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Aging related changes in circulating reactive oxygen species (ROS) and protein carbonyls are indicative of liver oxidative injury

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ABSTRACT

Oxidative stress, defined as an imbalance between the production of reactive oxygen species (ROS) and antioxidant defense mechanisms, plays a major role in inducing oxidative damage and cellular impairment, resulting in a general decline of the physiological functions. The aim of this work was to evaluate age-related changes in circulating ROS levels and plasma protein carbonyls, in very young (2 months aged), young (8 months aged) and in middle age (15 months aged) F344 rats. In addition, the DNA oxidative marker 8-hydroxy-2′-deoxyguanosine (8-OHdG) and the expression of the DNA repair enzymes *APE1, OGG1* and *UNG* genes were also measured in the liver of these animals. We also determined whether systemic oxidative stress reflects oxidative injury at organ level. Our results demonstrate that the increase in circulating ROS and protein carbonyl content occurs as early as middle age. Moreover, increased 8-OHdG in the liver of 15-month-old rats was at least in part associated with a reduced DNA damage repairing capacity as suggested by the down-regulation of *APE1* gene expression. In addition, we demonstrated for the first time, that plasma carbonyls and liver 8-OHdG are well correlated, suggesting that plasma protein carbonyls may be used as a surrogate marker of oxidative injury in target organs.

1. Introduction

The aging process is characterized by a gradual impairment in all physiological functions [1,2,3]. A key player in this process seems to be oxidative stress, which is defined as an imbalance between the production of reactive oxygen species (ROS) and the antioxidant systems. Free radicals, in absence of endogenous antioxidant defenses, elicit oxidative damage to cellular macromolecules (DNA, lipids and proteins) leading to aging and degenerative diseases [4]. Among a wide range of ROS-derived modifications, protein carbonyls are known to be a major hallmark of oxidative stress [5]; DNA base modifications are also common damages caused by oxidation, deamination or alkylation. In fact, there are > 100 types of oxidative base modifications that can potentially arise in DNA as the result of ROS attack. Among DNA lesions, 8-OHdG is one of the most abundant and well-characterized oxidatively modified lesion [6]. Consequently, oxidative stress may provide a mechanism leading to genomic instability and DNA damage, as well as oxidative protein modifications, both phenomena involved in the pathogenesis of age-associated diseases such as neurodegenerative and cardiovascular diseases [7]. Several major pathways of DNA repair have been described, the activation of which depends, in part, on the type of DNA damage to be repaired. The base excision repair (BER) is the main pathway for repairing small DNA modifications caused by alkylation, deamination or oxidation and it is estimated to be responsible for the repair of one million nucleotides per cell per day [8]. The BER pathway engages various enzymes and proteins, monofunctional DNA glycosylases, such as uracil-DNA glycosylase (UNG) and bifunctional DNA glycosylases, such as 8-oxoguanine DNA glycosylase (OGG1). The apurinic/apyrimidinic endonuclease 1/redox effector factor 1 (APE1/Ref-1) is a multifunctional protein which is essential in the BER pathway, being the rate-limiting enzyme. It is also a redox factor for transcription factors such as the early growth response protein-1 (Egr-1), the nuclear factor-kB (NF-kB), p53 and the hypoxia inducible factor-1α (HIF-1α) [9]. It has been reported that BER capacity declines with age [10]. Using a model of increased oxidative stress in the rodent brain, Edwards et al. [11] demonstrated that DNA repair processes were less responsive to oxidative stress in the aged rat brain compared to their young counterparts and, in particular, the APE/Ref-1 protein levels were no changed in 30-month-old rats, whereas they were increased in 3-month-old rats, after hyperoxia.

Paul et al. [12] investigating the consequences of aging on gene expression in rats pachytene spermatocytes and round spermatids, found an overall suppression of the key players in the BER pathway, including *APE1*. More recently, in human bone marrow derived

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mesenchymal stem cells aging, an increase in endogenous superoxide levels during senescence was reported, whereas, the expression of APE1/Ref-1, which is sensitive to intracellular redox state, was reduced [13].

Although it has been speculated that the liver does not undergo significant aging changes [14], many studies reported that the senescent liver exhibits a number of characteristics consistent with oxidative injury and ROS tissue levels impact liver functions and are intimately linked to most age-associated diseases [15].

