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Photoaging and chronological aging profile: Understanding oxidation of the skin

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ABSTRACT

The impact of chronological aging and photoaging on the skin is particularly concerning, especially when oxidative stress is involved. This article provides evidence of quantitative and qualitative differences in the oxidative stress generated by chronological aging and photoaging of the skin in HRS/J hairless mice. Analysis of the results revealed an increase in lipid peroxides as the skin gets older and in photoaged skin (10.086 ± 0.70 η MDA/mg and 14.303 ± 1.81 η MDA/mg protein, respectively), although protein oxidation was only verified in chronological aged skin (15.449 ± 0.99 η protein/mg protein). The difference between both skin types is the decay in the capacity of lipid membrane turnover revealed by the dislocation of older skin to the left in the chemiluminescence curve. Imbalance between antioxidant and oxidation processes was verified by the decrease in total antioxidant capacity of chronological and photoaged skins. Although superoxide dismutase remained unchanged, catalase increased in the 18 and 48-week-old skin groups and decreased in irradiated mice, demonstrating that neither enzyme is a good parameter to determine oxidative stress. The differences observed between chronological and photoaging skin represent a potential new approach to understanding the phenomenon of skin aging and a new target for therapeutic intervention.

1. Introduction

Aging of the skin occurs through two independent, biologically divergent mechanisms, sun exposure related aging and chronological aging. Among several oxidative stressors, reactive oxygen species (ROS) have been associated with the process of UV-induced skin damage, including photoaging, immunomodulation, melanogenesis and, ultimately, photocarcinogenesis [1]. Besides direct photochemical reactions, such as the generation of specific pyrimidine dimers, UVB generates a high level of ROS in the skin resulting in photooxidative damage of the cells and extracellular matrix [2]. The aging process has been the subject of numerous theories that fall into two groups: genetic and damage-accumulation theories. It has been proposed that oxidative damage is a common link between all the aging theories. Age-related oxidative phenomenology continues to provide evidence that there is a decline in the antioxidant enzymes and antioxidant defenses leading to an accumulation of oxidative damage end products [3]. These products are all markers of oxidative stress [4], and the influence of the environment, most notably solar UV irradiation, is of considerable importance for skin aging [5–7].

It is now well known that to counteract oxidative stress and maintain a redox balance within the cells, the skin is equipped with a network of antioxidant systems [8]. Ascorbic acid, α-tocopherol, uric acid and glutathione, are well-known nonenzymatic antioxidant molecules. Previous studies have also demonstrated the presence of several major enzymatic antioxidants, i.e. glutathione peroxidase (GPx), glutathione reductase, superoxide dismutase (SOD) and catalase (CAT), in the epidermis [8]. Among these enzymes, SOD and CAT are the major antioxidant enzymes protecting the epidermis [7]. SOD converts superoxide anions into hydrogen peroxide (H_2O_2), while CAT degrades H_2O_2 into water [9].

Despite the entire antioxidant defence, the skin is intensively subjected to exogenous and endogenous aggression; consequently, unsolved problems remain concerning chronological aging and photoaging. One of these is how oxidative stress is involved in each one of these aging processes. In the present work, evidence of quantitative and qualitative differences in the oxidative stress generated by chronological aging and photoaging was verified. Furthermore, the role of such differences in oxidative stress and antioxidant content between chronological aging and photoaging was investigated in the skin of HRS/J hairless mice.

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2. Materials and methods

2.1. Animals and UVB irradiation

Male and female hairless mice HRS/J were obtained from the University of São Paulo (USP). The mice were provided with water and balanced commercial feed (Nuvilab CR1; Nuvital Nutrients Ltda., Curitiba, Brazil) *ad libitum*, as recommended by National Research Council and National Institute of Health, USA. The mice used for the experiments were 8, 18 and 48-week-old, with 8 mice in each group. They were treated in accordance with the institutional ethical guidelines for animal experiments. A second group of 8-week-old mice was irradiated three times a week for 10 weeks, receiving a suberythemogenic dose of 54 mJ/cm^2 in week 1 and erythemogenic doses of 72 mJ/cm^2 in week 2, 90 mJ/cm^2 in week 3 and 108 mJ/cm^2 from weeks 4 to 10 [10], according to the method described by Moloney et al. [11]. The irradiation chamber was adjusted with a PHILIPS TL/12 40 W UVB fluorescent lamp, which emits irradiation from 270 to 400 nm with maximum peak around 313 nm. UVB output was measured using a Research Radiometer model IL-1700 (International Light, USA; calibrated by IL service staff) with a radiometer sensor for UV (SED005) and UVB (SED240), which detected that UVB was 73% of the total UV irradiation in the present experimental conditions. The UVB irradiation rate was 0.47×10^{-4} mW/cm^2.

