknown and controversial. The ambiguity rose in preliminary reports as to gender-dependent variations of the levels of main oxidative-stress parameters in liver tissue of aged subjects (Langley-Evans and Sculley 2005). However, the multiple relationships among gender and all these oxidized macromolecules in this regard could not be meaningfully correlated in previous studies (Langley-Evans and Sculley 2005; Justo et al. 2005).

In the current study, we investigated the relation between protein carbonyl (PCO) levels, which is a widely used marker of protein oxidation and other oxidative protein damage parameters such as advanced oxidation protein products (AOPP) and total thiol (T-SH) groups. Our study also covered other oxidative stress parameters, such as malondialdehyde (MDA), lipid hydroperoxides (LHP), and reduced glutathione (GSH) in liver tissue of the male and female aged rats.

Our aim was to bring out the difference between broad set of oxidative stress parameters of male and female rats of the same chronological age in order to understand the gender-dependent homeostatic redox-mechanisms in liver tissue.

Methods

Experimental animals and procedures

Aged Sprague–Dawley rats (24 months old) and their respective controls (5 months old) supplied by the Centre for the Experimental Animal Research and Breeding Laboratory, Istanbul University, Cerrahpaşa Medical Faculty, Istanbul, Turkey were used. Rats were divided into four groups: Group I (aged male rats, $n = 10$), Group II (aged female rats, $n = 10$), Group III (young male rats, $n = 10$), and Group IV (young female rats, $n = 10$). These animals were housed in conventional wire-mesh cages, four rats per cage, in a room with the temperature regulated at 21 ± 1°C, humidity 45–50%, and light/dark cycles (12 h). All animals were given ad libitum access to standard food and tap water throughout the course of the experiment. The experimental protocols were conducted under 3R's rule.

Apparatuses

All the samples were centrifuged at +4°C (Jouan G 412). Tissue PCO, T-SH, AOPP, LHP, MDA and erythrocyte GSH levels were measured by a UV–visible spectrophotometer (Heraeus 400, Kendro Laboratory Product, Osterode, Germany).

Chemicals

All kinds of chemical reagents were of analytical grade. Deionized water was used in entirety of analytical procedures. The reagents were stored at +4°C and equilibrated at room temperature for 0.5 h before use.

Preparation of tissue samples

The samples of liver from the rats were quickly removed, washed in cooled 0.15 M NaCl, and placed on an ice-cold plate. Tissue samples were then immediately frozen in liquid N_2 until experimentation. Liver tissue (200 mg) samples were homogenized manually in 2 ml of homogenizing buffer

(100 mM KH_2PO_4–K_2HPO_4, pH 7.4, plus 0.1% (w v^{-1}) digitonin) in a glass homogenizer to avoid disruptions of nuclear membranes. In this way, contamination by nucleic acids was minimized. Homogenates obtained from rats were centrifuged at 5,000×*g* for 10 min, and determination of various analytes was performed in the supernatant fraction.

Analytical methods

Assay of protein carbonyl levels

PCO groups were measured spectrophotometrically by using the method of Reznick and Packer (1994). PCO groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenylhydrazones. DNPH was dissolved in HCl, and after the DNPH reaction, proteins were precipitated with an equal volume of 20% (w v^{-1}) trichloroacetic acid and washed three times with 4 ml of an ethanol/ethyl acetate mixture (1:1). Washings were achieved by mechanical disruption of pellets in the washing solution using a small spatula, and re-pelleting by centrifugation at 6,000*g* for 5 min. Finally, the protein precipitates were dissolved in 6 M-guanidine-HCl solution and the absorbances were measured at 360 nm using the molar extinction coefficient of DNPH, $\varepsilon = 2.2 \times 10^4\ M^{-1} \times cm^{-1}$. Protein contents were determined on the HCl blank pellets spectrophotometrically using a Folin kit (Sigma Diagnostics, St. Louis, MO, USA). The coefficients of intra- and inter-assay variations for carbonyl assay were 5.2% ($n = 12$) and 9.3% ($n = 10$), respectively.

Assay of total thiol levels

T-SH groups were measured spectrophotometrically by using the method of Sedlak and Lindsay (1968). Aliquots of 250 µl of the supernatant fraction of the tissue homogenate were mixed in 5 ml test tubes with 750 µl of 0.2 M Tris buffer, pH 8.2, and 50 µl of 0.0 l M 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). The mixture was brought to 5 ml with 3,950 µl of absolute methanol. A reagent blank (without sample) and a sample blank (without DTNB) were prepared in a similar manner. The test tubes were stoppered with rubber caps, the color was developed for 15 min and the reaction mixtures were centrifuged at approximately 3,000*g* at room temperature for 15 min. The absorbance of supernatant fractions was read in a spectrophotometer at 412 nm. Molar extinction coefficient at 412 nm was 13,100 l mol^{-1} cm^{-1}.

Assay of advanced protein oxidation products

Spectrophotometric determination of liver tissue AOPP levels was performed by modification of Witko-Sarsat's method (1992). Samples were prepared in the following way: 200 µl of plasma was diluted 1:5 in PBS, 10 µl of 1.16 M potassium iodide was then added to each tube, 2 min later followed by 20 µl acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 2,000 µl of PBS, 100 µl of KI, and 200 µl of acetic acid. The coefficients of intra- and inter-assay variations were 1.6% ($n = 10$) and 2.4% ($n = 10$), respectively. The chloramine-T absorbance at 340 nm being linear within the range of 0–100 µmol l^{-1}, AOPP concentrations were expressed as micromoles per liter of chloramine-T equivalents.