In the present study, we focused on the evaluation of aging related changes in the oxidative-antioxidative status both at systemic and organ level. In order to explore if circulating oxidative-antioxidative status may reflect oxidative injury in the liver, circulating ROS, protein carbonyls and antioxidant status were determined in F344 rats aged 2, 8 and 15 months representative of very young, young and middle age animals. In the liver, 8-OHdG levels, as marker of oxidative DNA damage, protein carbonyls and antioxidant status were also measured. These data were then correlated with the expression of three genes involved in DNA repair, *APE1, OGG1* and UNG in hepatic tissue, with the further aim to evaluate the association among oxidative stress parameters and DNA damage response during the aging process.

2. Materials and methods

2.1. Chemicals

All chemicals and reagents used were of analytical grade and were purchased from Sigma Chemical Company

2.2. Animals and experimental design

Male F344 rats (aged 6 weeks) were purchased from Nossan (Milan, Italy). After their arrival from the supplier all 17 animals were quarantined for 1 week and fed standard lab chow and water, ad libitum during the entire experiment. The animals were checked for their general health status every day and body weight was measured every 2 weeks. Seven rats were sacrificed at 2 months of age (very young rats), 5 rats at 8 months of age (young rats), and a third group of 5 rats were sacrificed at 15 months of age (middle-age rats). All procedures were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) standard guidelines for the care of animals, and the experiments were conducted according to Italian regulations on the protection of animals used for experimental and other scientific purposes (DM 116/1992), after approval from the Italian Ministry for Scientific Research.

2.3. Plasma and tissue preparation

The animals (n = 17) were sacrificed by decapitation, 30 µl of whole blood was collected for ROS determination while another fraction was decanted into anticoagulant tubes and centrifuged at 1000*g* for 10 min to obtain plasma for oxidative stress parameter determinations (protein carbonyls and Ferric reducing ability of plasma (FRAP)). The livers were excised and frozen at −80° C until analysis. The liver was homogenized in 50 mM phosphate buffered solution (PBS) containing 0.1 M dithiothreitol and then centrifuged at 4° C for 20 min at 2000*g*. Liver supernatant was used for carbonyl residues and FRAP determinations. Liver pellets were used for 8-hydroxy-2′-deoxyguanosine (8-OHdG) assay.

2.4. ROS measurement

The levels of ROS were determined in the whole blood according to Pavlatou et al. [16] by using the Free Oxygen Radical Testing (Callegari 1930, Parma, Italy); briefly 30 µl of whole blood was added to acidic buffer and to phenylenediamine derivative [2CrNH2]. Samples were then centrifuged (5000 rpm), incubated for 6 min at 37 °C and for further 10 min at room temperature. Absorbance was determined at 505 nm. The amount of radical compounds was determined using H_2O_2 as standard for calibration curve and expressed as mM H_2O_2.

2.5. Oxidative protein damage (protein carbonyls) assessment

Protein carbonyls levels were determined by the method of Correa-Salde and Albesa [17] with few modification. Liver supernatant (0.35 ml) or plasma (0.25 ml) was treated for 1 h with 1 ml of 0.1% dinitro-phenylhydrazine in 2 M HCl and precipitated with 10% trichloroacetic acid before being centrifuged for 20 min at 10,000 x g. The pellets were extracted with 1 ml of an ethanol:ethyl acetate mixture (1:1) three times and then dissolved in 2 ml of 6 M guanidine HCl in 20 mM potassium phosphate buffer (PBS) pH 7.5. The solutions were incubated at 37° C for 30 min and insoluble debris was removed by centrifugation. The absorbance was measured at 364 nm.

2.6. Analysis of plasma antioxidant capacity (FRAP assay)

The FRAP assay was performed in the plasma and in the supernatant liver tissue according to the method of Benzie and Strain [18] with few modifications as described by Lodovici et al. [19].

2.7. DNA damage (8-OHdG assay)

The liver pellets were re-suspended and the DNA was isolated using the method recommended by ESCODD [20]. The purified DNA (about 50 µg) was hydrolyzed with P1 nuclease (10 IU) and alkaline phosphatase (7 IU). The hydrolyzed mixture was filtered using Micropure-EZ enzyme remover (Amicon, MA, USA) and 50 µl was injected into an HPLC apparatus. The nucleosides were separated by a C18 reverse-phase column (Supelco, 5 mm, I.D. 0.46 cm x 25 cm). The 8-OHdG and 2dG in the DNA were detected using an ESA Coulochem II electrochemical detector in line with a UV detector as previously described [6]. The 8-OHdG determinations has been carried out only in the liver of 9 animals, 3 per group.