The lamp was embedded in a 1.30 m × 0.43 m × 0.45 m box, in which the caged mice were placed, 35 cm beneath the lamp. Dorsal skin samples from control and experimental mice were removed 6 h after the last irradiation in the irradiated group and the homogenate was prepared in phosphate buffer was used for analysis. Control groups consisted of caged mice placed in the chamber with no exposure to irradiation.

2.2. Free radical formation analysis

The destabilization of cell membrane lipids formed peroxides that were measured by a very sensitive chemiluminescence (CL) method, which was initiated by the addition of tert-butyl hydroperoxide to the tissue homogenate in a Turner Designs luminometer, model TD-20/20, with a response range of 300–650 nm [12]. The CL generates curves that demonstrate the quantity of membrane peroxides formed as it shifts towards the *y* axis. The final lipoperoxidation products were analyzed by measuring the formation of thiobarbituric acid reactive species (TBARS), as described by Oliveira and Cecchini [13]. Briefly, low molecular weight aldehydes, such as malondialdehyde (MDA), react with thiobarbituric acid (TBA), generating a colored product that absorbs light at 532 nm. Carbonyl proteins were assayed using the method for detecting protein hydrazones, followed by reaction with dinitrophenylhydrazine, according to Reznick and Parker [14].

2.3. Chemiluminescence of skin oxidized with 2-azo-bis-2-amidinopropane (ABAP)

To obtain a reference for oxidized tissue, a sample of control skin was prepared using exactly the same process as hairless mice skin. Test skin was incubated with ABAP for 30 min at 37 °C, before initiating the experiments. The CL generates curves which demonstrate the quantity of lipid peroxides formed in the cell membrane as it shifts towards the *y* axis [12].

2.4. Antioxidant activity analysis

The total antioxidant capacity (TRAP) was also detected by CL. Initially, light emission in a reaction medium containing 2-azo-bis-(2-amidinopropane) and luminol, an alkoxyl generating system, was measured. Then, another curve was measured with the addition of a standard antioxidant, Trolox, a hydrosoluble vitamin E, which hinders the curve peak due to its antioxidant property. Finally, Trolox was replaced by the tissue homogenate and the peak hinder time was determined in comparison with the standard and results were expressed in μM Trolox [15].

Superoxide dismutase (SOD) and catalase (CAT) activities were assayed spectrophotometrically, as previous described [16]. Briefly, SOD quantification was based on the inhibition of pyrogallol autoxidation in aqueous solution by SOD and CAT present in the skin homogenate was determined using a standard H_2O_2 system.

2.5. Protein concentration

All protein concentrations were measured by the method of Lowry et al. [17], modified by Miller [18], except for protein carbonyl contents, which were quantified directly at 280 nm. Bovine serum albumin (BSA) was used as standard in both situations.

2.6. Statistical analysis

Reported values are presented as the mean ± SEM. All differences between groups were analyzed by the Student *t* test, except for the CL curves, which were qualitatively analyzed by two-way ANOVA and quantitatively analyzed by the Bonferroni *post hoc* test. Significance level was set as $p < 0.05$.

3. Results

Table 1 shows different results for each analysis in the different groups in terms of protein oxidation (CP), late lipid peroxide formation (TBARS) and the antioxidant enzyme activities (CAT and SOD). CP content was significantly lower in the 18-week-old group (3.847 ± 0.50 η protein/mg protein) compared to the 8-week-old group (7.919 ± 0.65 η protein/mg protein), but not compared to the irradiated (6.525 ± 0.96 η protein/mg protein) counterpart. In contrast, protein content oxidation was higher when comparing the 48-week-old group (15.449 ± 0.99 η protein/mg protein) to the remaining groups tested.

TBARS formation was greater in the irradiated group (14.303 ± 1.81 η MDA/mg protein) compared to the remaining groups. Although the 48-week-old group showed significantly lower lipid peroxide content (10.086 ± 0.70 η MDA/mg protein) compared to the irradiated group, it was still significantly higher than the 8 (3.650 ± 0.15 η MDA/mg protein) and 18-week-old groups (2.378 ± 0.67 η MDA/mg protein), which were not significantly different from each other.