Assay of lipid hydroperoxide levels

LHP levels were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange FOX2 (Ferrous Oxidation with Xylenol orange, version 2, Wolff 1994). Lipid hydroperoxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined by using ferric-sensitive dyes as an indirect measure of hydroperoxide concentration. Xylenol orange binds ferric ions with high selectivity to produce a coloured (blue-purple) complex. The recipe for measuring LHP is as follows: 100 µM xylenol orange, 250 µM ammonium ferrous sulfate, 90% methanol (HPLC grade), 4 mM butylated hydroxytoluene, 25 mM H_2SO_4 were used in the assay. Fifty microliters of sample was added to 950 µl (in a 1 ml microcentrifuge vial), vortexed, and incubated at room temperature for 30 min. The absorbance was read at 560 nm after removal of any flocculated material by centrifugation.

Determination of thiobarbituric acid reactive substances

The rate of lipid peroxidation was determined by the procedure of Buege and Aust (1978). One of the major

secondary products of lipid peroxidation is malondialdehyde (MDA). MDA along with other by-products reacts with thiobarbituric acid (TBA) to generate a colored product which absorbs at 535 nm representing the color produced by all the thiobarbituric acid reactive substances (TBARS). The coefficients of intra- and inter-assay variations for MDA assay were 3.5% ($n = 10$) and 5.4% ($n = 10$), respectively.

Determination of reduced glutathione levels

Tissue reduced glutathione (GSH) levels were measured by a commercially the available GSH assay kit (Cayman Chemical, Ann Arbor, MI, USA). GSH assay kit utilizes enzymatic recycling method using glutathione reductase for the quantification of GSH. The thiol group of GSH reacts with DTNB (5-5′-dithiobis-2-nitrobenzoic acid, Ellman's reagent) and produces yellow colored 5-thio-nitrobenzoic acid (TNB). The mixed disulfide, GSTNB that is concomitantly produced, is reduced by glutathione reductase to recycle the GSH and produce more TNB. The rate of TNB production is directly proportional to the concentration of GSH in the deproteinized sample. Measurement of the absorbance of TNB at 414 nm provides an accurate estimation of GSH in the sample.

Statistical analysis

Descriptive statistics were given as mean ± SEM. Differences between groups were assessed by analysis of variance (ANOVA) using the SPSS software package for Windows. Post-hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test. Post hoc tests were conducted with the Bonferroni–Dunn test. The relationships among the analyzed parameters were investigated using Pearson's correlation coefficients. Probability value of less than 0.05 was considered statistically significant for all comparisons.

Results

In the observations of our experiments conducted during 2005–2007 within the animal husbandry conditions specified by the Centre for the Experimental Animal Research and Breeding Laboratory, Istanbul University, Cerrahpaşa Medical Faculty, Istanbul, Turkey, the median life-span of the male Sprague–Dawley rats were found to be 26 months and their maximum life-span 29 months; on the other hand, the median life-span of the female Sprague–Dawley rats turned out to be 28 months and their maximum life-span was 30 months.

Protein oxidation parameters in the study groups of male rats are given in Fig. 1. PCO and AOPP levels of male rats were significantly higher compared with those of the young male rats ($P < 0.001$, $P < 0.01$; respectively). Although there was a trend toward lower T-SH levels in male rats compared to young male rats, but this did not reach statistical significance (Fig. 1c; $P > 0.05$). Concentrations of the lipid peroxidation markers in the liver tissue of male rats are given in Fig. 2. MDA levels of the liver tissue were significantly higher in the aged male rats compared with those of the young male rats (Fig. 2a; $P < 0.01$). On the other hand, liver LHP levels were not found to be different among groups (Fig. 2b; $P > 0.05$). GSH levels were found to be significantly lower in old male rats when compared to those in young male rats (Fig. 3; $P < 0.01$).

Protein oxidation parameters in the study groups of female rats are given in Fig. 4. PCO and AOPP levels of female rats were significantly higher compared to those of the young female rats ($P < 0.001$; for both parameters). Although there was a trend toward lower liver T-SH levels in old female rats compared to those in young female rats, T-SH levels were not found to be significantly lower in old female rats when compared with those in young female rats (Fig. 4c). Concentrations of the lipid peroxidation markers in the liver tissue of female rats are given in Fig. 5. MDA and LHP levels of old female rats were significantly higher compared with those of the young female rats [$P < 0.001$ (Fig. 5a) and $P < 0.05$ (Fig. 5b), respectively]. Figure 6 shows the effect of aging on liver tissue GSH levels of old female rats wherein there was a significant decrease in GSH ($P < 0.05$) levels in old female rats.