2.8. Semi-quantitative RT-PCR

Total RNA was extracted from liver tissue samples using the NucleoSpin® RNA kit (Machery-Nagel, Germany); RNA concentration and purity were determined by using a NanoPhotometer spectrophotometer (IMPLEN). For first-strand cDNA synthesis, 100 ng of total RNA from each sample was reverse-transcribed using the RevertAid RT Reverse Transcription Kit (Thermo Scientific™, Italy).

Primers, listed in Table 1I, were designed on the basis of the rat GenBank sequences for APE1 nuclease 1 (*APE1*), uracil-DNA glycosylase (*UNG*) and 8-oxoguanine DNA glycosylase (*OGG1)*. For each target gene we performed a duplex PCR, co-amplifying the b-actin as reference gene [21]. The PCR reactions were carried out on aliquots of the cDNA preparation, in a 20 µl volume containing 1x PCR buffer, 0.5 mM dNTPs, 5 ng/µl of each target gene primer, 0.5 ng/µl of the β-actin primers and 1.25 units of DreamTaq (Thermo Scientific™, Italy). The PCR conditions were the same: 30 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 55. Gel images were captured by a digital photocamera (UviDoc) and the intensity of the bands was analyzed with Quantity-

Table 1
List of primers used for gene expression analyses.

Gene	Primer forward	Primer reverse	bp
UNG	TCCGGACCCCGACTCCTGGC	GCGGGGGTGGAACTGGCCTC	419
OGG1	CCTGGCTGGTCCAGAAGTAG	TTTCCCAGTTCTTTGTTGGC	345
APE1	GCTCAGAGAACAAACTCCCG	TTGTTTCCTTTGGGGTTACG	385

One software (Bio-Rad, Segrate, Milan, Italy). For each target gene, the relative amount of mRNA in the samples was calculated as the ratio of each gene to the co-amplified b-actin.

PCR products were separated on 1.6% agarose gel and visualized by SafeView™ staining (ABM). Gel images were captured by a digital photocamera (UviDoc) and the intensity of the bands analyzed with the Quantity-One software (Bio-Rad).

2.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Newman-Keuls post hoc test. All analyses were carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Results are expressed as means ± standard error (SEM), $p < .05$ was considered significant.

3. Results

3.1. ROS levels, oxidative protein damage (protein carbonyls) and plasma antioxidant capacity (FRAP)

Plasma ROS levels increased with the age of animals with values significantly higher in 15- month-old rats in comparison to younger animals (2 and 8 month-old) (Fig. 1a). Protein oxidative damage in the plasma, evaluated as protein carbonyls content, was significantly higher in 15 month-old rats in comparison to both 2 and 8 month-old, and 8 month-old rats showed the lowest levels (Fig. 1b). The antioxidant capacity, measured as FRAP levels in the plasma was not significantly affected by age (Fig. 1c). Furthermore, a medium correlation between ROS and protein carbonyls in the plasma of all analyzed rats was found (Fig. 1d). In the liver, protein oxidative damage and antioxidant capacity were similar among rats of different ages (Fig. 2, panels a and b).

3.2. Oxidative DNA damage (8-OHdG) and expression of DNA damage repair genes in the liver

Oxidative DNA damage, measured as 8-OHdG levels, was significantly higher (about four fold) in the liver of older rats compared to 2 and 8 month-old (Fig. 3a). The expression of *APE1* is shown in Table 2: animals at 8 and 15 months of age had a significantly lower *APE1* expression in comparison to young rats; on the contrary, *OGG1* and *UNG* liver expression, were similar among groups, independently of age (Table 2).

3.3. Correlation between ROS, oxidative stress parameters and DNA damage repair

Interestingly, a correlation between plasma protein carbonyls and oxidative DNA damage (8-OHdG) in the liver was found (Fig. 3b). In addition, 8-OHdG levels also correlated with ROS plasma levels in rats of 8–15 months of age (Fig. 3c). Despite the small sample size, we observed that 8-OHdG levels correlated with *APE1* gene expression measured in the liver (Fig. 3d).

4. Discussion

Reactive oxygen species (ROS) are capable of oxidatively modify many biological macromolecules such as protein, lipids and nucleic acids which may result in genetic mutations and cellular senescence.

