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# Analysis of oxidative stress in sun-exposed and unexposed skin

[1]Raluca E. Diaconu, [2]Antonia E. Macarie, [3]Remus Orăsan

**[1] "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; [2] Department of Geriatrics and Gerontology, Faculty of Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania; [3] Department of Physiology, Faculty of Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania.**

**Abstract.** Aim: This study aims to describe the differences in oxidant/antioxidant levels based on patient age and sun exposure. Material and methods: The study included 44 patients. In all patients, skin biopsies were taken from the surplus area from routine excisions, undergoing histopathological examination. Patients were selected from those admitted to the surgery ward of the Municipal Clinical Hospital of Cluj-Napoca between January 2014 and June 2014 for different types of surgery. Average age of patients in the sun-exposed group was 54.2 ± 12.9 years and 52.1 ± 10.2 years in the unexposed group. Biopsy samples helped to determine MDA (nmol/mg protein), PC levels (nmol/mg protein), GSH (nmol/mg protein), GSSG (nmol/mg protein), SOD (U/g protein), CAT (U/g protein) and GPx (U/g protein) levels. Results: There was a statistically significant difference in MDA levels between the sun-exposed group (0.41 (0.24, 0.54) and the unexposed group (0.27 (0.19, 0.36) (p=0.03). Although PC levels were higher in the sun-exposed group (0.49 (0.38, 0.57) than in the unexposed group, there was no statistically significant difference between the groups (0.26 (0.14, 0.73) (p=0.1). GSH levels were statistically significantly higher in the unexposed group (2.63 (1.71, 2.91) than in the sun-exposed group (1.43 (1.12, 1.82) (p<0.001). GSSG levels were statistically significantly higher in the unexposed group (0.22 (0.19, 0.27) than in the exposed group (0.17 (0.14, 0.21) (p=0.001). SOD levels were statistically significantly higher in the unexposed group (394.89 (377.19, 449.85) than in the exposed group (286.85 (259.45, 330.54) (p<0.001). CAT levels were statistically significantly higher in the unexposed group (44.34 (38.54, 54.47) than in the exposed group (22.14 (17.25, 40.64) (p=0.001). GPx levels were statistically significantly higher in the unexposed group (69.26 (63.07, 77.21) than in the exposed group (22.14 (17.25, 40.64) (p<0.001) Conclusions: The levels of antioxidants were higher in sun-exposed skin area. The levels of oxidants were higher in unexposed skin area.

**Key Words:** age, skin, sun, oxidants/antioxidants.



**Corresponding Author:** A. E. Macarie, macarieantonia@yahoo.com

## Introduction

Skin aging is a complex process determined by genetic and environmental factors, hormonal changes and metabolic processes. Changes occur at both macro and microscopic scale, being mainly due to oxidative stress. This occurs despite the presence of effective antioxidant systems. When the formation of oxygen free radicals exceeds the working ability of anti-oxidants, there is excessive and uncontrolled accumulation of free radicals, leading to various skin diseases, including skin cancers (Black 2004). Oxidative stress is primarily caused by external factors such as ultraviolet radiation, food additives, cosmetics, certain drugs, etc. Oxidative stress-producing substances include the superoxide radical, the hydroxyl radical and hydrogen peroxide. Defense mechanisms against oxidative stress are provided by superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione transferase, or non-enzymatic materials (alpha-tocopherol, beta-carotene, etc.).

Malondialdehyde (MDA) is a biomarker for oxidative stress. MDA is the end-product of the radical-initiated oxidative decomposition of polyunsaturated fatty acids. MDA levels are predictive for the occurrence of cardiovascular events (myocardial infarction, stroke), metabolic diseases (diabetes mellitus) (Grune & Berger 2007). Lipids are the primary targets of photo-oxidative stress. Structural and functional changes associated with skin alteration as a result of UV radiation were correlated with lipid peroxidation (Dissemond et al 2003). It has been established that MDA is produced in the skin by lipid peroxidation under UV radiation (Morliere et al 1995).

Proteins are other important targets of oxidative changes. Oxygen radicals or other activated oxygen species cause changes in the constituent amino acids, which lead to functional or structural protein alterations (Stadtman 1992). Protein carbonyls are formed by the direct oxidation of lysine, arginine, proline, or by reactions with MDA produced by lipid peroxidation (Berlett & Stadtman 1997). The determination of carbonyl group content in human skin can be used in order to emphasize the oxidation of proteins by reactive oxygen species (Dimon-Gadal et al 2000). GSH, CAT and SOD enzymes play a central role in the antioxidant defense system of the human body. CAT and GPx are enzymes that break down H2O2 and turn it into water and O2, and at the same time, GPx breaks down peroxides and turns them into relatively non-toxic alcohol species. These enzymes require GSH during peroxidation, GSH being converted to its oxidized form, further recycled by glutathione reductase (Bickers & Athar 2006).