Pearson's rank correlation coefficients among various oxidative stress markers in the whole series of male and female rats are given in Figs. 7, 8, and 9, respectively. In liver tissue of male and female rats, MDA levels were significantly correlated with PCO levels (Fig. 7a, b, respectively). LHP levels were significantly correlated with PCO levels in male rats

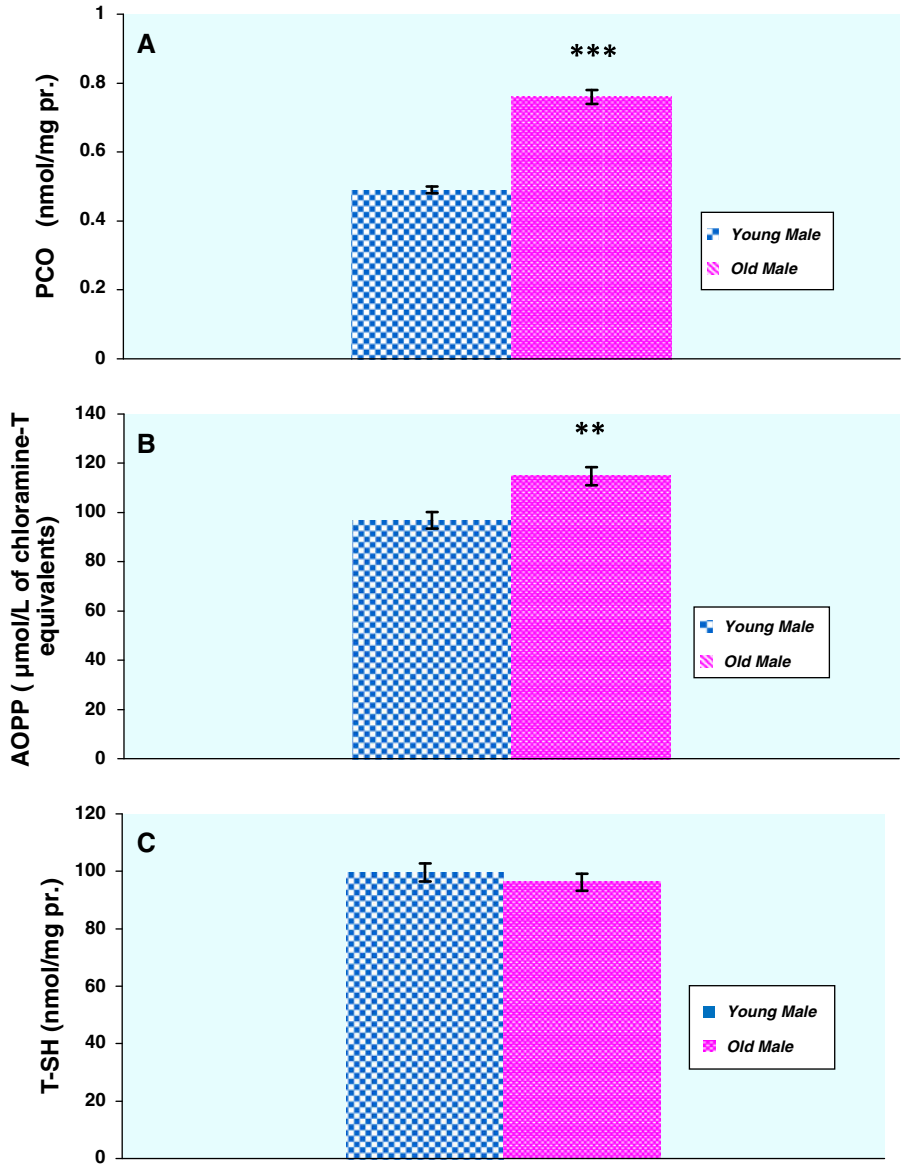

Fig. 1 Protein oxidation parameters in the liver tissue of male rats. Results are expressed as mean ± SEM. Data are statistically different between groups. (**) $P < 0.01$, (***) $P < 0.001$, *PCO* protein carbonyl; *AOPP* advanced oxidation protein products; *T-SH* total thiol; *pr* protein

(Fig. 7c). On the other hand, tissue LHP levels were not significantly correlated with PCO levels in female rats (Fig. 7d). MDA and LHP levels were not significantly correlated with tissue AOPP levels in male rats (Fig. 8a, c). There was a significant correlation between MDA and AOPP in female rats (Fig. 8b). On the other hand, LHP levels of the female rats were significantly correlated with tissue AOPP levels (Fig. 8d). In liver tissue of male and female rats, GSH levels were negatively correlated with protein oxidation parameters such as PCO and AOPP (Fig. 9a–d respectively).

Discussion

The search for a single cause of aging has recently been replaced by the view of aging as an extremely

