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Age-related changes in the activity and expression of manganese superoxide dismutase, and mitochondrial oxidant generation in female and male rats

[Dişi ve erkek sıçanların mangan superoksit dismutaz aktivitesi ve ekspreyonu ile mitokondriyal oksidan üretiminde yaşla ilişkili değişiklikler]

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ABSTRACT

Objective: The aim of this study was to examine the age- and gender-related differences in peroxide production, manganese superoxide dismutase (MnSOD) activity and expression in liver mitochondria of Wistar rats at 12 and 24 months of age.

Methods: The chemiluminometric method for peroxide production, fluorometric method for malondialdehyde (MDA) levels, cumene hydroperoxide assay for glutathione peroxidase (GPx), the nitroblue tetrazolium assay for MnSOD activity, and Western-blotting for MnSOD expression were used.

Results: Mitochondrial peroxides are increased significantly in both genders as aging proceeded, and females exhibited more profound increment than the males. Mitochondrial SOD and GPx activities remained unaltered between 12 and 24 months of age, with no difference between two genders. The gender- and age-related differences were observed in MnSOD expression ($p<0.01$). The SOD activity per expressed enzyme protein was significantly decreased in 24-month-old animals of both genders ($p<0.01$). Female rats had a significantly lower ratio than their male counterparts ($p<0.05$). In females, the expression was not associated with the activity of MnSOD, while a positive correlation existed between these parameters in males ($r=0.573$, $p=0.001$). Enzyme expression was found to be significantly higher in female rats as compared to their male counterparts. Liver mitochondria are less prone to oxidative damage in female rats compared to males as observed at 12 and 24 months of age
Conclusions: An involvement of factors other than estrogen seems to be relevant for the difference in the MnSOD activity and expression pattern between two genders.

Key Words: oxidative stress, aging, gender differences, MnSOD, rat liver

Conflict of interest: The authors declare that there is no conflict of interest.

ÖZET

Amaç: Çalışmamızda, 12 ve 24 aylık Wistar cinsi sıçanların karaciğer mitokondrilerinde yaş ve cinsiyet ile ilişkili olarak peroksit üretimi, mangan süperoksit dismutaz (MnSOD) aktivitesi ve ekspresyonundaki ortaya çıkan değişikliklerin incelenmesi amaçlanmıştır.
Yöntemler: Peroksit üretimi kemilümünometrik, malondialdehit (MDA) düzeyi florometrik, glutatyon peroksidaz (GPx) kümen hidroperoksit, MnSOD aktivitesi nitroblue tetrazolium yöntemleri ile MnSOD ekspresyonu ise western blotting yöntemi ile ölçülmüştür.
Bulgular: Her iki cinsiyette de yaş ilerledikçe SOD ve GPx aktivitelerinde anlamlı bir değişiklik oluşmadığı, mitokondriyal peroksit üretiminin ise anlamlı şekilde arttığı ve bu artışın dişi sıçanlarda daha belirgin olduğu saptanmıştır. MnSOD ekspresyonun da yaş ve cinsiyete bağlı olarak değişiklikler gösterdiği ($p<0.01$) ve eksprese edilen enzim proteini başına düşen enzim aktivitesinin 24 aylık sıçanlarda her iki cinsiyette de anlamlı olarak azalmakla birlikte ($p<0.01$) bu azalmanın dişi sıçanlarda erkek sıçanlara göre daha belirgin ($p<0.05$) olduğu görülmüştür. Erkek sıçanlarda MnSOD aktivitesi ile ekspresyonu arasında pozitif bir korelasyon ($r=0.573$, $p=0.001$) olduğu saptanmış, dişi sıçanlarda ise böyle bir ilişki saptanmamıştır.
Sonuç: 12 ve 24 aylık sıçanlar karşılaştırıldığında dişi sıçanların MnSOD ekspresyonunun akranı olan erkek sıçanlara göre anlamlı olarak yüksek olduğu, karaciğer mitokondrilerinin oksidatif strese duyarlılığının ise daha az olduğu görülmüştür. Yaş ilerledikçe iki cinsiyet arasında MnSOD aktivitesi ve ekspresyon paterninde ortaya çıkan bu değişikliklerin sadece östrojen etkisi ile açıklanamayacağı, başka faktörlerin de katkısının olduğu sonucuna varılmıştır.

