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Sunscreen protection against ultraviolet-induced oxidative stress: Evaluation of reduced glutathione levels, metalloproteinase secretion, and myeloperoxidase activity

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Several studies have demonstrated the skin protection by sunscreens considering the aspects skin penetration, photostability, and protection against erythema and sunburn. However, little is known about the effect of topically applied sunscreen formulations on the antioxidant defense, metalloproteinases, and inflammatory processes of skin in response to UVR exposure. Therefore, this study aimed to investigate the use of a cream gel formulation containing the UV filters benzophenone-3, octyl methoxycinnamate, and octyl salicylate to prevent skin damage from a single dose of UVR (2.87 J/cm^2). This protective effect was evaluated *in vivo* by measuring the following biochemical parameters: reduced glutathione levels, secretion of matrix metalloproteinases, and myeloperoxidase activity. The results showed that the sunscreen formulation, despite having sun protection factor (SPF) 15, was not completely effective to protect the skin against GSH depletion, MMP-9 secretion and the inflammatory process induced by UVR. These results demonstrate the importance of analyzing UV-altered biochemical parameters of skin in order to propose new sunscreen formulations that can completely protect skin against UVR-induced damage.

1. Introduction

Exposure to ultraviolet radiation (UVR) is a very important factor in the pathogenesis of many skin disorders such as hyperpigmentation, erythema, photoaging, immunosuppression and cancer (Afaq et al. 2003; Ndiaye et al. 2011). UVR can be absorbed by endogenous photosensitizers, such as DNA, porphyrins, urocanic acid and aromatic amino acids, leading to the generation of reactive oxygen species (ROS) (Young 1997; Wondrak et al. 2006).

To protect against the deleterious effects of solar UVR, skin is equipped with a complex defense system consisting of skin pigment, skin thickening and several enzymatic and non-enzymatic antioxidants that eliminate damage and toxic substances. However, this defense can be overwhelmed when UV-induced oxidative stress exceeds the antioxidant capacity (Svobodová and Vostálová 2010).

To limit sun exposure, the government advises that people cover up with loose-fitting, tightly woven clothing, stay in the shade between 11 a.m. and 3 p.m., and use a sunscreen with a sun protection factor (SPF) of 15 or higher (IARC 2001). Sunscreens attenuate the transmission of solar radiation into the skin by absorption, reflection or scattering. Organic UV filters in sunscreens are capable of absorbing UVR and converting it into harmless radiation (Flor et al. 2007). Ideally, sunscreens should protect against skin cancer, the sun's effects on the immune system and photoaging of the skin (Duale 2010).

Several studies have demonstrated the photoinstability of UV filters and sunscreen's ability to penetrate the skin and protect it against erythema and sunburn (Gaspar and Maia Campos 2006; Janjua et al. 2004; Treffel and Gabard 1996). The increasing use of sunscreens has promoted interest in the photostability of these products, and it has been shown that upon UV exposure, organic sunscreens may decrease their protective capability and behave as photooxidants (Duale 2010).

It is well known that UVR exposure can decrease levels of endogenous reduced glutathione (GSH), increase matrix metalloproteinase (MMP) secretion and increase myeloperoxidase (MPO) activity (an inflammation biomarker) in the skin. GSH is one of the most important endogenous defense mechanisms against UV-induced ROS and matrix metalloproteinases is a family of proteolytic enzymes that contribute to skin photoaging and to the spreading of metastatic cells in skin carcinoma by specifically degrading skin collagen and elastin (Arrigo 1999; Carini et al. 2000; Casagrande et al. 2006; Fonseca et al. 2010; Vicentini et al. 2008; Xu and Fisher 2005).

However, little is known about the effect of UV filters on these parameters when sunscreen formulations are applied to skin and exposed to UVR. Evaluating the effect of UVR exposure on the biochemical parameters of skin is of great importance for the development of new sunscreen formulations because using erythema as the only response against UVR may not reflect the real protective effectiveness of sunscreens.