The effect of the aging process on systemic redox homeostasis of plasma proteins, lipids, DNA and antioxidants was previously documented both in rats [22,23] in humans, and in age-related diseases [24,25,26]. However, most of these research analyzed the oxidative-antioxidative status of old rats (mostly at 24 months of age) and none of these studies associated systemic redox homeostasis to oxidative damage in organs.

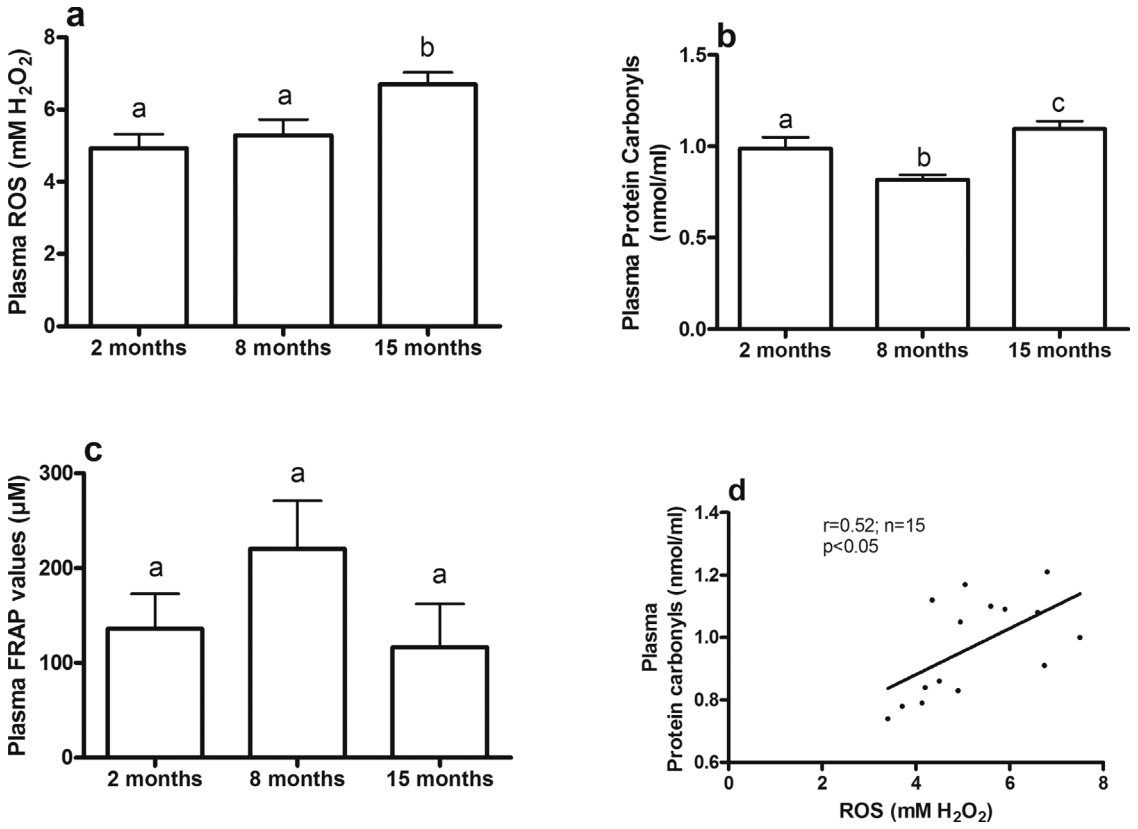

Fig. 1. ROS levels (a), protein carbonyls (b) FRAP levels (c) in the plasma of rats of 2-, 8- and 15-month-old. Correlation between ROS and protein carbonyls in the plasma of rats 2-, 8- and 15-month- old (d). Values are means ± SEM from 5 to 7 animals per group. ANOVA bars with different lower case letters (a, b, c) $p < .05$.

