Role of Oxidative Stress and Adenosine Nucleotides in the Liver of Aging Rats

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Summary

We studied the response of several parameters related to oxidative stress in the liver of aging rats. Male Wistar rats aged 1.5, 3, 18 and 24 months were used. Livers showed an increase in superoxide anion (O_2^-) concentration at 1.5 and 18 months of age compared to the 3-month-old group; a decrease in superoxide dismutase (SOD) was seen at 1.5 months and catalase concentrations remained unaltered throughout the aging process. Nitric oxide (NO) progressively declined with age; a significant decrease was particularly apparent at 18 and 24 months of age. Thiobarbituric acid reactive substances (TBARS) decreased significantly at 1.5 months, whereas it increased at 18 and 24 months of age. Concentrations of prostaglandin E_2 (PGE_2), and adenine nucleotides, and their metabolites, remained unchanged throughout the aging process. Although the mitochondrial damage caused by oxidative stress can result in reduced ATP production and compromised cell function, our results on adenosine nucleotides and their metabolites support the notion that the integrity of mitochondria and enzymatic activity remain mostly unchanged with aging. In conclusion, we observed a significant decrease in the levels of NO in the older groups of rats and hence in its antioxidant activity. This could explain the observed increase in lipid peroxides which suggests an important role for NO in oxidative stress in the liver of older rats.

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Introduction

Aging can be defined as an intrinsic biological process from which there is no way out. It is characterized by the degeneration of basic functions in the late or post-reproductive phase of all multicellular organisms (Sohal 2002, Ehrenbrink *et al.* 2006).

The extent of cellular damage and aging is related to a balance between the production of oxidants and their removal by the antioxidant system. Superoxide anions (O_2^-) are the first oxygen free radical generated and are formed by the univalent reduction of oxygen. This reduction is mediated by several enzyme systems, including NADPH oxidase, xanthine oxidase and cyclooxygenase. In healthy livers, hepatocytes produce small amounts of reactive oxygen species (ROS) and Kupffer cells, the resident macrophages in the liver, release ROS in response to bacterial stimuli. Hepatic stellate cells contain a non-phagocytic form of NADPH oxidase. This form is constitutively active, producing relatively low levels of ROS under basal conditions (De Minicis *et al.* 2006). ROS is also involved in the pathophysiology of inflammatory liver diseases (Jaeschke 2000, De Minicis *et al.* 2006).

Steady-state levels of O_2^- are dependent on both the production rate and the activity of the various superoxide dismutase (SOD) isoforms (Faraci and Didion 2004). The O_2^- generated is captured by SOD and metabolized to hydrogen peroxide (H_2O_2) in a cellular antioxidant reaction; catalase and glutathione peroxidase then detoxify the H_2O_2 generated. Lipid peroxides which are expressed as thiobarbituric acid reactive substances (TBARS), reflect a condition of oxidative stress. Nitric oxide (NO) is a free radical that has a beneficial effect on the liver by protecting it against both tissue injury and the cytotoxic effects of invading microorganisms, parasites, and tumor cells. Thus, NO may prevent liver tissue damage caused by oxidative stress (Suzuki *et al.* 1995). Prostaglandin E_2 (PGE$_2$) is generated through the activation of constitutive cyclooxygenase-1 (COX-1) in hepatocytes and plays a crucial role in liver pathophysiology through essentially hepatoprotective functions such as inhibiting the generation of ROS, preventing leukocyte migration, improving hepatic insulin and lipid metabolism and regulating the production of inflammatory cytokines (Park *et al.* 1986, Hossain *et al.* 2006).

Mitochondria are the main cell components responsible for events involving oxidative stress. Thus, a vicious cycle may occur in which damaged mitochondria produce progressively greater amounts of ROS, leading, in turn, to progressively greater damage to mitochondrial, cytosolic and nuclear compartments and finally resulting in dysfunctional or defective mitochondria, which is the basis of the mitochondrial theory of aging (Miquel *et al.* 1980, Harper *et al.* 2004). The mitochondrial damage caused by oxidative stress could result in reduced ATP production and compromised cell function (Van Remmen and Richardson 2001). These classical considerations together with a recent discussion on the weaknesses of the mitochondrial theory of aging (Krause 2007) and the data that question whether the *in vitro* studies of oxidative damage to mitochondria reflect what occurs *in vivo* (Van Remmen and Richardson 2001) prompted us to determine the concentrations of adenosine nucleotides, nucleosides and purine bases in the liver of aging rats.