Anahtar Kelimeler: oksidatif stres, yaşlanma, cinsiyet farklılıkları, MnSOD, sıçan karaciğeri

Çıkar Çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Aging is a natural and progressive process for all living species, which contributes to age-related decline in health and causes susceptibility to diseases. It is suggested that age-related decline in health conditions is the result of accumulated damage over time [1, 2]. In the "Free Radical Theory of Aging" proposed by Harman in 1950s, it has been suggested that the cumulative effect of free radicals produced during aerobic metabolism ultimately causes aging and death of all living things through oxidative damage [3]. Over the years, Harman improved his theory to the "Mitochondrial Theory of Aging", and suggested that mitochondria are both the major source and the target of reactive oxygen species (ROS) in the cell [4]. Further studies have established convincing data for the role of mitochondria in aging process [5-8]. In a cell, the electron transport chain that produces 90% of ROS, is located on the inner membrane of mitochondria, thus making mitochondria the first potential target of ROS attack. Under physiological conditions, a small portion of oxygen consumed by mitochondria is constantly converted to ROS, i.e. superoxide anions, hydrogen peroxide, and hydroxyl radical [9, 10]. At a limited concentration, ROS modulate some cellular functions, but whenever they exceed this level, devastating interactions with macromolecules take place in the mitochondria [8, 11, 12]. In order to prevent this oxidative damage, cells have antioxidant defense systems including manganese-dependent superoxide dismutase (MnSOD), copper-zinc superoxide dismutase, catalase, glutathione peroxidase (GPx) and glutathione reductase. Any decrements in the activities of these antioxidant enzymes may lead to accumulation of ROS, which are generated at a higher rate during the aging process, thus leading to oxidative damage to biological macromolecules [13]. Oxidative damage to polyunsaturated fatty acids of membrane lipids generates malondialdehyde (MDA) as one of the end-products [14, 15]. Studies on age-related changes in antioxidant defense system suggest that MnSOD is an essential front line defense against oxidative damage and its expression rate is important for cells to cope with ROS-mediated damage [11, 16].

Aging studies are mainly focused on gender-related differences in life span [10, 17-20]. Numerous studies have reported that females live significantly longer than males both in mammals and other vertebrates, as well as in some invertebrate species [18, 20-22].

The present study was designed to examine the activities of MnSOD and GPx, the levels of MDA and peroxide production, and the MnSOD expression in liver mitochondria of 12- and 24- month-old rats, and to see whether age-related changes in the oxidant/antioxidant state may be affected by the gender.

Methods

Experimental design

Male and female Wistar albino rats comprised two study groups, 17 animals in each. Nine-week-old rats were obtained from Istanbul University, Institute of Experimental and Medical Research, and included in the study. They were kept at 22 ± 2°C, and 12 h light/dark cycle with 50±10% relative humidity and were fed a standard diet containing 590 g carbohydrate, 30 g lipid and 160 g protein per kilogram rat chow during the study period that lasted for 24 months. Two animals (one male and one female) did not survive after 20 months.

At two time points (12 and 24 months), eight animals from each gender were sacrificed. Following ether anesthesia, rats were perfused from the heart with PBS, pH 7.4, containing 0.16 mg/ml heparin to remove red blood cells and clots from tissues. Livers were quickly removed and divided into portions. All tissue samples were frozen in liquid nitrogen and stored at -80°C until analysis. The Committee of Animal Care and Use approved the study protocol.