Therefore, the present study aimed to investigate the use of a cream gel formulation containing the UV filters benzophenone-3 (BP3), octyl methoxycinnamate (OMC), and octyl salicylate (OS) to prevent UVR-induced skin damage. This protective effect was evaluated *in vivo* by measuring the following bio-

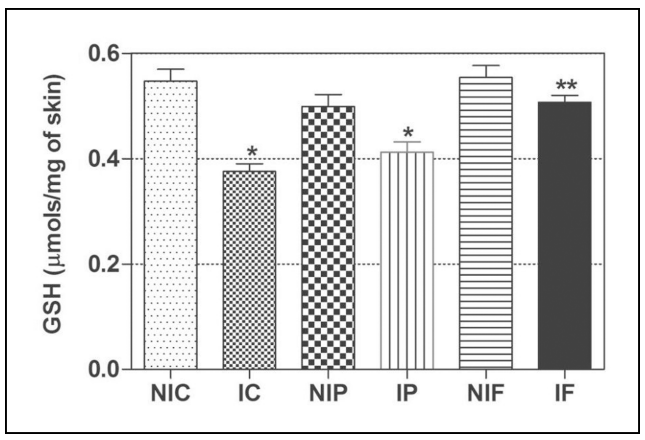

Fig. 1: Effect of cream gel formulation containing BP-3, OMC, and OS on the decrease of UVB irradiation-induced GSH levels. NIC = non-irradiated control, IC = irradiated control, NIP = treated with a placebo formulation and non-irradiated, IP = treated with a placebo formulation and irradiated, NIF = treated with a formulation containing the UV filters and non-irradiated, and IF = treated with a formulation containing the UV filters and irradiated. Bars represent means ± S.E.M. of three separated experiments, 3–4 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test of multiple comparisons. *$p < 0.05$ compared to the NIC group. **$p < 0.05$ compared to the IC group.

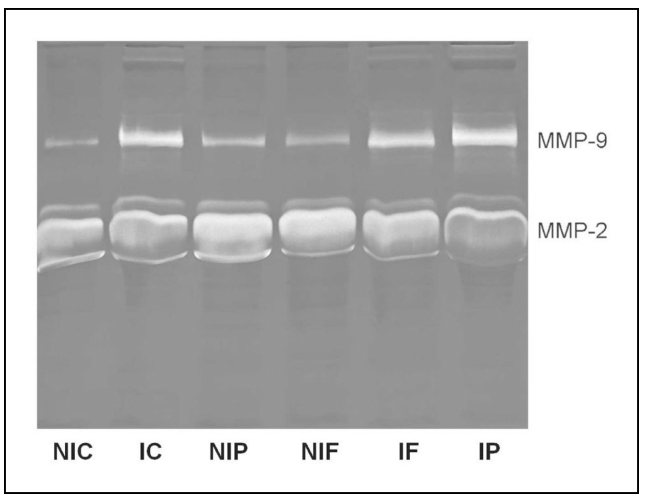

Fig. 2: Effect of cream gel formulation containing BP-3, OMC, and OS on the increase of UVB irradiation-induced metalloproteinase activity. NIC = non-irradiated control, IC = irradiated control, NIP = treated with a placebo formulation and non-irradiated, IP = treated with a placebo formulation and irradiated, NIF = treated with a formulation containing the UV filters and non-irradiated, and IF = treated with a formulation containing the UV filters and irradiated. The data are representative of three separated experiments, 3–4 animals per group.

chemical parameters: endogenous reduced glutathione levels, matrix metalloproteinase secretion and myeloperoxidase activity.

2. Investigations and results

2.1. GSH assay

Glutathione is one of the most important endogenous defense mechanisms against UV-induced reactive oxygen species. It directly scavenges radicals by hydrogen transfer and acts as a cofactor for the enzyme GSH-peroxidase, which scavenges peroxides and regenerates vitamins E and C (Carini et al. 2000). The results showed a decrease of 31.3% in GSH levels in the IC group when compared to the NIC group (Fig. 1). This result reinforces other studies showing that GSH levels are decreased after UV exposure, which indicate that the glutathione antioxidant system is strongly affected by photo-oxidative stress (Fuchs et al. 1989; Ho et al. 2005). We have also demonstrated that UVB radiation induced a dose-dependent (0.96 to 2.87 J/cm^2) decrease in GSH levels in the skin of hairless mice (Casagrande et al. 2006; Vicentini et al. 2008).
Additionally, the topical treatment containing the UV filters BP-3, OMC and OS inhibited 76% of the GSH depletion induced by UVB radiation, as shown by a comparison of the IF group with the IC group. The same result was not obtained in the IP group, which suggests that the protective capability is due to the presence of the UV filters in the formulation, not due to the physical protection afforded by the placebo formulation.
As shown in Fig. 1, there was not a statistically significant difference among the GSH levels observed in the non-irradiated groups (NIC, NIP and NIF), demonstrating that the formulation components did not interfere with the regular levels of GSH.