Adenosine nucleosides (adenosine and inosine) have an anti-inflammatory potential, which is based mainly on the inhibition of pro-inflammatory cytokines and the release of free radicals. Increased purine nucleosides are found in ischemic and inflammatory tissues and these compounds prevent oxidative damage to DNA and protect mice against γ-radiation-induced death

(Gudkov *et al.* 2006, Schneider *et al.* 2006).

Here we studied the occurrence of oxidative stress in rats aged 1.5, 3, 18 and 24 months. These represent a broad range of ages, from very young to old rats. The presence of oxidative stress was determined on the basis of the production of O_2^-, two antioxidant enzymes: SOD and catalase, and TBARS as an indicator of lipid peroxidation. The production of two hepatoprotective agents, PGE$_2$ and NO, was also examined as were the concentrations of adenosine nucleotides and their metabolites caused by the involvement of mitochondria in oxidative stress. The rats were not manipulated; age was the only parameter considered.

Materials and Methods

Animals and sample preparation

Male Wistar rats (Harlan Interfauna Iberica S.L.) aged 1.5, 3, 18 and 24 months were used. All the animals were fed standard laboratory chow and had access to water *ad libitum*.

Experiments were conducted in accordance with the Guide for the Care of Laboratory Animals (EEC Council Directive 86/609/EEC). All the studies were approved by the Research and Development and Animal Care Committee of the University of Barcelona.

The rats were anesthetized with sodium pentobarbital and killed by exsanguination. The liver was perfused with 20 ml of saline through the suprahepatic vein. The liver was then excised and after removing the border, the main lobe was cut into fragments 3 mm wide and 10 mm long. These samples were frozen in liquid nitrogen and kept at –80 ºC. All the *ex vivo* assays were performed in duplicate in 6 different animals. The 3-month-old rats were used for comparison with the other age groups.

Superoxide anion assay

The technique developed by Pagano *et al.* (1995) was used. Liver samples (150-160 mg) from each rat were defrosted by immersion in a Tyrode solution (in mM, NaCl 137; KCl 3; CaCl$_2$ 1.36; MgSO$_4$ 1.2; NaH$_2$PO$_4$ 0.5; NaHCO$_3$ 12; glucose 5.5) at 4 °C. Each experiment was performed in duplicate. The fragments were incubated in 1 ml Tyrode solution for 30 min at 37 °C with continuous air bubbling and then rinsed with Tyrode solution. The samples were placed in 1 ml of the same solution in a luminescence cuvette containing a

non-redox cycling concentration of lucigenin (5 μM) (Sigma). The cuvettes were then placed in a luminometer (Bio-Orbit 1251, Turku, Finland) and maintained at 37 ºC. The luminometer was set to report arbitrary units of emitted light, and integrated over a 30-s interval for 3 min. A blank Tyrode solution gave low and constant readings that were comparable to those obtained from a blank lucigenin solution. The results are expressed as units of luminescence/min/g tissue. The possible differences in the luminescence response to fresh and frozen samples of liver were also examined. The behavior of both types of samples was similar (data not shown).

Thiobarbituric acid reactive substances

Liver samples were homogenized with 120 mM KCl (1 ml/50 mg of wet tissue) and 50 μl of 0.5 % butylated hydroxytoluene (BHT). After centrifugation at 1000 x g for 10 min, 100 μl of supernatant was collected. We then added 100 μl trichloroacetic acid (TCA) 40 % (w/v) (Aldrich), and 400 μl of thiobarbituric acid (TBA) 0.67 % (w/v) (Merck) in 0.05 M NaOH was added to the supernatant. The mixture was then heated in a water bath at 97 ºC for 10 min using a glass ball as a condenser. After cooling, 200 μl of glacial acetic acid and 400 μl of chloroform were added and shaken with a vortex. After centrifugation at 1700 x g for 30 min, the organic layer was collected for spectrophotometric measurements at 530 nm. The results are expressed as ng TBARS/mg protein. A standard curve was drawn by preparing a malondialdehyde (MDA) solution (10 mM) by hydrolysis of 1,1,3,3-tetraethoxypropane (Sigma) with hydrochloric acid. Protein quantification was performed following the method described by Lowry et al. (1951) using serum albumin as standard.