This study aims to describe the differences in oxidant/antioxidant levels based on patient age and sun exposure.

## Material and method

This is an observational, analytical, cross-sectional, case-control study.

The study included 44 patients. In all patients, skin biopsies were taken from the surplus area from routine excisions, undergoing histopathological examination. Patients were selected from those admitted to the surgery ward of the Municipal Clinical Hospital of Cluj-Napoca between January 2014 and June 2014 for different types of surgery. Patients were divided into two groups: the sun-exposed group (20 patients, 45.5%) and the unexposed group (24 patients, 54.5%).

Average age of patients in the sun-exposed group was 54.2 ± 12.9 years and 52.1 ± 10.2 years in the unexposed group.

Subjects were included after signing the informed consent form for inclusion in the study and genetic determinations.

The study protocol was approved by the Ethics Committee of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca.

Patients older than 18 years who signed the informed consent form and who were about to undergo upper extremity, lower extremity, forehead or abdomen surgery were enrolled in the study.

The following were not included in the study: patients younger than 18 years, patients who did not sign the informed consent form, patients diagnosed with cutaneous manifestations of systemic diseases or affections of the abdomen, lower or upper extremities, forehead, cancers and autoimmune diseases.

The following demographic data were recorded for each patient: age and gender.

Biopsy samples helped to determine MDA (nmol/mg protein), PC levels (nmol/mg protein), GSH (nmol/mg protein), GSSG (nmol/mg protein), SOD (U/g protein), CAT (U/g protein) and GPx (U/g protein) levels.

Tissue homogenization was performed using a PT 1200E Polytron homogenizer. The homogeneous medium was obtained using 50 mM Tris-buffered saline + 10 mM EDTA, pH 7.5. A given volume of Tris-buffered saline was added to a tissue sample while stirring in an ice bath. The content was centrifuged for 10 minutes at 1000 x g, 400C, and the supernatant was separated. The total protein concentration of the supernatant was determined using the Bradford method.

MDA was determined by fluorescence lipid peroxidation. Through this process, the resulting MDA reacts with thiobarbituric acid to form a fluorescent adduct. For the determination of tissue homogenate, the sample was boiled for one hour with 10 mM of 2-thiobarbituric solution in 75 mM K2HPO4, pH 3. After quenching, the reaction product was extracted with n-butanol. The concentration was measured in the organic phase after its separation by centrifugation. Emission intensity was measured at 534 nm with a Perkin Elmer spectrofluorimeter using the synchronous fluorescence technique to a 14 nm difference between the excitation and emission wavelengths (Δλ). MDA concentration is determined based on a calibration curve consisting of common MDA concentrations determined using the same measurement technique (Conti et al 1991).

Catalase activity adjustment was done by means of tissue homogenate. The method consisted in marking the changes in absorbance for a 10 mM solution of H2O2 in 0.05 M potassium phosphate buffer (pH 7.4) at 240 nm. One unit of this kind of activity was arbitrarily defined as the amount of enzyme that induces a 0.43 decrease in absorbance at 2500C, for 3 minutes. The activity is expressed in U/mg protein using the formula derived from working conditions: CAT = A240/0.43x0.02 (mg/ml), where A240 is the absorbance at 240 nm (Pippenger et al 1993).

Protein concentration was determined using the Bradford method. The method is a colorimetric protein assay using Coomassie Brilliant Blue G-250 (Bradford reagent). The determination was performed on tissue homogenates that were diluted to reach a protein content of 5-100 µg protein/100 µl homogenate. A quantity of 2.5 ml of Bradford reagent was added to a volume of 50 µl tissue homogenate dilution. The absorbance at 595 nm was read after 5 minutes, together with the protein content (µg/100 µl) in the sample, on the calibration curve (Bradford 1976).