The enzymatic profile of the skin samples showed an increase in catalase activity for 18-week-old (0.650 ± 0.08 abs/mg protein) and 48-week-old skin (0.577 ± 0.03 abs/mg protein) compared to 8-week-old skin (0.391 ± 0.01 abs/mg protein). Irradiated skin (0.489 ± 0.08 abs/mg protein) showed no significant difference compared to 8-week-old skin. SOD content was not significantly different in any of the groups analyzed.

Membrane lipid peroxide exposure (Fig. 1B) was significantly increased for 18-week-old skin (2.75 RLU/mg protein ± 0.32) compared to 8-week-old (2.01 RLU/mg protein ± 0.28), 48-week-old (1.83 RLU/mg protein ± 0.19) and irradiated skin samples (1.54 RLU/mg protein ± 0.13). Irradiated skin was also different from all the other groups, though in contrast, it showed a reduction in membrane lipid peroxide exposure formation. A different pattern of early lipid peroxide formations was observed for 48-week-old skin, which was significantly different from 8 and 18-week-old and irradiated skin, showing peculiar displacement of the curve

Table 1
Oxidation parameters and enzyme profile of the skin.

Week-old skin samples	8	18	48	Irradiated
CP (η protein/mg protein)	7.919 ± 0.65[a]	3.847 ± 0.50[b]	15.449 ± 0.99[c]	6.525 ± 0.96[b]
TBARS (η MDA/mg protein)	3.650 ± 0.15[a]	2.378 ± 0.67[a]	10.086 ± 0.70[b]	14.303 ± 1.81[c]
Catalase (abs/mg protein)	0.391 ± 0.01[a]	0.650 ± 0.08[b]	0.577 ± 0.03[b]	0.489 ± 0.08[a]
SOD (U SOD/mg protein)	1.117 ± 0.18	1.331 ± 0.11	1.471 ± 0.11	1.285 ± 0.12

[a,b,c] Different letters represent statistical difference.

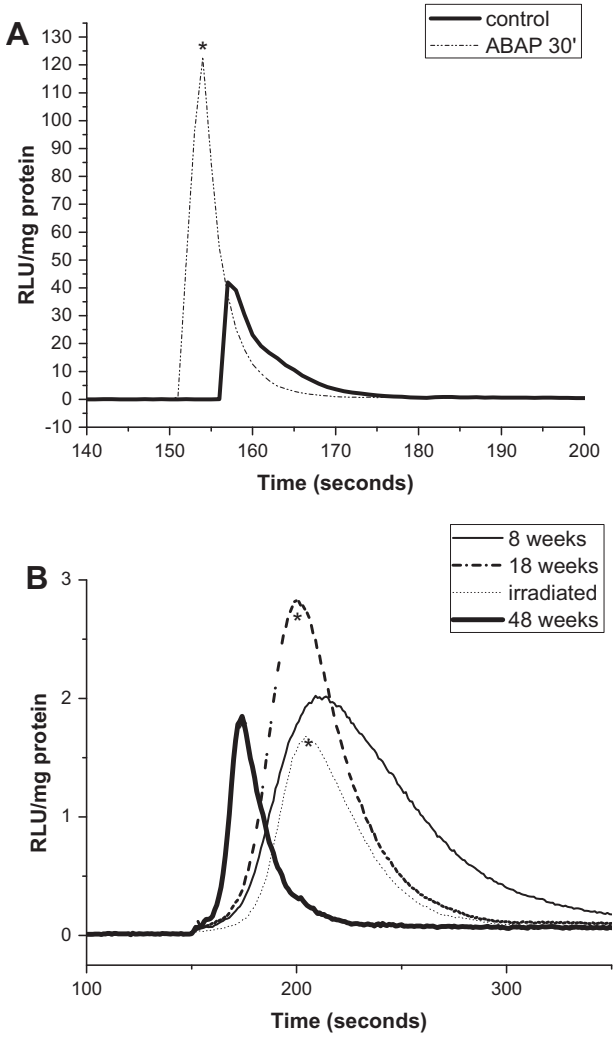

Fig. 1. Levels of early lipid peroxidation measured by chemiluminescence expressed in relative light unit/mg protein (RLU/mg protein). (A) Analysis of oxidation of control skin induced by ABAP. The concentration of the homogenate used was 0.1 mg tissue/mg protein. Two-way ANOVA test was applied and showed significance ($p < 0.0001$). (B) Lipid peroxidation levels of aged skins and UVB irradiated skin. Two-way ANOVA test and the Bonferroni *post hoc* test was applied and only the 8 and 18 week-old skin samples were not considered different. $^{*}p < 0.05$.

to the left. This displacement was compared to the displacement obtained by skin oxidation due to ABAP (Fig. 1A).