Antioxidant enzymes assays

Liver samples were homogenized by Polytron (Kinematica, AG, Littau, Switzerland) in 2 ml of phosphate buffer (50 mM, pH 7.0) containing 10 mM ethylenediaminetetraacetic acid (EDTA), 0.13 mM BHT, and 0.13 mM desferrioxamine (iron chelator) to minimize oxidation during the homogenization. The minced tissue/buffer mixture was homogenized in two passes of 20 s at 37 °C. The homogenates were centrifuged at 300 x g for 10 min at 4 ºC to remove cellular debris. The supernatant was then collected to analyse SOD and catalase antioxidant enzyme activity.

SOD activity was assayed by the inhibition of pyrogallol autooxidation (Marklund 1985). We then added 1.2 ml of ethanol-chloroform (5:3 v/v) to 750 μl of supernatant. After centrifugation at 3400 x g for 5 min at 4 °C, several volumes of the supernatant were added to 25 μl of Tris-HCl. Finally, 20 μl of pyrogallol (Sigma) was added to start the reaction. Results are expressed as units/g tissue (one unit induces an inhibition of 50 % pyrogallol auto-oxidation).

Catalase activity was assayed by H_2O_2 consumption, following Aebi's (1984) method and modified by Pieper et al. (1995). Briefly, ethanol was added (1:100 v/v) to the supernatants and incubated for 30 min in an ice bath. We then added 1 % Triton X-100 (1:10 v/v) (Sigma) to the homogenates. This solution was placed in an ice bath for an additional 15 min. 500 μl of this solution was placed into a glass cuvette and 250 μl of 30 mM H_2O_2 (Sigma) in a phosphate buffer was then added to start the reaction. After 15 s the absorbance at 240 nm was read every 15 s for 45 s. The first-order reaction rate (k) of H_2O_2 consumption by catalase was calculated and the results are expressed in k/g tissue. The assay was performed at 25 °C.

Prostaglandin E_2 assay

Liver samples were incubated for 30 min at 37 ºC. After centrifugation the supernatant was stored at –80 ºC until the day of the analysis. The production of PGE_2 was determined with a PGE_2 monoclonal antibody (PGE_2 EIA kit, Cayman Chemical Co) following the manufacturer's protocol. The results are expressed as pg/mg protein.

Nitric oxide assay

NO was detected as described by Vanin (1999). Liver samples were preincubated at 37 °C for 20 min in Ringer solution (pH 7.4) and exposed to the spin trapping agents diethyldithiocarbamic acid (DETC, 5 nmol/l) (Aldrich) and $FeSO_4$ (50 μmol/l) for 30 min. Finally, the samples were weighed, frozen in liquid nitrogen and stored at –80 °C for later ESR analysis. The ESR-detectable paramagnetic complex was evaluated in a Bruker 300E spectrometer (Bruker Instruments, Billerica, MA). Given the complex, the signal corresponded to the difference in intensity between a maximum at 3440 [G] and a minimum at 3470 [G]. A standard curve of Fe-NO-DETC was generated by diethylamine NONOate (Cayman) (10 nmol/l to 100 μmol/l). This curve was used to extrapolate the Fe-NO-DETC signal and also to estimate the linearity of the assay. Results are expressed as relative intensity units/mg tissue.