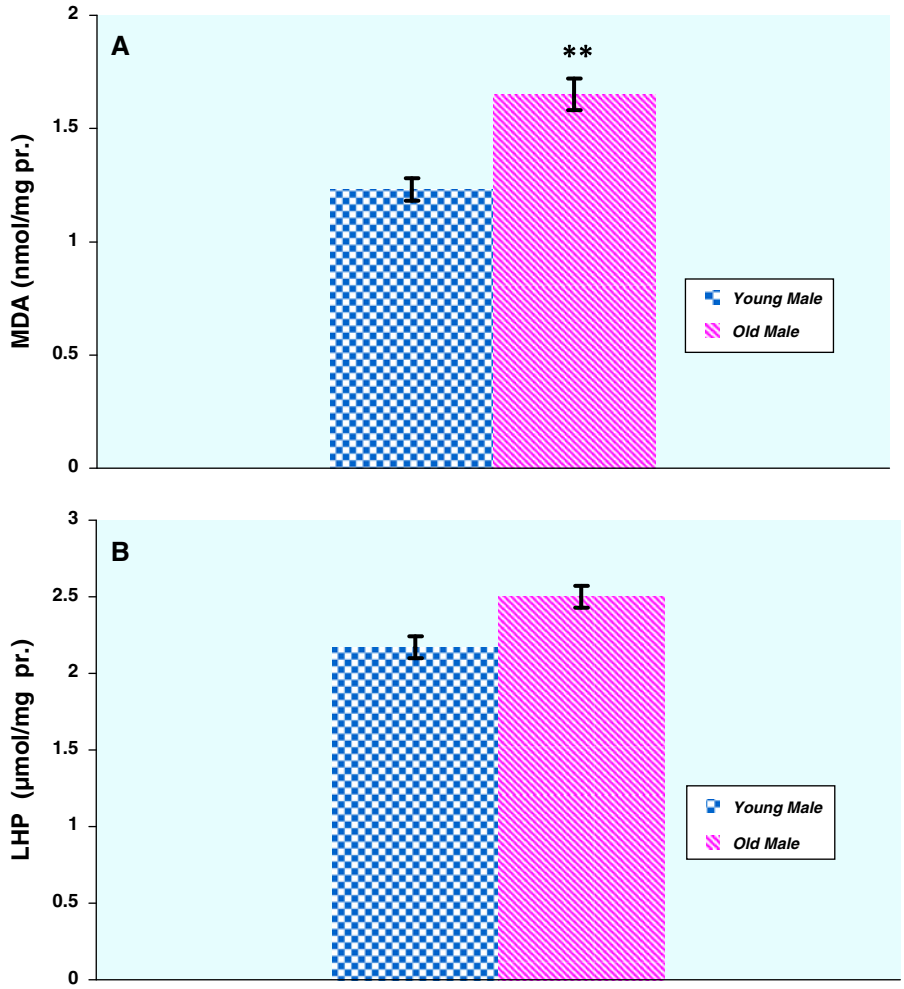

Fig. 2 Concentrations of the lipid peroxidation markers in the liver tissue of male rats. Results are expressed as mean ± SEM. Data are statistically different between groups. (**) $P < 0.01$. *MDA* malondialdehyde; *LHP* lipid hydroperoxides; *pr* protein

complex, multifactorial process. Each of them being insufficient stand-alone, many theories, which have brought partially new points of view and new questions to be answered, have been postulated to explain the phenomenon of aging. Some of the recent experimental studies (Pérez et al. 2009; Brink et al. 2009), support the theory of metabolic stability of aging, which question the general validity of the oxidative stress theory of aging proposed by Harman. Transcriptional changes in gene expression constitute a biomarker of metabolic stability. Comparative studies of transcriptional gene regulation in glutathione metabolism, insulin signaling and oxidative phosphorylation, networks commonly associated with aging, have provided support for the metabolic stability theory (Brink et al. 2009). It is our belief that although these studies reject the free radical theory of aging, they are insufficient in revealing the effects of age-related oxidative stress on transcriptional gene regulation relevant to the metabolic pathways mentioned above.

The majority of mammalian aging research has been focused on males, and only recently are the survival data for females being characterized in studies which directly compare the genders under similar conditions. Increased oxidation of hepatic proteins was apparent with aging. Differences in the level of protein oxidation between the genders suggest that programming of mechanisms of aging could be occurring through gender-specific processes,

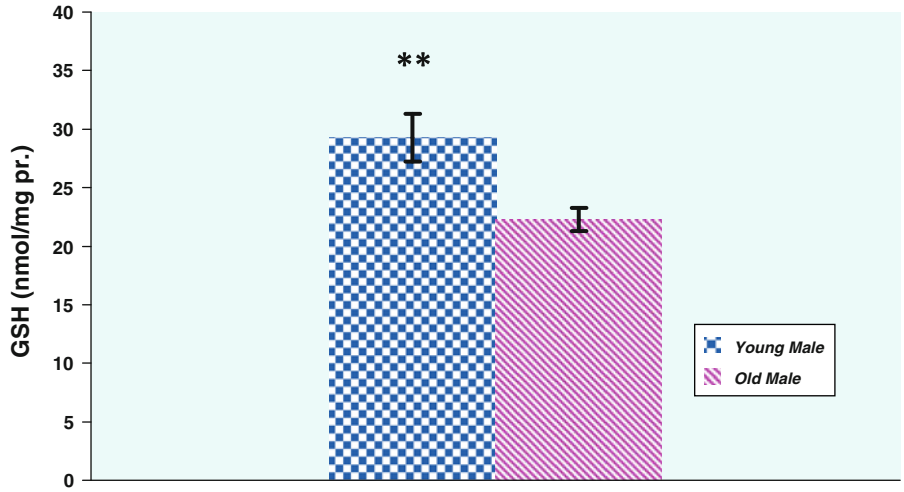

Fig. 3 GSH levels in the male study groups. Results are expressed as mean ± SEM. Data are statistically different between groups. (**) $P < 0.01$, *GSH* glutathione

or that such programming occurs via multiple mechanisms that operate in a gender-specific manner (Langley-Evans and Sculley 2005).