The determination of protein carbonyl was carried out by means of a technique which is based on the reaction with a classic carbonyl reagent: 2,4-dinitrophenylhydrazine. The reaction leads to the formation of 2,4-dinitrophenylhydrazone, yellow in color, that can be determined spectrophotometrically. Serum samples were reacted with 10 mM of 2,4-dinitrophenylhydrazine solution in 2.5 N HCl for 1 hour at room temperature in the dark. After being treated with 20% trichloroacetic acid and after the separation of the precipitate obtained by centrifugation, the sample was washed three times with a 1:1 mixture of ethyl acetate and absolute ethanol (v/v). Further, the precipitate was dissolved in 6 M guanidine hydrochloride. From the samples obtained, protein concentration was determined by measuring the extinction at 280 nm. Later, on the same samples, the extinction at 355 nm was also read (wavelength corresponding to the absorption spectra of hydrazones). The protein concentration of the samples analyzed was determined based on a calibration curve consisting of common concentrations of albumin solutions in 6 M guanidine hydrochloride. Simultaneously with the samples treated with 2,4-dinitrophenylhydrazine, blank samples were also processed, only treated with 2.5 N HCl. Extinction was read based on these samples. Carbonyl concentration assessment is done according to the following formula: C = Abs 355 x 45.45 nmol/ml. Results were expressed as nmol/mg protein, taking into account the protein concentration of the sample expressed in milligrams (Reznick & Packer 1994).

Determination of SOD was done by the following technique. The superoxide radical is generated by the xanthine-xanthine oxidase system in the presence of oxygen. The superoxide reacts with ferricytochrome C that can be continuously monitored by recording the absorbance at 550 nm. SOD reduces superoxide ion levels and thereby inhibits the reduction of cytochrome C. SOD levels can thus be calculated based on the degree of inhibition of the reduction of cytochrome C using a calibration curve consisting of common SOD standards. One unit of SOD is defined as the amount of enzyme able to inhibit the rate of reduction of cytochrome C under the conditions specified by 50%. Dosing was performed on lysed erythrocytes at 2500C. Results are expressed in U/g protein (Flohe & Otting 1994).

Determination of GSH and GSSG was performed using the technique described. A 50 µl tissue homogenate sample was treated with 450 µl of 10% M phosphoric acid solution and centrifuged for 10 min at 1000 x g. The determination of GSH

was accomplished using 0.1 ml of the supernatant obtained, diluted with 1.8 ml of 0.1 M phosphate buffer (pH 8) containing 5 nmol/L EDTA, further adding 0.1 ml of o-phthalaldehyde solution in methanol (1 mg/ml). After 15 min of incubation, fluorescence at 420 nm was measured with an excitation of 350 nm. The determination of GSSG was done in 250 µl of supernatant, incubated for 30 minutes with N-ethylmaleimide 40 nmol/L. Subsequently, 0.65 ml of 0.1 N NaOH were added. Then, the procedure was similar to that of GSH, but instead of phosphate buffer, 1.8 ml of 0.1 N NaOH and 0.1 ml of o-phthalaldehyde solution were added to 0.1 ml of reaction mixture. The determination of the concentration of GSH and GSSG was based on calibration curves consisting of common concentrations of GSH and GSSG processed in the same way (Vats et al 2008).

The determination of GSH-Px is made by an indirect method based on monitoring the decrease in NADPH concentration, in whose presence GSSH that formed in the reaction is converted to GSH by glutathione reductase (GSSG-R). The working method consisted in tracking the change in NADPH extinction at 340 or 365 nm, in a reaction medium containing 2.4 U/ml glutathione reductase, 10 mM GSH, 1.5 mM NADPH, 1.5 mM H2O2 in PBS, 0.1M, pH 7, for 6 minutes. Enzyme activity is defined as the amount of glutathione peroxidase that can induce a net decrease in GSH of 10% of the initial concentration, in one minute, at 370 nm and pH 7. The calculation takes into account the reaction's stoichiometry and molar extinction coefficient of NADPH. Therefore: A = 0.868 (Δ[NADPH]/[GSH]0t) (Vi/Vs); where [NADPH] is the molar concentration of NADPH, [GSH] 0 - initial concentration of GSH, t - reaction time, Vi - volume of the incubation mixture, and Vs - volume of the sample to analyze. The activity is reported to 1 mg protein for tissue homogenates (Flohe & Gunzler 1984).

Statistical analysis was performed using MedCalc software version 14.8.1.

Data were labeled as quantitative and ordinal variables. Ordinal variables were defined by calculating frequencies and percentages. Continuous variables were determined using the mean and standard deviation or the median and the 25th and 75th percentiles, depending on the normality of the distribution - tested using Kolmogorov-Smirnov test.