Adenosine nucleotide assay

Liver samples were placed in 10 volumes of 3.6 % $HClO_4$ and then immediately homogenized. Following homogenization, tissues were extracted for 30 min at 0.5 °C and then centrifuged at 850 x g for 15 min. Supernatants were adjusted to pH 6.0-6.5 and centrifuged at 14000 x g. Then, we injected 50 µl of the supernatant into a Waters 717 Plus Autosampler for liquid chromatography. Nucleotide profiles were obtained using a reversed-phase Spherisorb ODS column (column C_{18}, 5 µm particle size, 15 x 0.4 cm; Teknokroma, Sant Cugat, Spain) coupled to a 600 HPLC system (Waters, Milford, MA) equipped with a Waters 996 Photodiode Array Detector. The absorbance was monitored at 254 nm. Nucleotide separation was allowed to proceed in an isocratic fashion with 100 mmol/l ammonium phosphate (pH 5.5), until the point when ATP, ADP, hypoxanthine, xanthine, and AMP were separated. At this point, a mixture of water-methanol (96:4) was introduced into the column, thereby eluting inosine. Another mixture of water-methanol (60:40) was introduced after inosine in order to elute adenosine. Calibration chromatograms were generated for the following standards by injecting 50 µl of a mixture of known concentrations: ATP, ADP, AMP, adenosine, inosine, hypoxanthine, and xanthine (Sigma). The profiles were processed by a Millennium 32 system (Waters, Milford, MA). The results are expressed as pmol/mg tissue.

Statistical analysis

All experiments were carried out at least in duplicate. Every age group consisted of 6 rats. One-way analysis of variance (ANOVA) was performed and Dunnett's *post-hoc* test was used to compare all the groups against the 3-month-old group. Statistical analyses were performed using the InStat 3.0 program. The results are expressed as mean ± S.E.M. Linear correlation tests were also performed. Differences of $p<0.05$ were considered significant.
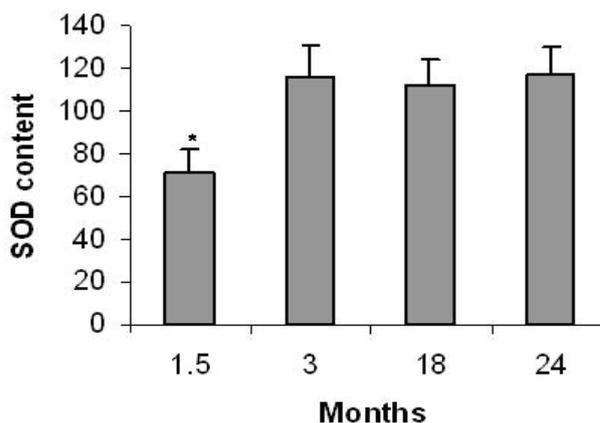
Results

One-way ANOVA revealed a significant effect of aging on O_2^- production ($p<0.001$), NO ($p<0.001$), TBARS ($p<0.001$) and SOD ($p<0.05$), whereas age did not significantly affect the levels of catalase, PGE_2, adenosine nucleotides, adenosine nucleosides or purine bases.

We examined the production of O_2^- – the first
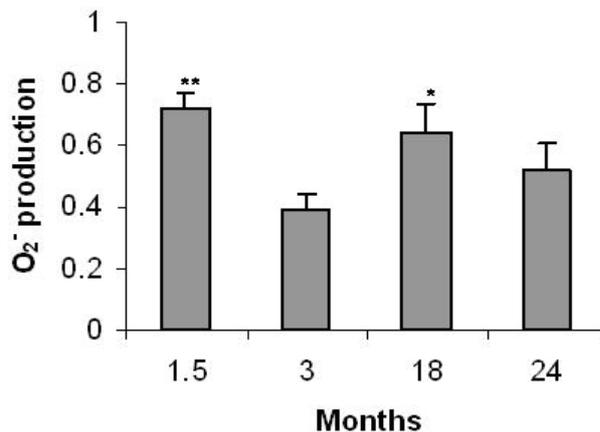
Fig. 1. Effects of aging (1.5 - 24 months) on superoxide anion (O_2^-) production in rat liver. Results are expressed as luminescence units/min/g tissue. Values are shown as mean ± S.E.M. (n = 6 per group). * $p<0.05$, ** $p<0.01$ vs. 3-month-old group.
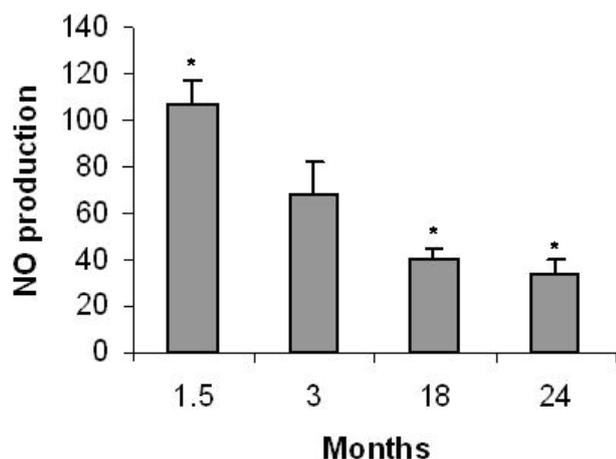