All amino acids can be attacked by ROS and RNS, but sulphur-containing and aromatic amino acids are the most susceptible to protein oxidation in an aging cell. PCO groups may be formed by peptide backbone fragmentation (I); extraction of hydrogen from alpha carbon atom (II); or an oxidative attack on several amino acid side-chains (III), the formation of adducts between some amino acid residues and the products of lipid peroxidation such as MDA (IV). On the other hand, PCO groups are also generated by glycation/glyoxidation of lysine amino groups (Stadtman and Levine 2006). Disruption of redox regulation is likely to contribute to the exponential age-related rise in the level of oxidized protein and occurrence of disease (Humphries et al. 2006). Several recent papers point out the role of protein oxidation in gender-related redox homeostasis (Kayali et al. 2007a, b; Uzun et al. 2009). Our results regarding PCO levels are entirely consistent with those of the other authors, which demonstrated greater susceptibility to hepatic PCO formation in females than in males (Gasbarrini et al. 2001; Langley-Evans and Sculley 2005). This appears as a contradiction the experimental observation that females are less susceptible to oxidative injury than males (Borrás et al. 2003). Our experimental findings appear to be inconsistent with Borras et al. at the first glance. The seemingly different cause's probable reason is that Borras et al. have studied isolated liver mitochondria, whereas we have studied the liver homogenate. On the other hand, we also have the idea that the presence of extramitochondrial oxidative stress on establishment of redox homeostasis should not be overlooked. In addition, previous research has indicated that females have greater global O_2 consumption, which is indicative of a higher energy expenditure than that of male rats (Rodriguez-Cuenca et al. 2002).

We are of the conviction that the increased MDA levels determined in aged male and female rats may be an accelerating factor in propagation of protein oxidation, as the PCO and AOPP levels in their liver tissue were increased. Spearman's rank correlation coefficients between the various lipid peroxidation parameters and PCO groups except that the female LHP/PCO couple were significantly correlated in aged rats. At the same time high level of positive MDA–PCO correlation in both gender of rats demonstrates the activation of the formation of adducts between some amino acid residues and the products of lipid peroxidation such as MDA.

Moreover, there is no study investigating oxidative protein damage in terms of AOPP and T-SH in aged liver tissue. For reasons stated above, we opted to perform the protein oxidation marker determinations in liver. AOPP are defined as dityrosine-containing cross-linked protein products and are considered to be