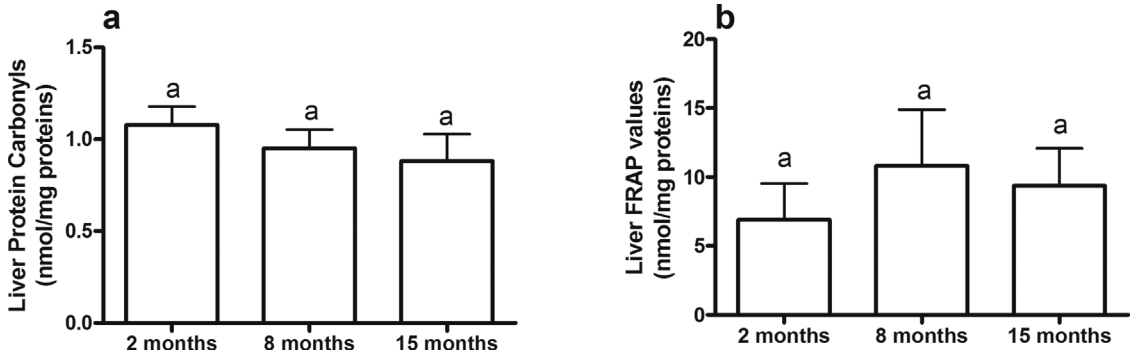

Fig. 2. Protein carbonyls (a) and FRAP levels (b) in the liver of rats of 2-, 8- and 15-month-old.

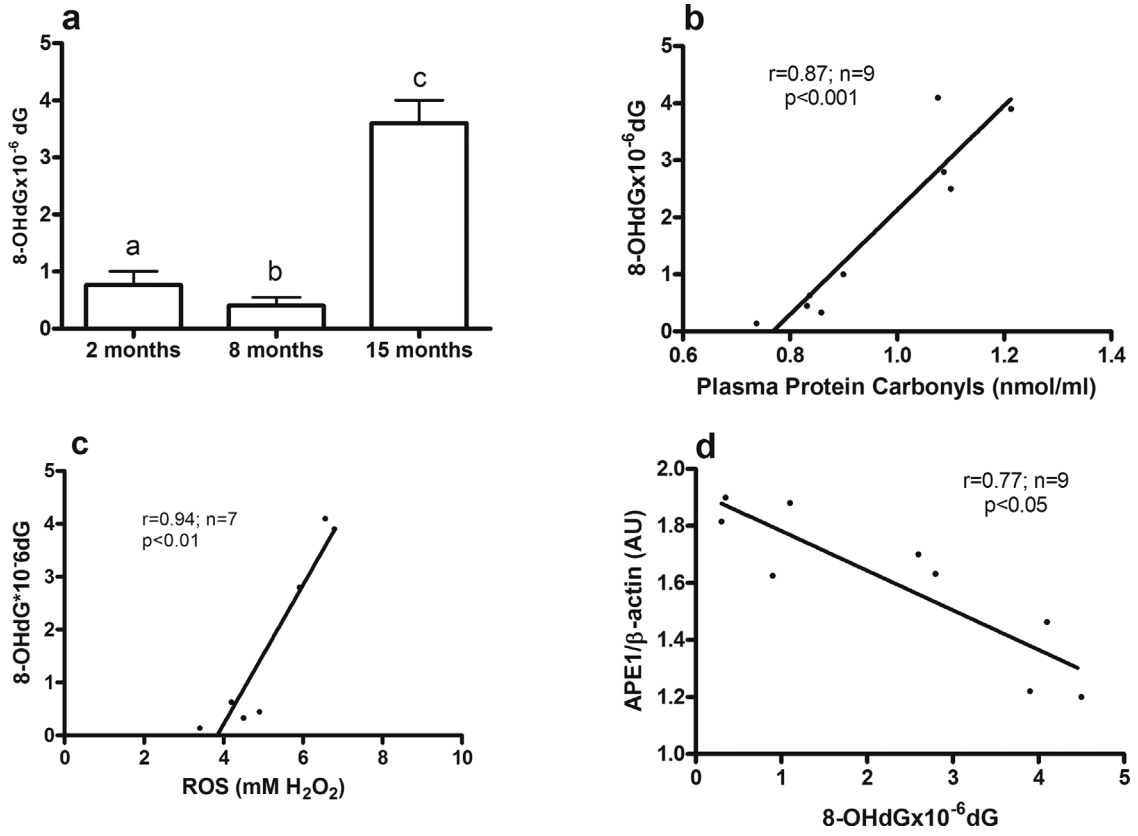

Fig. 3. Levels of 8-OHdG in the liver of rats 2-, 8-, and 15-month-old (a). Values are means ± SEM from 5 animals per group. ANOVA bars with different lower case letters (a, b, c) $p < .05$. Correlation between 8-OHdG in the liver and protein carbonyls in the plasma of rats 2-, 8- and 15-month-old (b). Correlation between 8-OHdG in the liver and ROS in the plasma of rats 8- and 15-month-old (c). Correlation between *APE1* gene expression and 8-OHdG levels in the liver of rats 2-, 8- and 15-month-old (d).