2.2. Qualitative analyses of skin proteinases

This study qualitatively evaluated the effect of topically added cream gel sunscreen on the activity/secretion of the proteolytic enzymes MMP-2 and MMP-9 using zymography in polyacrylamide gel with SDS. These proteolytic enzymes degrade the collagen that is already partially degraded by collagenases in the extracellular matrix of skin in mice exposed to UVB radiation (Varani et al. 2001).
In the non-irradiated groups, it is possible to identify the presence of the constitutive enzyme MMP-9 in the skin, but in smaller intensities than those observed in the irradiated groups. The basal expression of this enzyme is relatively low; however, it is increased by radiation both *in vivo* and in cell culture (Jenkins 2002).
Fig. 2 shows an increased intensity in proteinase activity bands in the irradiated groups when compared with the non-irradiated ones. This result corroborates other studies showing that UVR induces the secretion and action of gelatinases, especially MMP-9 (Fonseca et al. 2010; Vicentini et al. 2008, Xu and Fisher 2005).
A comparison of the activity/secretion of MMP-9 (92 kDa) in the IF, IP and IC groups revealed similar electrophoretic profiles. This result shows that the presence of sunscreen was not able to protect the skin against the increase in the activity/secretion of UVR-induced MMP-9 (Fig. 2).

2.3. MPO activity in the cutaneous tissue

MPO is a hydrogen peroxide oxidoreductase enzyme that is specifically found in granulocytic leukocytes, including polymorphonuclear leukocytes (PMN), monocytes, basophils and eosinophils and contributes to the bactericidal capacity of these cells. MPO has been used as a biomarker to assess the inflammatory response to a number of well-characterized skin irritants and tumor promoters (Trush et al. 1994).
Studies have indicated that UVB irradiation induces a dose-dependent increase (0.61–3.69 J/cm^2) in MPO activity in the skin of hairless mice (Casagrande et al. 2006). In the present study, the UVB dose of 2.87 J/cm^2 induced an increase of 41.22% in MPO activity in the IC group when compared with the NIC group (Fig. 3).
The results of this study also revealed that MPO activity in the irradiated groups IC and IF was similar, which demonstrates that the topical application of the cream gel formulation containing BP-3, OMC, and OS was not able to inhibit the MPO increase induced by UVB irradiation. Moreover, the MPO activity observed for the non-irradiated groups NIC, NIP and NIF was

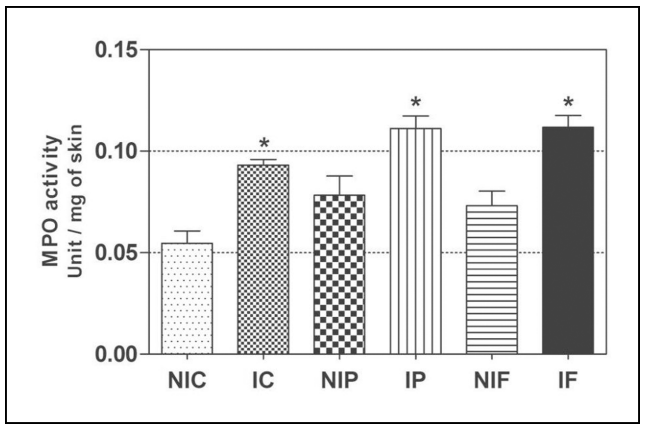

Fig. 3: Effect of cream gel formulation containing BP-3, OMC, and OS on the increase of UVB irradiation-induced myeloperoxidase activity. NIC = non-irradiated control, IC = irradiated control, NIP = treated with a placebo formulation and non-irradiated, IP = treated with a placebo formulation and irradiated, NIF = treated with a formulation containing the UV filters and non-irradiated, and IF = treated with a formulation containing the UV filters and irradiated. Bars represent means ± S.E.M. of three separated experiments, 3–4 animals per group. Statistical analysis was performed by one-way ANOVA followed by Tukey's test of multiple comparisons. *$p < 0.05$ compared to the NIC group.

statistically similar, demonstrating that neither the formulation components nor the non-irradiated UV filters interfered in the normal MPO activity (Fig. 3).