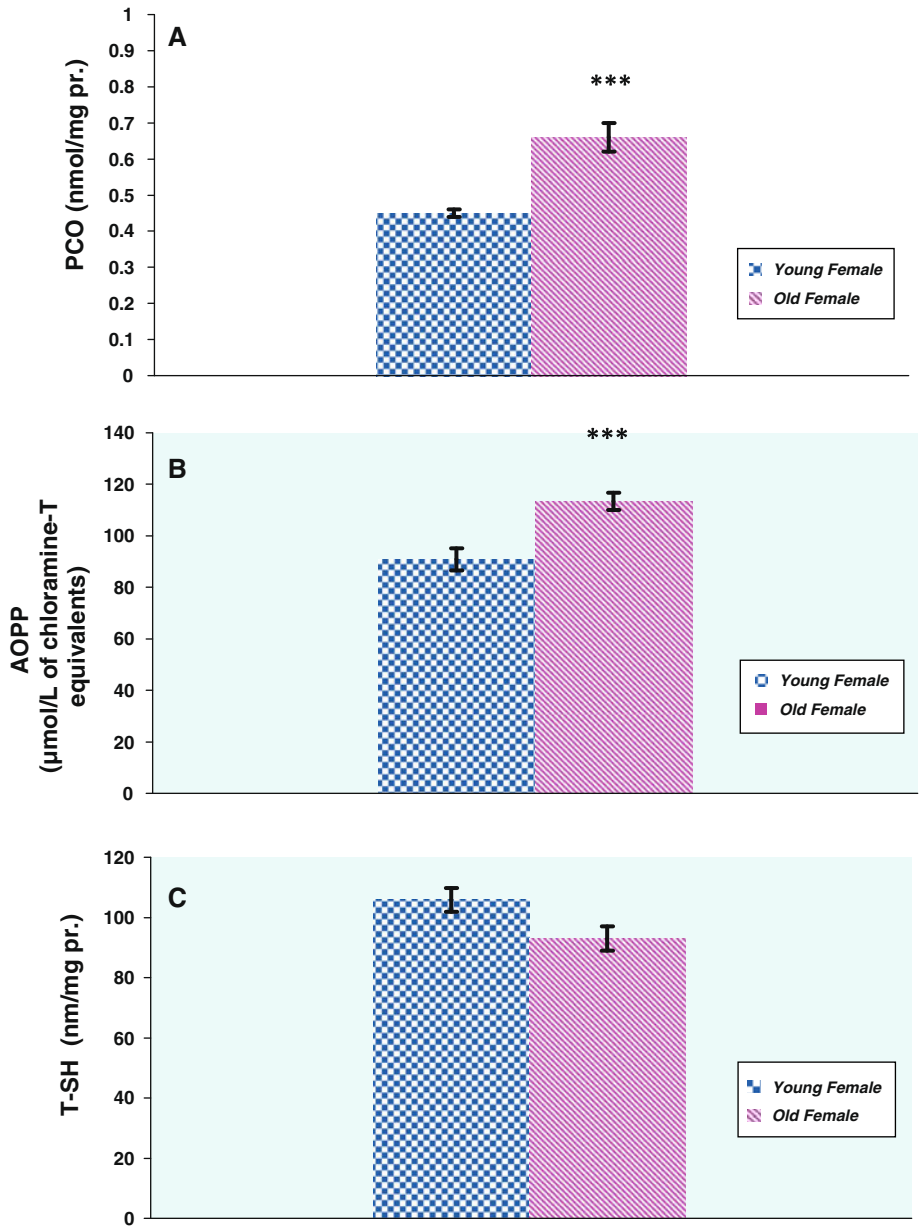

Fig. 4 Protein oxidation parameters in the liver tissue of female rats. Results are expressed as mean ± SEM. Data are statistically different between groups. (***) $P < 0.001$, *PCO* protein carbonyl; *AOPP* advanced oxidation protein products; *T-SH* total thiol; *pr* protein

reliable markers to estimate the degree of protein oxidation. In the present study, the increased occurrence of protein oxidation in liver tissue of aged female rats was also confirmed by a novel marker (AOPP assay) that provides information on the degree of oxidative damage to proteins, and the data obtained support those found with detection of PCO. Mitochondria contain thiol enzymes acting as antioxidants both by removing mitochondrial H_2O_2 and by repairing protein thiol oxidation. There were no significant differences in tissue T-SH levels in both genders of rats with respect to their corresponding controls in our study. To be able to continue their critical functions mentioned above for thiol groups,

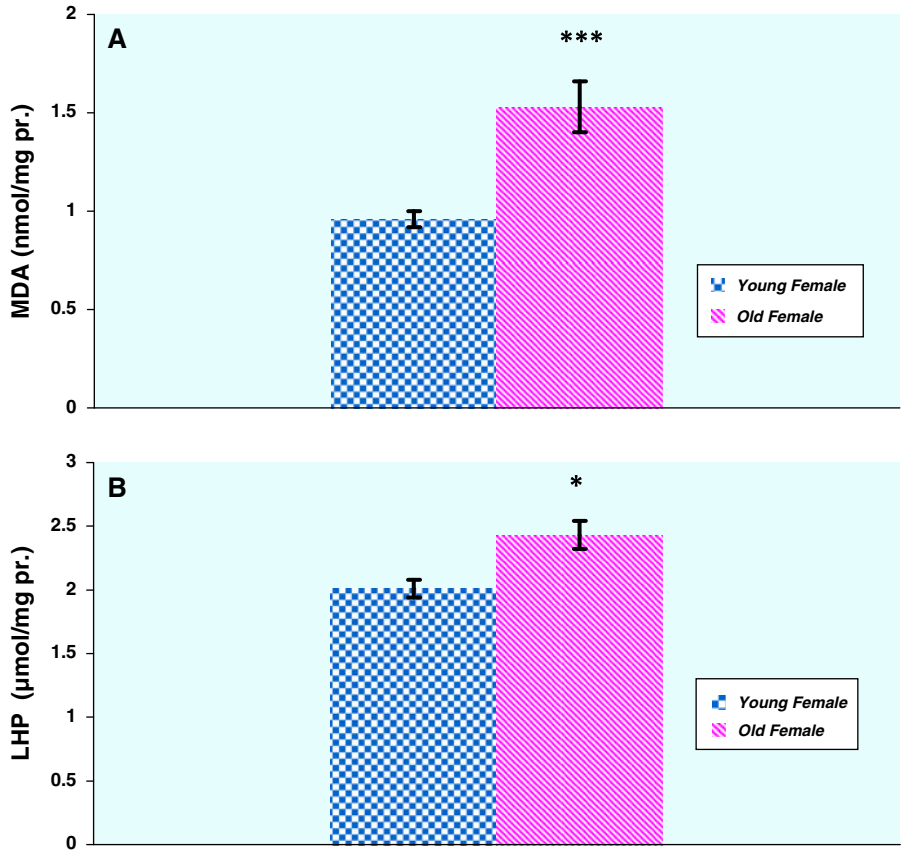

Fig. 5 Concentrations of the lipid peroxidation markers in the liver tissue of female rats. Results are expressed as mean ± SEM. Data are statistically different between groups. (*) $P < 0.05$, (***) $P < 0.001$. *MDA* malondialdehyde; *LHP* lipid hydroperoxides; *pr* protein