Isolation of Liver Mitochondria

Liver mitochondria were prepared using differential centrifugation method with some modifications [23]. Briefly, liver tissue was minced in ice-cold buffer containing 10 mM Tris–HCl pH 7.4, 250 mM sucrose, 1 mM EGTA, 0.2% BSA, 1 mM phenylmethylsulphonyl fluoride, 1 mM dithiothreitol, 10 µg/ml aprotinin, 10 µg/ml leupeptin and 10 µg/ml soybean trypsin inhibitor. The samples were homogenized on ice for 1 min followed by centrifugation at 800 *g* for 5 min at 4°C. The supernatant was re-centrifuged at 10 000 *g* for 12 min at 4°C, and then the mitochondrial pellet was resuspended in a small volume of the homogenization buffer without BSA. Protein content of the samples was determined by the bichinconinic acid method [24].

MDA Assay

MDA levels in liver mitochondria were measured by the fluorometric method using 1,1,3,3-tetraethoxypropane as a standard, at wavelengths of 532 nm for excitation and 547 nm for emission. Results were expressed as nmol/mg protein [25].

Peroxide Production

For the measurement of free oxygen radicals in the liver mitochondria, chemiluminometric method was employed. The measurements were carried out with Mini Lumat LB 9506 Luminometer (EG&G, Berthold, Germany) at room temperature using luminol as enhancer. The chemiluminescence corresponding to total value for hydroxyl radical, hydroperoxyl radical and peroxyl radical was measured. Counts were obtained by one minute intervals for a counting period of 10 min, and the area under curve (AUC) was determined. Counts were

corrected for protein content of samples and results were expressed as relative light unite (rlu)/mg protein [26].

MnSOD Activity Assay

MnSOD activity of the liver mitochondria was measured by the spectrophotometric procedure depending on the inhibition of nitroblue tetrazolium (NBT) reduction with superoxide generated by the xanthine–xanthine oxidase system. One unit of SOD activity is defined as the amount of protein that inhibits the rate of NBT reduction by 50%. Results were expressed as U/mg protein [27].

MnSOD Expression Assay

Mitochondria isolated by differential centrifugation were suspended in 0.5 mM phosphate-buffered saline (PBS) containing aprotinin, PMSF and leupeptin. Mitochondria samples (50 µg protein) were heated at 95°C for 5 min. Samples and MnSOD standard (4□g/mL) were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using the Bio-Rad Mini Protean III gel system. Resolved samples were transferred to the polyvinylidine difluoride (PVDF) membranes. Membranes were treated with 5% non-fat dry milk in PBS containing 0.01% Tween 20 (PBS-T) to block any non-specific antibody binding sites, and then incubated for 16 h at 4°C with rat monoclonal antibody against MnSOD protein (1:500, Santa Cruz Biotechnology, CA, USA). Membranes were washed with PBS-T and incubated further for 1.5 h at room temperature with horseradish peroxidase-conjugated anti-sheep IgG secondary antibody (1:2500, Santa Cruz Biotechnology, CA, USA). The chemiluminescence signals were visualized by Biorad® GS800 Calibrated Densitometer and the bands were then quantified using the image analysis software Quantity One 4.6 (Bio-Rad, CA, USA).

GPx activity

GPx activity in liver mitochondria was measured using cumene hydroperoxide as substrate. Enzymatic activity was performed at 37° C by measuring the oxidation of NADPH to $NADP^+$ at 340 nm. Results were expressed as U/mg protein [28].

Statistical analyses

All statistical analyses were performed with SPSS version 11.0 for Windows. Data were expressed as means±SD. Mann-Whitney U test was used to compare the data obtained from male and female rats in the same age, as well as to evaluate the age-related differences in the same gender. $p< 0.05$ was considered statistically significant. The correlations between the measured parameters were searched using Spearman test.

Results

Mitochondrial peroxide production showed a profound increase in female rats as the animals grow older ($p=0.001$), whereas in male rats the age-related difference between 12 months and 24 months of age was not significant. When the gender-related difference in peroxide production was examined, female rats had significantly lower peroxide production than male rats in both 12 months and 24 months of age ($p=0.001$ and $p=0.015$, respectively). Mitochondrial MDA levels tended to increase both in female and male rats at 24 months, as compared to the levels in 12 months, but the difference did not reach a statistical significance.