3. Discussion

Sunscreens are applied over large areas of the body and for long periods of time, producing a constant input of UV filters into the skin and even into the circulatory system (Touitou and Godin 2008). Thus, although there are many studies evaluating the effect of different sunscreens on skin penetration, photostability and protection against erythema and sunburn, to our knowledge this is the first work aimed at evaluating the effect of a formulation containing widely used UV filters on biochemical parameters altered by UVR exposure.

Studies conducted by our group showed that topical treatment with a formulation added to Marigold extract was able to inhibit the GSH depletion by 100% under the same irradiation conditions as the present study (Fonseca et al. 2010). This extract exhibited similar efficacy to quercetin, epigallocatechin-3-gallate and green tea polyphenols (Vayalil et al. 2003; Vicentini et al. 2008).

Considering that the sunscreen formulation used in the present study does not act as an antioxidant, the 76% protection against UV-induced GSH depletion was likely conferred by the studied formulation's ability to block UVR (primarily UVA), leading to a lower GSH consumption in the IF group, as compared to the IC group. UVA is the major component of the solar spectrum that induces oxidative stress in the skin. It penetrates deeply into the skin, reaching the basal layer of the epidermis and even dermal fibroblasts (Marrot and Meunier 2008; Wang et al. 2008).

A previous study demonstrated the ability of the UV filters present in this cream gel to penetrate the stratum corneum and reach the viable epidermis (Vilela et al. 2012). Therefore, the UV filters in the epidermis were able to significantly protect the skin against UV-induced GSH depletion.

Despite the sunscreen formulation's partial protection against GSH depletion, it is important to consider strategies to create sunscreen formulations that completely prevent the depletion of GSH in the skin, such as the incorporation of antioxidant compounds in sunscreen formulations. Sunscreens and antioxidants act by different mechanisms and are therefore expected to be complementary (Oresajo et al. 2010). However, we emphasize the importance of skin penetration studies that examine the penetration of the antioxidant compounds to the viable epidermis to protect against oxidative stress.

Regarding metalloproteinase activity, the treatment of animals with the sunscreen formulation did not inhibit the MMP-9 secretion/activity induced by UVR, as shown by a comparison of the IF and IC groups. This result might indicate that this formulation was not enough to inhibit the penetration of UVR in the skin.

Several studies have shown an increase in proteinase secretion induced by UVR and its relationship to photoaging and photocarcinogenesis (Fisher et al. 1996). The increase in the expression of metalloproteinases has been observed in almost all diseases in which inflammation is present, and recent studies suggest that MMP evolved to operate in a wide range of defensive functions, such as lesions, inflammation and repair processes (Manicone and McGuire 2008).

In the present study, the increase in MMP-9 activity in all the irradiated groups was accompanied by an increase in the inflammatory process induced by UVR exposure as observed by the MPO assay. The topical application of the cream gel formulation containing BP-3, OMC, and OS was not able to inhibit or even decrease the UVR-induced MPO activity.

Once again it is evident that the formulation did not effectively block UVR, allowing the generation of free radicals and the activation of inflammation mediators. This study shows that this sunscreen formulation, despite having SPF 15, was not adequate to completely protect the skin against GSH depletion, MMP-9 secretion and the inflammatory process induced by UVR. Because the UV lamp used in this study emitted more UVB than UVA, we suggest that the increase in MMP-9 and MPO activities in the IF group may be due primarily to the lack of protection against UVB. Therefore, this formulation containing only organic UV filters did not completely block the radiation. This shows the importance of adding physical filters into sunscreen formulations to increase their effectiveness.

Additionally, these results suggest that the combination of sunscreens with antioxidant compounds, whose effectiveness in inhibiting the UVR-induced oxidative stress has been demonstrated, might be a useful approach for reducing skin diseases associated with UVR exposure, such as photoaging and skin cancer (Afaq and Mukhtar 2006; Kang et al. 2003; Vayalil et al. 2004; Vicentini et al. 2008).