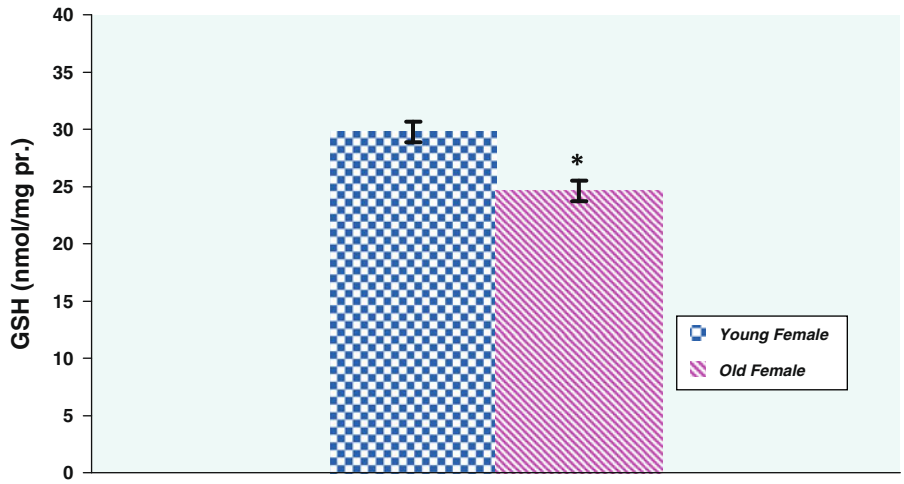

Fig. 6 GSH levels in the female study groups. Results are expressed as mean ± SEM. Data are statistically different between groups. (*) $P < 0.05$, *GSH* glutathione

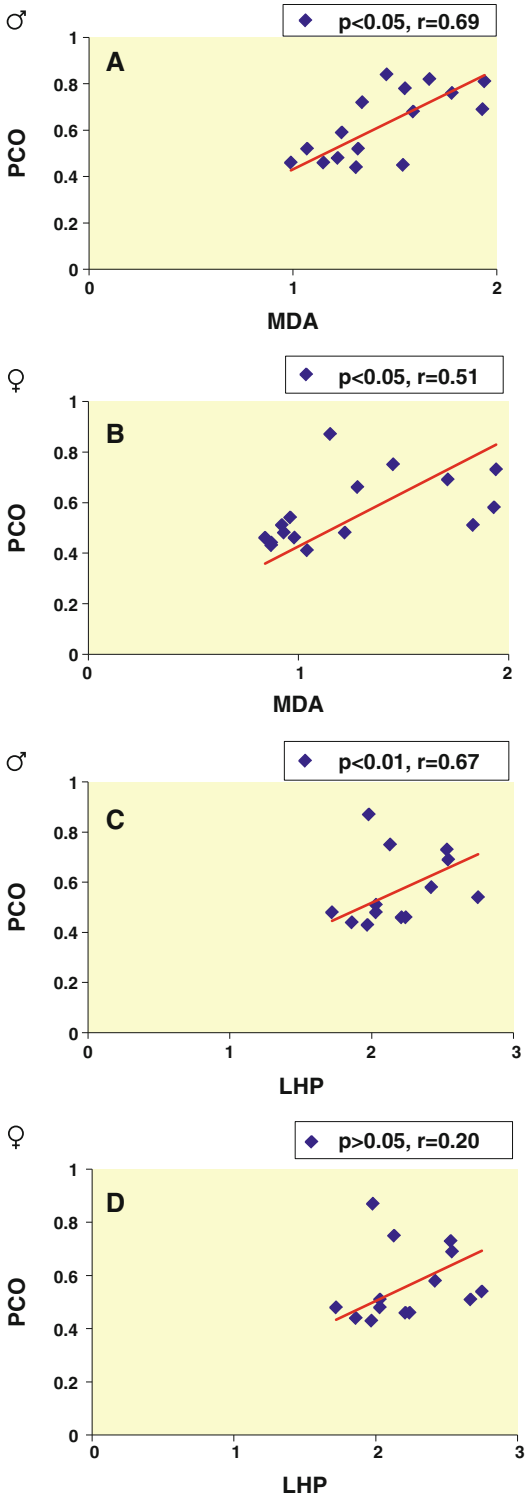

Fig. 7 Spearman's rank correlation coefficients between lipid peroxidation markers and PCO in the whole series of rats