Also, mitochondrial SOD and GPx activities were unaltered between 12 and 24 months of age. No difference in enzymatic activity and MDA levels were seen between two genders.

The gender- and age-related differences were evident in MnSOD expression (Table 1). When the SOD activity per expressed enzyme protein was calculated, the ratio significantly lowered in 24-month-old animals of both genders ($p<0.01$). Female rats had a significantly lower ratio than their male counterparts ($p=0.021$ for 12 months, Figure 1 and $p=0.006$, for 24 months, Figure 2). As a result, in female rats, the expression was not

Table 1. Mitochondrial peroxide production, Mn-SOD activity and expression, GPx activity and MDA levels in rat liver (means±SD).

Parameters	12-month-old		24-month-old	
	Female (n=8)	Male (n=8)	Female (n=8)	Male (n=8)
Peroxide production (RLU/mg protein)	53.7 ± 24.4	221 ± 60.0[a]	174±44.7*	255 ± 47.0[c]
Mn-SOD Activity (U/mg protein)	2.7 ± 0.8	1.9 ± 0.8	2.7 ± 0.9	2.8 ± 0.8
Mn-SOD Expression (mg protein/mL)	1.87 ± 0.3	0.8 ± 0.3[a]	7.3 ± 0.9*	2.7 ± 1.4*,[b]
GPx Activity (U/mg protein)	39.2 ± 15.0	32.4 ± 13.4	36.8 ± 12.3	45.1 ± 10.8
MDA (nmol/mg protein)	1.1 ± 0.3	1.4 ± 0.4	1.4 ± 0.3	1.8 ± 0.5

*$p < 0.001$, *$p < 0.01$; when compared with 12- and 24-month-old rats of the same gender.
[a]$p < 0.001$, [b]$p < 0.01$, [c]$p < 0.05$; when compared with female and male rats of the same age.

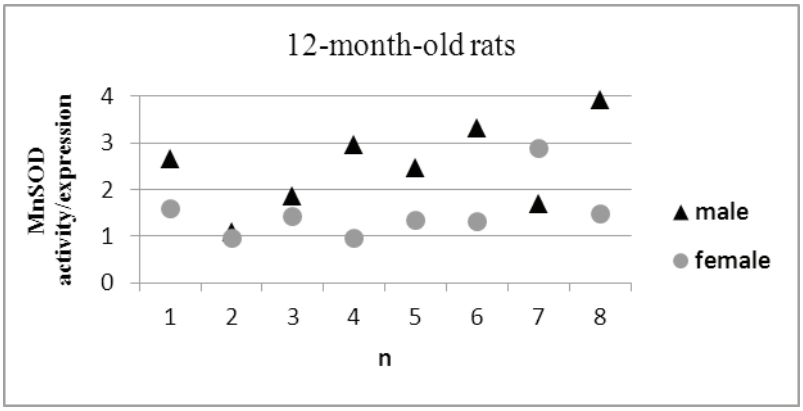

Figure 1. MnSOD activity /expression ratio in the livers of 12-month-old rats.

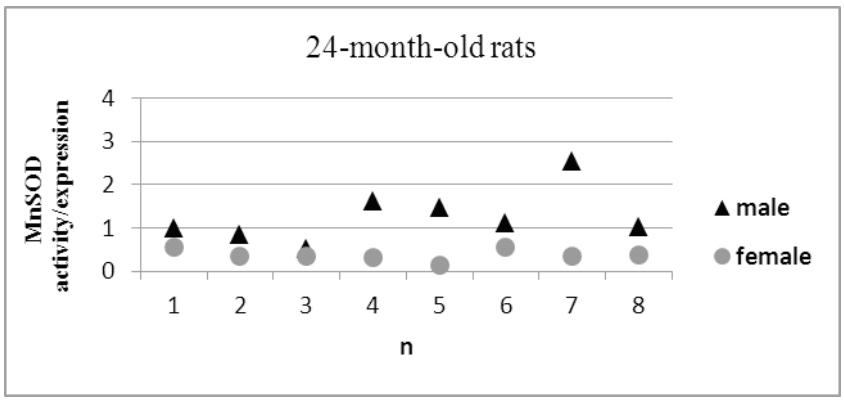

Figure 2. MnSOD activity /expression ratio in the livers of 24-month-old rats.

associated with the enzyme activity, while a positive correlation existed between the activity and expression of MnSOD in male rats (r=0.573, p=0.001).