Finally, the UV filters, such as endogenous photosensitizers, can be excited by the capture of UVR photons and transfer the energy of UV light to molecular oxygen, leading to ROS generation (Diaz-Cruz et al. 2008). Moreover, our previous study demonstrated that when the presence of the proposed sunscreen formulation was associated with ultraviolet exposure in the skin, a significant decrease in superoxide dismutase (SOD) activity, greater than that naturally produced under UVR, was observed (Vilela et al. 2012). Therefore, the lack of photoprotection, as measured by the biochemical parameters in the present study, might be explained by the photoinstability of these UV filters, which may have degraded under radiation, decreasing their protective capability. Thus, the production of photostable products is extremely important (Hojerová et al. 2011).

In conclusion, these results show the importance of studying UV-altered biochemical parameters in the skin to propose new sunscreen molecules, stable combinations of sunscreen agents, and the combinations of UV filters and antioxidant substances. These studies could lead to sunscreen formulations that completely protect the skin against damages induced by UVR. In addition, it is important to consider lines of skin protection besides the use of sunscreens with high SPF, such as avoiding sun exposure and wearing clothing.

4. Experimental

4.1. Materials

3-Benzophenone (99.9%, Eusolex® 4360), octylmethoxycinnamate (99.9%, Eusolex® 2292), and octylsalicylate (99.8%, Eusolex® OS) were purchased from Merck (Darmstadt, Germany). All of the raw materials used for the formulations were purchased from Galena (Campinas, SP, Brazil) or Clariant (Sao Paulo, SP, Brazil). Reduced glutathione, protease inhibitor cocktail, ethylene glycol bis (-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA), o-phthalaldehyde (OPT), sodium dodecyl sulphate (SDS) and acrylamide were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

4.2. Formulation

This study was performed using a cream gel formulation that is an emulsion containing a high percentage of aqueous phase and a low percentage of oily content stabilized by hydrophilic colloids (Leonardi 2004). The cream gel was added by a mixture of the three organic UV filters at concentrations of 4.0% (w/w) BP3, 7.5% (w/w) OMC, and 5.0% (w/w) OS. The selection of UV filters and their concentrations were determined based on the concentrations usually employed in commercial formulations. A placebo formulation was prepared containing all of the components without the UV filters. According to the BASF Sunscreen Simulator, the prediction of outdoor SPF for this formulation was 15.4, based on UV filter composition. A homemade formulation was used because commercial products usually contain physical filters, such as zinc oxide, titanium dioxide, vitamin E, and plant extracts, in addition to the chemical filters. These additional physical filters could have interfered with the results.

4.3. Irradiation of animals

In vivo experiments were performed on 3-month-old, sex-matched hairless mice. All experiments were conducted in accordance with the National Institutes of Health guidelines for the welfare of experimental animals and with the approval of the Ethics Committee of the Faculty of Pharmaceutical Science of Ribeirao Preto (University of Sao Paulo, Ribeirao Preto, SP, Brazil – Process # 08.1.1399.53.4).

The animals were divided into six groups as follows: NIC = non-irradiated control, IC = irradiated control, NIP = treated with a placebo formulation and non-irradiated, IP = treated with a placebo formulation and irradiated, NIF = treated with a formulation containing the UV filters and non-irradiated, and IF = treated with a formulation containing the UV filters and irradiated. Experiments included three or four animals per group and were repeated three times.

The treatment protocol consisted of applying 30 mg of the formulations topically on the dorsal surface of the animals 1 h before irradiation. We have previously reported that BP3, OMC and OS are able to penetrate the skin of hairless mice 1 h after application of the sunscreen formulation used in this study. Therefore, to assess the *in vivo* effect, the formulation was applied 1 h before UVR exposure to ensure that the UV filters were present in the epidermis and dermis of the animal at the beginning of irradiation (Vilela et al. 2012).

The groups exposed to UVR were placed inside a wooden enclosure containing the lamp and were irradiated for 3 h, which corresponds to a total dose of 2.87 J/cm^2. The UVR source was a Philips TL/12RS 40 W lamp (Medical-Holland). This source emits in the range of 270–400 nm with an output peak at 313 nm, resulting in an irradiation of 0.27 mW/cm^2 at a distance of 20 cm, as measured by an IL 1700 radiometer (Newburyport, MA, USA) equipped with UVB and UV detectors. UVB output accounts for 78% of the total UVR.