The independent samples t-test, ANOVA, Mann-Whitney test or Kruksall-Wallis test were used for univariate analysis of normally distributed quantitative variables between two or more groups. The correlation between two quantitative variables was done using Spearman's correlation.

A p value of $<0.05$ was found statistically significant.

# Results

There were no significant differences in patient age between the two groups (p=0.5).

The study consisted of 22 (50%) women and 22 (50%) men.

Patient distribution into groups according to age was not statistically significantly different (p=0.5).

Table 1 shows the sites of skin biopsies.

There was a statistically significant difference in MDA levels between the sun-exposed group (0.41 (0.24, 0.54) and the unexposed group (0.27 (0.19, 0.36) (p=0.03).

There was no statistically significant correlation between MDA levels and patient age (r=-0.005, p=0.9), nor between MDA levels and different age groups (p=0.8). There was no statistically significant difference in MDA levels between different biopsy sites (p=0.1).

Table 1 . Skin biopsy sites

| Site | Number | Percentage |
|---|---|---|
| **Abdomen** | 24 | 54.5 |
| **Anterior forearm** | 4 | 9.1 |
| **Forearm/hand** | 4 | 9.1 |
| **Radiocarpal joint** | 4 | 9.1 |
| **Forehead** | 4 | 9.1 |
| **Calf** | 4 | 9.1 |
| **Total** | 44 | 100 |

Although PC levels were higher in the sun-exposed group (0.49 (0.38, 0.57) than in the unexposed group, there was no statistically significant difference between the groups (0.26 (0.14, 0.73) (p=0.1). There was no statistically significant correlation between PC levels and patient age (r=0.083, p=0.5), nor between PC levels and different age groups (p=0.3). There was no statistically significant difference in PC levels between different biopsy sites (p=0.4).

GSH levels were statistically significantly higher in the unexposed group (2.63 (1.71, 2.91) than in the sun-exposed group (1.43 (1.12, 1.82) (p<0.001).

There was a statistically significant negative correlation between GSH levels and patient age (r=-0.301, p=0.04). The correlation was also maintained during sun exposure control (r=-0.310, p=0.04).

There were statistically significant differences in GSH levels between different age groups (p=0.05).

There were statistically significant differences in GSH levels between different biopsy sites (p=0.001).

GSSG levels were statistically significantly higher in the unexposed group (0.22 (0.19, 0.27) than in the exposed group (0.17 (0.14, 0.21) (p=0.001).

There was no statistically significant correlation between GSSG levels and patient age (r=-0.255, p=0.1). There were no statistically significant differences in GSSG levels between different age groups (p=0.3). There were statistically significant differences in GSH levels between different biopsy sites (p=0.001).

SOD levels were statistically significantly higher in the unexposed group (394.89 (377.19, 449.85) than in the exposed group (286.85 (259.45, 330.54) (p<0.001).

There was no statistically significant correlation between SOD levels and patient age (r=-0.062, p=0.6). There were no statistically significant difference between age groups in terms of SOD levels (p=0.3). There were statistically significant differences in SOD levels between different biopsy sites (p<0.001).

CAT levels were statistically significantly higher in the unexposed group (44.34 (38.54, 54.47) than in the exposed group (22.14 (17.25, 40.64) (p=0.001).

There was no statistically significant correlation between CAT levels and patient age (r=-0.042, p=0.7). There were no statistically significant difference between different age groups in terms of CAT levels (p=0.5). There were statistically significant differences in CAT levels between different biopsy sites (p<0.001).

GPx levels were statistically significantly higher in the unexposed group (69.26 (63.07, 77.21) than in the exposed group (22.14 (17.25, 40.64) ($p<0.001$).

There was no statistically significant correlation between GPx levels and patient age ($r=-0.181$, $p=0.2$). There were no statistically significant differences between different age groups in terms of GPx levels ($p=0.3$). There were no statistically significant differences in GPx levels between different biopsy sites ($p=0.1$).

## Discussion

Prolonged exposure to ultraviolet radiation can cause skin cancer and accelerates the process of skin aging. Understanding the mechanisms by which UV radiation affects the skin has been an important step in recent medical research. These studies helped develop products that offer a better protection against UV radiation. Considering the fact that ultraviolet radiation promotes the production of free radicals, it is important to study them, as well as the antioxidant skin protection mechanisms. In this study, we analyzed the differences between sun-exposed and unexposed skin in terms of the oxidizing agents and antioxidant substances found in the skin.

The two groups (sun-exposed and unexposed skin groups) did not differ significantly in terms of patient age and gender.