The total antioxidant capacity was measured and all groups studied showed a reduction in water soluble antioxidant molecules compared to the 8-week-old control (Fig. 2).

4. Discussion

Unlike chronological aging, which depends on the passage of time per se, photoaging depends on the degree of sun exposure

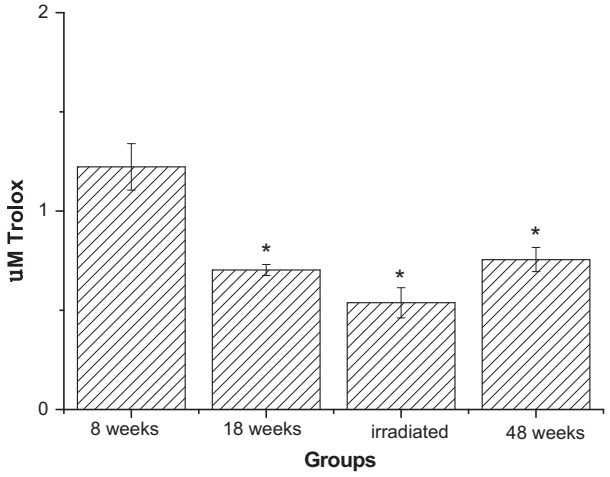

Fig. 2. Total antioxidant capacity in normal aging and photoaged skin measured by the chemiluminescence method. The results are the mean ± SEM. $^{*}p < 0.05$.

and an individual's skin pigmentation. UVB may directly damage DNA by producing cross-linkage [19], but the damage mediated by ROS is an important mechanism leading to photooxidative damage of skin cells. Thus, the purpose of this study was to investigate the oxidative stress involvement in chronological aging and photoaging alone. Although many biochemical mechanisms of chronological aging and photoaging seem alike with regard to oxidative stress, the skin cells behave differently in each case. The development of the hairless mouse model for photoaging studies contributes enormously to the identification of histological and biochemical alterations [20], confirming the characteristic changes observed in photoaged skin in humans [21].

One of the most important early events in cell degeneration leading to necrosis is the lipid peroxidation process that occurs mainly in the cell membrane. In addition, lipid peroxidation represents the most frequent event resulting from free radical attack of biological structures. *Tert*-butyl hydroperoxide-initiated chemiluminescence was used to evaluate the integrity of cell membrane and the degree of exposure to lipid peroxides, leading to membrane destabilization [12,13,22,23]. A possible relationship between chemiluminescence and tissue damage has been demonstrated [13,22,23] and in the present study, mouse skin homogenates at three different ages and young photoaged skin (irradiated) were compared regarding oxidative stress.

Protein oxidation and MDA (late formed lipid peroxides) formation are also parameters that indicate free radical formation and the modification of lipids in tissue. They also represent the final stage of cell destruction by oxidative stress.

The present results indicate that cells of the epidermis are gradually submitted to lipoperoxidation as the skin gets older (8, 18 and 48-week-old skin samples). This is verified not only by the increasing values of MDA (Table 1), but also by the increase in protein oxidation (Table 1). CL is a very sensitive method that takes into account a kinetic analysis of the ascending part of the chemiluminescence curve under the assumption that variation in V_0

(initial velocity) values depends on the level of preexisting lipid peroxide in the tissue [23]. Moreover, consider the very fast overall rate constant of the lipoperoxidation propagation reaction of about $10^9\ M^{-1}\ s^{-1}$ [24]. This information is useful when considering the dislocation of the CL curve to the left in the groups studied (Fig. 1B). In Fig. 1A, it is clear that this dislocation occurs according to the time that skin homogenates remain exposed to oxidation. The displacement to the left of the CL chart obtained by skin oxidation due to ABAP (Fig. 1A) represents the modification of the lipid structure of the cell membrane. The exposure or destruction of lipids in the cell layer facilitates the degradation chain reaction [24].

The extent of oxidative changes that occurs under these conditions, leading to increased ROS production, was evaluated by the levels of light emission resulting from *in vitro* exposure to ABAP of skin preparation [25]. The *Vo* of the ABAP oxidized skin was 41.28 RLU mg $prot^{-1}$ min^{-1}, showing the efficiency of the skin oxidation and that the displacement of the curve is due to the same.