Table 2
Expression of *UNG*, *OGG1* and *APE1* in the liver of rats of 2, 8 and 15 months of age.

Groups	*UNG*	*OGG1*	*APE1*
2 months old rats (n = 5)	1.92 ± 0.166	1.10 ± 0.085	1.77 ± 0.076
8 months old rats (n = 5)	2.54 ± 0.215	1.15 ± 0.114	1.42 ± 0.021*
15 months old rats (n = 5)	2.86 ± 0.263	1.33 ± 0.181	1.43 ± 0.085*

The relative amount of mRNA in the samples was calculated as the ratio of each gene to the housekeeping gene, β-actin. Data are expressed as mean ± SEM. *$p < .05$ vs 2-months old.

Our findings suggest that a systemic increase of ROS during aging occurs already in middle age rats of 15 months, resulting in systemic oxidative stress, as pointed out by the increased amount of protein carbonyls in plasma of 15 months-aged animals. We also analyzed the levels of plasma antioxidant defenses, finding no variations during the aging process, at least looking at variations from young to middle-aged animals. Likewise, Wang et al. [27] reported no age-dependent variation in FRAP values in human plasma. In the liver, we observed that the oxidative DNA damage increased with the age, becoming very high already, in middle aged animals. It is interesting to note that systemic oxidative damage (as indicated by the plasma protein carbonyls) and liver oxidative damage (as indicated by 8-OHdG levels) in the same animal, were significantly correlated. These data suggest an overall disturbance in the balance between pro-oxidant and antioxidant status during aging, associated with the decreased ability to repair, as supported by the reduction of *APE1* expression in the liver of 15-months-old rats and by its inverse correlation with 8-OHdG levels.

ROS overproduction increases genomic instability and the DNA repair mechanisms play a central role in preventing the accumulation of DNA mutations and in maintaining of DNA stability. We did not find any statistically significant variations in the hepatic expression of *UNG*

and *OGG1* among groups, however, we measured for all of them, increased values from the youngest to the oldest animals, data that suggest an increased need to counteract the higher level of ROS inducing DNA damage. The expression of *APE1* was on the contrary significantly lower in 15-months-old rats compared to the youngest animals. A decline of *APE1/Ref-1* expression at both mRNA and protein levels, was also reported in models of replicative and oxidative stress-induced cellular senescence [13]. A comprehensive, microarray-based, analysis performed in pachytene spermatocytes isolated from rats at 4 and 18 months of age, revealed that some of the most predominant gene sets affected by aging were those related to DNA repair/DNA damage and that the BER pathway showed the highest change, in terms of percent of genes changed, and were overall downregulated. In addition, *APE1* gene expression and protein levels were also significantly lower by comparing young and aged pachytene spermatocytes and the number of 8-oxodG-positive spermatozoa in the aged samples was significantly higher. A decrease in BER activity in nuclear extracts from brain, spleen and testes of old mice was reported by Cabelof et al. [10], who observed a 50% reduction as compared to extracts from young animals; in liver extracts, this reduction was even higher (75%).

The higher level of 8-OHdG measured in liver DNA of 15-month-old rats can be, at least partially, associated with an increased ROS formation and a reduced capacity to repair DNA damage during the aging process. It should be noted that these alterations, previously described by comparing young with old animals are detectable already in middle-age animals, in physiological condition, without any treatment or the use of stress-inductors. We previously reported that protein oxidation, measured as carbonyl residues and 4-hydroxynonenal, progressively increased in an in vitro model of cellular senescence and that these effects were reduced by the antioxidant resveratrol [28]. We also demonstrated in vivo that the aging process is associated to increased oxidative stress at central level and that antioxidant polyphenols counteract age-related impairments [29,30] supporting “the free radical hypothesis” of aging [31,32,33].

In conclusion, we observed that during the aging process ROS overproduction leads to a state of oxidative damage at both liver and systemic level which occurs as early as middle age. In addition, notwithstanding the small size of our study, we demonstrated for the first time, that systemic (plasma protein carbonyls) and local damage (hepatic 8-OHdG) are correlated, suggesting that the measure of the plasma protein carbonyls may be used as surrogate biomarker of oxidative injury in target organs.

Conflict of interest

We declare that there are no conflicts of interest.

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