MDA and PC were the oxidizing agents studied in this research. In our study, MDA levels were statistically significantly higher (approximately 40%) in patients with sun-exposed tissue samples than in those with biopsies from unexposed skin. Ultraviolet radiation causes phototoxic skin reactions by generating reactive oxygen species and free radicals (Wondrak et al 2006). MDA production in the skin has been described by cell cultures, the correlation between MDA levels and ultraviolet radiation exposure dose being demonstrated in people (Morliere et al 1995; Katiyar et al 2001). Trommer et al have shown that UV radiation induces lipid peroxidation in the skin, thus creating a prerequisite for the production of large amounts of MDA (Trommer et al 2001). Williams et al have shown that MDA-derived epitopes occur in healthy skin exposed to ultraviolet radiation on both short and long term. It has also been established that MDA acts as a chemical carcinogen in the occurrence of non-melanoma skin-cancer (Williams et al 2014).

Although MDA levels were higher in intensely sun-exposed skin (forehead and forearm) than in less exposed skin (calf), this difference was not statistically significant, probably due to the small number of patients.

PC levels assessed in patients in our study were higher in the sun-exposed group than in the unexposed group, but the difference was not statistically significant. The coefficient p value close to statistical significance (0.1 vs. 0.05), as well as the higher PC levels, indicate the possibility of attaining closer results to the literature in a study that would consist of a larger number of patients. Reactive carbonyl species, as well as reactive oxygen species, cause DNA, protein and lipid damage, while collagen structures are important targets for carbonyl stress in the skin (Berlett & Stadtman. Studies in the literature have shown increased PC levels mainly in the outermost skin layer in subjects with extended exposure to ultraviolet radiation (Podda et al 1998; Sander et al 2002).

Out of the antioxidant agents found in the skin, GSH, GSSG, SOD, CAT and GPx levels were determined.

GSH and GSSG levels were significantly higher in patients with biopsies obtained from unexposed skin than in those with biopsies obtained from sun-exposed skin. Reduced efficiency of skin antioxidant activity has been proposed as one of the determinants of skin aging (Kohen & Gati 2000; Wei et al 2001). Our study supports this hypothesis. We determined reduced GSH levels in aging patients. Patients with skin biopsies obtained from the abdomen showed higher GSH and GSSG levels than the values obtained for any other area. GSH and GSSG are part of the antioxidant defense system that the body uses against oxidative stress. Research on animals has shown that GSH levels in their skin are higher in those whose skin is exposed to ultraviolet radiation (Tülüce et al 2012). Rhie et al have determined that skin areas not exposed to sunlight contain large amounts of GSH and GSSG (Rhie et al 2001).

Animal studies have shown reduced SOD, CAT and GPx levels in the skin of mice with both short and long term exposure to ultraviolet radiation (Iizawa et al 1994). It has been shown that SOD, CAT and GPx activities are also reduced in sun-exposed human skin (Rhie et al 2001; Thiele 2002). Similarly, in our study, SOD, CAT and GPx levels were higher in patients with biopsies obtained from unexposed skin. SOD, CAT and GPx levels were not correlated with patient age. Neither Rhie et al nor Lopez-Torres et al were able to show any correlation between antioxidants and patient age (Lopez-Torrez et al 1994; Rhie et al 2001).

Some of the limitations of the study mainly include the small number of subjects, the impossibility of determining more oxidative agents / antioxidants, as well as the inability to obtain biopsies only from one sun-exposed skin area.

## Conclusions

The levels of antioxidants were higher in sun-exposed skin area. The levels of oxidants were higher in unexposed skin area.

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## Authors

•Raluca E. Diaconu, "Iuliu Haţieganu" University of Medicine and Pharmacy, 8 Victor Babes Street, Cluj-Napoca, Cluj, Romania, EU, email: deraluca2005@yahoo.com

•Antonia E. Macarie, Department of Geriatrics and Gerontology, "Iuliu Haţieganu" University of Medicine and Pharmacy, Geriatric Unit, Municipal Clinic Hospital, 11 Tăbăcarilor Street, 400139, Cluj-Napoca, Cluj, Romania, EU, email: macarieantonia@yahoo.com

•Remus Orăsan, Department of Physiology, Faculty of Medicine, "Iuliu Hatieganu" University of Medicine and Pharmacy, 1 Clinicilor Street, Cluj-Napoca, Cluj, Romania, EU, email: remus.orasan@umfcluj.ro

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