Fig. 2. Effects of aging (1.5 - 24 months) on superoxide dismutase (SOD) content in rat liver. Results are expressed as units/g tissue. Values are shown as mean ± S.E.M. (n = 6 per group). * $p<0.05$ vs. 3-month-old group.

ROS generated in the univalent reduction of oxygen. Our results show significantly higher O_2^- production at 1.5 (82 %, $p<0.01$) and at 18 months of age (63 %, $p<0.05$) compared to the 3-month-old group (Fig. 1). The content of SOD, which is the main enzyme responsible for maintaining the O_2^- homeostasis, showed a significant decrease (38 %, $p<0.05$) only when the results for the 1.5-month-old group were compared with the 3-month-old rats. All other age groups were unaltered (Fig. 2).

The univalent reduction of O_2^- gave rise to H_2O_2 a stable metabolite that can generate hydroxyl radicals in the presence of divalent metals. This metabolite was neutralized by the catalase enzyme. The activity of catalase enzyme in the liver did not significantly change throughout aging (Fig. 3).
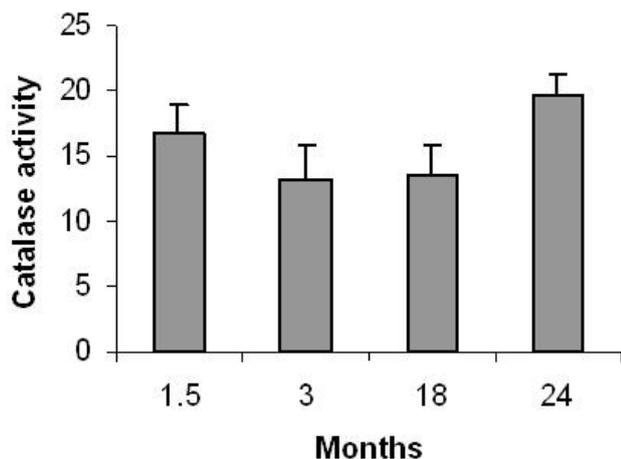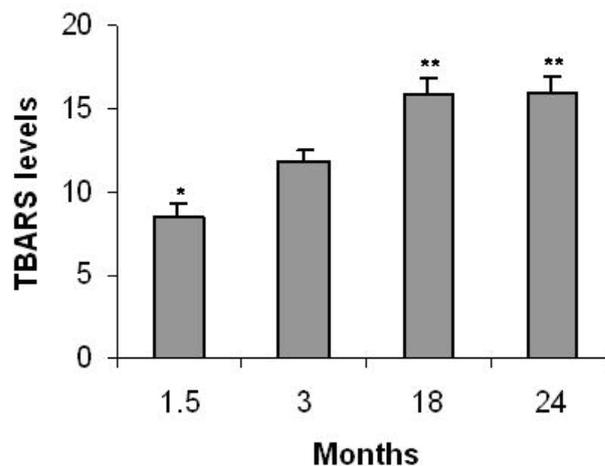
Fig. 3. Effects of aging (1.5 - 24 months) on catalase activity in rat liver. Results are expressed as k/g tissue. Values are shown as mean ± S.E.M. (n = 6 per group).