This particular behavior of the curve is due to the increase in hydroperoxide exposure to the hydrophilic side of the cell due to prior oxidation of the membrane. As the cell gets older, an increase in the susceptibility to phospholipase attack also occurs [24], leading to membrane degradation. Although the displacement represents the age of the skin, the height of the peak determines the concentration of the lipid peroxides formed. In young skin (18-weeks-old), larger quantities of lipoperoxides that are not susceptible to oxidation are observed and cell membrane turnover is well established (Fig. 1B). When compared to 8-week-old skin, 18-week-old skin has a high ability to convert lipid peroxides back to functional membrane lipids (Fig. 1B), a fact supported by the low MDA content (Table 1). This basically occurs because of the presence of antioxidants and transitions metals in the medium [26], which convert the transition form of monocyclic peroxide back to a more stable form of aldehyde.

Catalase was increased in the 18 and 48-week-old skin groups compared to the 8-week-old skin group (Table 1). To maintain a redox balance within the cells, it is favorable for the epidermal cells of the skin to be equipped with a network of enzymatic and/or nonenzymatic antioxidant systems to counteract oxidative stress caused by H_2O_2 [27]. In a study on new born rat skin, Muramatsu et al. [27] showed that catalase protein predominantly colocalized with H_2O_2 in the cytoplasm of cells in the *stratum granulosum*. Therefore, the overall distribution of catalase protein seemed to almost correspond to that of H_2O_2 accumulation, indicating the role of normal skin catalase as a marker of epidermal differentiation, as well as its role in redox damage. In the same work, analysis verified that in the upper epidermal layers, catalase expression decreased 24 h after exposure to UVB. Previously, Fuchs et al. [28] documented how photooxidative stress reduced catalase activities. Jeon et al. and Vayalil et al. [29,30] also indicated that catalase activity decreased in mouse skin after UVB irradiation at doses of 200 and 180 mJ/cm^2, respectively. These studies suggest that UVB impairs endogenous antioxidant mechanisms and that catalase is one of the most sensitive components of such mechanisms [28]. Despite these findings concerning catalase activity following acute UVB exposure, in the present work, observation verified no difference between irradiated skin and 8-week-old skin, suggesting that no changes occur in the profile of catalase activity under chronic exposure, which indicates that it is not a good marker for oxidative stress in this particular experimental model. The increase in catalase activity of 18 and 48-week-old skin is related to cell differentiation and the maintenance of the redox balance.

Besides the increase in catalase activity, the 48-week-old group showed high cellular lesion, represented by the MDA of TBARS (Table 1). An increase in MDA has been reported in numerous studies involving acute [31,32] and chronic [33–35] UVB exposure and it is one of the parameters that indicates lipid peroxidation cell damage. The imbalance between the antioxidant status and the injury related to ROS leads to oxidative stress.

Superoxide dismutase activity remained unchanged in all groups. A single exposure of UVB light has been reported to reduce the amount of Mn–SOD and increase Cu–Zn–SOD in human keratinocytes, with full recovery to normal values after 24 h [36]. On the other hand, chronic UVB irradiation was shown to be accompanied by the induction of epidermal SOD activity *in vivo*, while the activities of the other antioxidant enzymes were not significantly altered [37]. Okada et al. [38] showed that SOD activity was increased by UVB 36-week irradiation and gradually returned to control levels, but that continual cumulative stress may overwhelm the capacity of this system. The total antioxidant capacity (TRAP) was significantly diminished in all groups compared to 8-week-old skin (Fig. 2), even though Punnonen et al. [37] observed no significant change in this parameter following chronic epidermal exposure.

This indicates that besides enzymatic and water soluble antioxidants, other antioxidants of the skin protect the photoaged skin from membrane destabilization and lipid peroxides. Fig. 1B clearly shows that chronological aging (48-week-old skin) produces uncompensated oxidative stress compared to irradiated photoaged skin. This uncompensated oxidative stress state also occurs in other pathologies [39].

Finally, the alterations in cell membrane structure according to age (48-week-old) revealed by the displacement of the curve to the left (Fig. 1B) and the increase in late formed lipid peroxides represented by the increase in MDA-TBARS, associated with the reduction in total antioxidant capacity, indicates that an imbalance occurs as the skin ages. Moreover, these same parameters are observed in photoaged skin, with one exception, shown in Fig. 1B. No shift to the left of the curve occurred for irradiated skin, indicating that in adult skin, cell membrane lipid exposure occurs to less extent than in old skin and that although the total antioxidant capacity is diminished, the skin possesses the ability to promote efficient turnover.

The different behavior towards cell oxidation in chronological aging or photoaging generates new insight concerning the mechanisms of cell adaptation and response under oxidative stress. The increase in lipid peroxide exposure in chronological aging skin, which facilitates free radical attack, is a novel target for developing potential therapeutic approaches for photoaging and chronological skin aging indications.

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