In female rats, MnSOD expression was correlated with the mitochondrial MDA levels and peroxide production (r= 0.578, p=0.001, and r=0629, p=0.000, respectively).

Discussion

This study was carried out in 12 and 24 months old rats under natural environmental and physiological conditions, and searched the age- and gender-related differences in liver mitochondrial oxidant/antioxidant status. The amount of mitochondrial peroxides was found elevated significantly in both genders at 24 months of age. In female rats, the peroxide production per milligram of mitochondrial protein was less than the males, reflecting the sex-dependent differences in free radical homeostasis. Accordingly, the previous studies carried out in rats have reported that females had lower oxidative stress and mitochondrial dysfunction than the males [29-31]. However, these reports concerning the gender differences in mitochondrial oxidant status have either dealt with the 4-6 months old animals [32-33], or applied some interventions such as ovariectomy and estrogen replacement therapy [30, 32, 34, 35].

The effect of age and gender on the activity and expression of manganese superoxide dismutase (MnSOD), a key antioxidant enzyme specifically localized in the mitochondria was also examined in this study. No significant difference in enzymatic activity was found with regard to age. When the gender-related differences were evaluated, MnSOD activity in hepatic mitochondria was found approximately 30% lower in male rats than in female rats at the age of 12 months. However, the difference in enzymatic activity between two genders disappeared at the age of 24 months.

An increment in SOD expression due to aging was evident in both genders, but the degree of increase was less in male rats than females. The researchers have shown that antioxidant enzymes, namely SOD and GPx, are overexpressed in females [32, 33, 36]. Since their findings were restricted to the species in which females live longer, it was thought to be due to estrogenic effect on longevity-associated genes [32, 37, 38]. In a previous study, estrogen treatment restored both SOD and GPx activity and decreased MDA levels in aged rats [39]. If estrogenic activity had been the sole factor, expressions of SOD and other enzymes in antioxidant defense system would have been lowered with advancing age in female rats. On the contrary, we observed a four-fold increase

in the SOD expression in 24-month-old females as compared to 12-month-old females. This finding suggests the influence of some other mechanisms or endogenous factors on MnSOD expression. It has been reported that in aged rats, female brain is less prone to oxidative damage than male brain [33]. Furthermore, estrogen treatment enhanced MnSOD activity whereas it had no effect on enzyme protein in brain mitochondria of female rats [35]. This study suggests that the expression and activity of MnSOD may not be well-correlated, and some factors may independently influence MnSOD activity.

Lysine acetylation has recently emerged as an important, and perhaps the primary, posttranslational modification employed to regulate mitochondrial proteins [40]. MnSOD protein contains a reversibly acetylated lysine residue that is deacetylated by caloric restriction and 36 hours of fasting [41]. *In vitro* experiments have shown that purified MnSOD was directly deacetylated by recombinant SIRT3 protein and the wild-type *Sirt3* gene decreased MnSOD acetylation thereby increasing the MnSOD activity. Their results strongly suggested that deacetylation activity of SIRT3 regulates the enzymatic properties of MnSOD through modulating its acetylation status [41].

Finally, the findings of the present study clearly indicate that oxidant production in liver mitochondria is less in female rats than in males, and this situation is carried out throughout the life-span, as observed at 12 and 24 months of age. It can be concluded that differences between two genders related to oxidative stress are still evident when female hormonal activity is diminished. A possible involvement of endogenous factors on gender-related differences as aging proceeded should also be evaluated by further studies.

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