Fig. 5. Effects of aging (1.5 - 24 months) on thiobarbituric reactive substances (TBARS) levels in rat liver. Results are expressed as ng TBARS/mg protein. Values are shown as mean ± S.E.M. (n = 6 per group). [*] $p < 0.05$, [**] $p < 0.01$ vs. 3-month-old group.
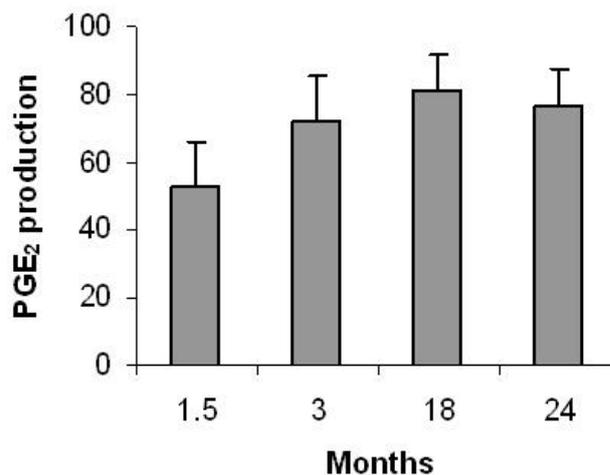

Fig. 4. Effects of aging (1.5 - 24 months) on nitric oxide (NO) production in rat liver. Results are expressed as units/mg tissue. Values are shown as mean ± S.E.M. (n = 6 per group). [*] $p < 0.05$ vs. 3-month-old group.
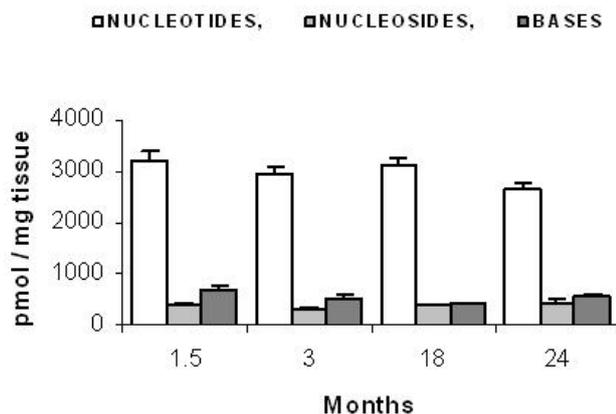

Fig. 6. Effects of aging (1.5 - 24 months) on prostaglandin E_2 (PGE_2) production in rat liver. Results are expressed as pg/mg protein. Values are shown as mean ± S.E.M. (n = 6 per group).

The cytoprotective action of NO on liver prompted us to address its production throughout aging. NO production progressively decreased over time. When the results for the distinct age groups were compared with the 3-month-old rats, a significant increase at 1.5 months (40 %, $p < 0.05$) and a decrease at 18 months (47 %, $p < 0.05$) and 24 months (55 %, $p < 0.05$) were observed (Fig. 4).

Given that lipid peroxidation is widely accepted as a sign of oxidative stress, we used lipid peroxides content, expressed as TBARS, to determine the degree of lipid peroxidation. Our data revealed significantly low levels of peroxidation in the 1.5-month-old group (29 %, $p < 0.05$), whereas higher levels were detected at 18 and 24 months of age (35 % for both groups of rats,

$p < 0.01$) than in the 3-month-old rats (Fig. 5). NO showed a significant negative correlation with TBARS ($r = -0.9902$, $p < 0.01$).

PGE_2 production did not change significantly throughout the aging process (Fig. 6) which contributed to the hepatoprotective function of PGE_2 in aged animals.

It has been reported that mitochondria are the main source of ROS and hence of oxidative stress. On the basis of this observation, we determined the content of nucleotides and their metabolites during aging. No alteration was observed in any of the parameters studied during aging (Fig. 7).

Fig. 7. Effects of aging (1.5 - 24 months) on adenosine nucleotides (ATP, ADP and AMP), nucleosides (adenosine and inosine), and purine bases (xanthine and hypoxanthine) content in rat liver. Results are expressed as pmol/mg tissue. Values are shown as mean ± S.E.M. (n = 6 per group).