The mice were sacrificed by inhalation of carbon dioxide 6 h following UVR exposure, and full dorsal skins were removed and stored at −80 °C until analysis (Fonseca et al. 2010; Vicentini et al. 2008; Vilela et al. 2012).

4.4. GSH assay

GSH skin levels were determined using a fluorescence assay (Hissin and Hilf 1976). The dorsal skin (1:3, w/w dilution) was homogenized in 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA using a Turrax TE-102 (Turratec). Whole homogenates were treated with 30% trichloroacetic acid, centrifuged at 1,900 × *g* for 6 min at 4 °C and the fluorescence of the resulting supernatant measured in a Hitachi F-4500 fluorescence spectrophotometer. Briefly, 100 μL of sample supernatant was mixed with 1 mL of 100 mM NaH_2PO_4 (pH 8.0) containing 5 mM EGTA and 100 μL of OPT (1 mg/mL in methanol). The fluorescence was determined after 15 min (λ_{exc} = 350 nm; λ_{em} = 420 nm) and the values compared to a standard curve prepared with GSH. The results are presented as μmols of GSH per mg of skin.

4.5. Qualitative analyses of skin proteinases by substrate embedded enzymography

SDS–PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) substrate-embedded enzymography (zymography) was used to detect enzymes with gelatinase activity. Assays were carried out as previously reported by Gerlach et al. (2007).

The skin (1:4, w/w dilution) was homogenized in 50 mM Tris–HCl buffer (pH 7.4) containing 10 mM $CaCl_2$ and 1% of protease inhibitor cocktail in a Turrax TE-102 (Turratec). Whole homogenates were centrifuged at 12,000 × *g* for 10 min at 4 °C. The Lowry method was used to measure protein levels in skin homogenates (Lowry et al. 1951). Supernatant aliquots (50 μL) were mixed with 10 μL of 100 mM Tris–HCl buffer (pH 7.4) containing 4% SDS, 20% glycerol, and 0.001% bromophenol blue. Fifty microliters of the mixture (40 μg of protein) was taken for electrophoresis. After electrophoresis, the gels were washed with 2.5% Triton X-100 for 1 h with constant shaking, incubated overnight in 50 mM Tris–HCl (pH 7.4) 10 mM $CaCl_2$ at 37 °C and stained the following day with Coomassie Blue 350-R (Phast gel blue R-Pharmacia Biotech). After destaining in 20% acetic acid, zones of enzyme activity were detected as regions of negative staining against a dark background. The proteolytic activity was qualitatively analyzed by comparing the bands from all groups of animals. The identification of metalloproteinases was performed by comparing the electrophoretic mobility of the samples with molecular weight standards (PageRuler prestained protein ladder plus SM 1819, Fermenta®).

4.6. MPO activity in the cutaneous tissue

The UVB-induced leukocyte migration into the skin was evaluated using the MPO kinetic–colorimetric assay (Bradley 1982; Casagrande et al. 2006). The dorsal skin (1:20 dilution) was collected and placed in 50 mM K_2HPO_4 buffer (pH 6.0) containing 0.5% hexadecyltrimethylammonium bromide (HTAB). The skins were then homogenized using a Turrax TE-102 (Turratec). The homogenates were centrifuged at 16,100 × *g* for 2 min and the resulting supernatant assayed spectrophotometrically for MPO activity determination at 450 nm (μQuantTM; BioTek Instruments Inc.,Winooski, Vermont) after 10 min. Briefly, 10 μL of sample were mixed with 200 μL of 50 mM phosphate buffer (pH 6.0) that contained 0.167 mg/mL *o*-dianisidine dihydrochloride and 0.015% hydrogen peroxide. The MPO activity of samples was compared with a standard enzyme curve and the results were presented as units of MPO per mg of skin.

4.7. Statistical analysis

Data were statistically analyzed using one-way analysis of variance followed by Tukey's test of multiple comparisons (GraphPad Prism® software, San Diego, California) and the level of significance was set at a p value of less than 0.05.

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