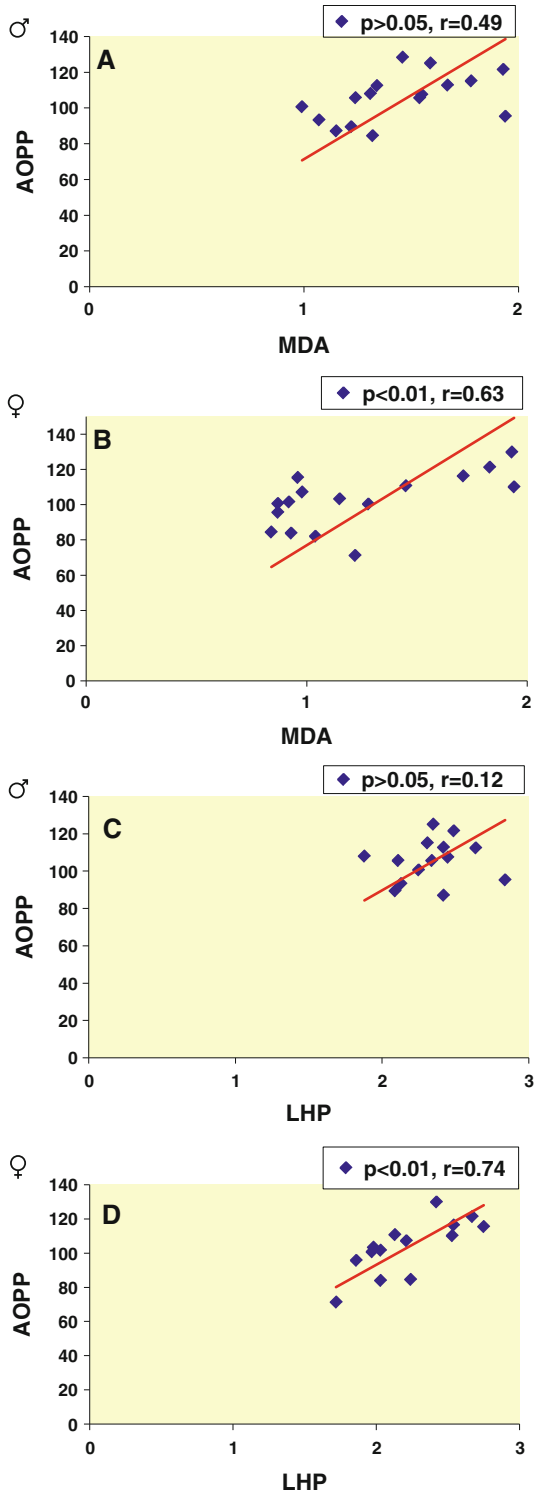

Fig. 8 Spearman's rank correlation coefficients between lipid peroxidation markers and AOPP in the whole series of rats

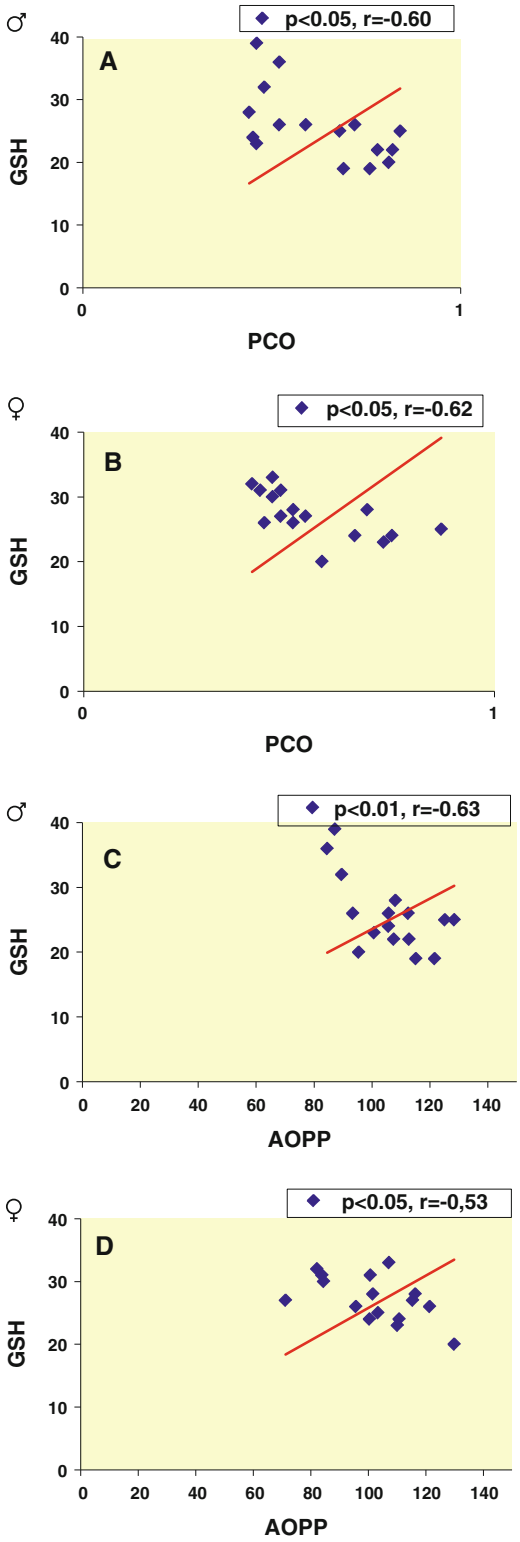

Fig. 9 Spearman's rank correlation coefficients between GSH and protein oxidation markers in the whole series of rats

this finding may be explained by the strict maintenance of redox homeostasis of the liver tissue.

It has been well established that a decrease in GSH concentration is associated with gender dimorphism in aged rats (Kayali et al. 2007a, b; Uzun et al. 2009). Furthermore a high blood GSH level was correlated with long life span in the mouse, rat, and in the healthy elderly human beings (Lang et al. 2000, 2002). Our current results are in accordance with the previous findings as regards GSH levels. PCO and AOPP levels in liver tissue were negatively correlated with GSH levels in both genders. An explanation for the negative correlation may provide evidence for a protective role of GSH against protein oxidation.

The exact molecular mechanisms leading to these experimental findings are not yet in entirety known and further studies are therefore required. In the current study, it is important to note that the gender-dependent changes in correlation of the parameters of oxidative protein damage indicate that different regulatory mechanisms might play an important role in gender dependency.

Acknowledgments This work was supported in part by funding from a grant from the Research Fund of The University of Istanbul (UDP-4/2010). The corresponding author is grateful to linguistic expert *Mr. Burak Alkan* for reading the manuscript and for improvements in the linguistic style.

Declaration of interest The authors declare that there have been no competing interests with respect to the contents of this article.

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