Discussion

Appropriate levels of O_2^-, the first ROS generated, are maintained by SOD in order to prevent the cascade of events that gives rise to oxidative stress, which is known to play a crucial role in regulating the activity and lifespan of many species (Orr and Sohal 1994, Cutler 1995, Orr and Sohal 2003). At 1.5 months of age a decrease in SOD was observed together with a simultaneous increase in O_2^- production, thereby indicating oxidative stress at this age. The increase in O_2^- production was related to low levels of SOD, which may be a consequence of rat immaturity at this age; similar results were observed by Jung and Henke (1996) and this observation is attributed to developmental changes. The slight improvement in O_2^- production at 18 months of age compared to 24 months of age confirm in liver the findings observed in rat aorta and gastrointestinal tract (Marmol et al. 2007, 2009). This improvement can be attributed to a greater susceptibility of the 18-month-old group to oxidative stress than the oldest rats. These results are consistent with the observations, in very old mice, in which immunological parameters were more similar in adult mice than aged ones (Puerto et al. 2005). Similar results were obtained in rat brain and were attributed to hormonal alterations (andropause) at this age (Ehrenbrink et al. 2006).

The observation that the antioxidant enzymes assayed (SOD and catalase) remained constant in the aged animals is consistent with the data that do not demonstrate any alteration in the levels of antioxidant enzymes in the liver of aged rats (Cand and Verdetti

1989, Semsei et al. 1989, Barja de Quiroga et al. 1990, Santa Maria et al. 1996, Dogru-Abbasoglu et al. 1997).

The observed resistance to lipid peroxidation, expressed as TBARS, at 1.5 months of age could be due to the low availability of substrate and a high cholesterol/phospholipid ratio (Devasagayam 1986). The increase in lipid peroxides at 18 and 24 months of age reflects oxidative stress in both groups. However, this condition was not associated with changes in antioxidant enzyme activity. Nevertheless, this increase in lipid peroxides in the oldest groups of rats (18 and 24 months) may be related to the significant antioxidant activity of NO (Hummel et al. 2006). The decrease in NO at 18 and 24 months of age and hence a decrease in antioxidant activity could explain the increase in lipid peroxides observed at these ages. The significant negative correlation between NO and TBARS supports the mechanisms proposed. The significant increase in NO at 1.5 months of age may be related to the immaturity of the rats and should be interpreted as beneficial because of its cytoprotective action (Suzuki et al. 1995, Farzaneh-Far and Moore 2001). In contrast the significant decrease in NO observed at 18 and 24 months of age is a contributory factor to aging (Di Massimo et al. 2006) and has previously been related to a decrease in the endogenous substrate L-arginine (Reckelhoff et al. 1994).

Our results show similar production of PGE_2 at 1.5, 3, 18 and 24 months, thereby indicating no changes in its protective role in the liver. Furthermore, the PGE_2 production observed in aging rats indicate that the liver is more resistant than other organs to the pro-inflammatory status. These findings also indicate that there is no relationship between oxidative stress and inflammatory parameters in aged animals.

Given that mitochondrial respiration is essential for the production of ATP, damage to mitochondria as a result of oxidative stress could result in reduced energy production and compromised cell function. It is currently unclear whether in vitro studies of oxidative damage to mitochondria are an accurate indication of what occurs in vivo. The question of whether the accumulation of oxidative damage under normal physiological conditions in vivo leads to mitochondria dysfunction and increased generation of ROS remains to be answered (Van Remmen and Richardson 2001). Recently has questioned the commonly accepted idea that ROS generation by the mitochondrial respiratory chain is the main source of ROS involved in aging (Krause 2007). In this regard ultrastructural analysis of the human liver has shown that

the integrity of mitochondria and enzymatic activity and ATP content and production remain mostly unchanged with aging (Anantharaju *et al.* 2002, Drew and Leeuwenburgh 2003). Our results corroborate these findings and no alteration in the levels of nucleotides or their metabolites was observed during aging. This may be related to the fact that the levels of oxidative stress were not sufficient to alter liver function.

In conclusion, our results demonstrate a significant decrease in the levels of NO in the older groups of rats and hence in its antioxidant activity. This could explain the observed increase in lipid peroxides in these groups of rats, and therefore suggests an important role for NO in oxidative stress in the liver of older rats.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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