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The Antioxidant Activity of Peptides Isolated from *Amaranthus* on Normal Human Skin *in vitro* and Inflammatory Cytokines Detection

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Abstract

Oxidative stress has been implicated in various skin diseases through the generation of reactive oxygen species and the depletion of endogenous antioxidant systems. Evidence exists indicating that oxidative stress injury of the skin caused by UVB irradiation is mediated predominantly by reactive oxygen species (ROS) immediately after irradiation and by reactive nitrogen species (RNS) at later points. We investigated the protective effect of peptides (albumin and hydrolysates) isolated from Amaranthus (pA) against UVB irradiation, lipid peroxidation (malondialdehyde, MDA) and endogenous antioxidant defense enzymes such as glutathione peroxidase (GPX), superoxide dismutase (SOD) and catalase (CAT). Supernatant levels of pro-inflammatory and anti-inflammatory cytokines were also investigated. We used human skin organ culture as an *in vitro* model via 24, 36 and 48 hrs of culture. The pA has a significant protective effect via the inhibition of damaged caused by UVB irradiation. The treatment of skin with pA inhibited the UVB-induced lipid peroxidation. Additionally, this pA-inhibited UVB-induced depletion of antioxidant defense components, such as, CAT, SOD, and GPX, and the final antioxidant treatment also reduced levels of pro-inflammatory cytokines: IL-1beta, IL-12, TNF and INF-gamma. We conclude that pA could be useful in attenuating UVB-induced oxidative stress and mitigating skin diseases in human skin due to antioxidant properties.

Keywords: Reactive oxygen species; Reactive nitrogen species

Abbreviations: ROS: RACTIVE Oxygen Species; RNS: Reactive Nitrogen Species; pA: Peptide isolated from *Amaranthus*; GPX: Glutathione Peroxidase; SOD: Superoxide Dismutase; CAT: Catalase; MAD: Malondialdhehyde

Introduction

In recent years, biopeptides with antioxidative activity have attracted the attention of researchers due to their low molecular weight, easy absorption and high activity [1,2]. Amaranth (*Amaranthus hypochondriacus*) is a traditional Mexican plant that produces grains and flavorful leaves of high nutritional value. The complete plant is high in protein [3] and contains other substances that play various biological roles in the human diet, such as protease inhibitors, antimicrobial peptides, lectins, and antioxidant compounds [4-6]. Amaranth also possesses desirable agricultural properties, including drought resistance and amenability to cultivation in climatic changes [7].

Grain amaranth is a pseudo-cereal and is rich in essential amino acids such as lysine, tryptophan, and sulfur-containing amino acids. In amaranthus, 50% of the total seed proteins at maturity are globulin and albumin [8]. Various authors characterized the extraction method of peptides from amaranths [9,10]. Additionally, it has been predicted that amaranth may be a source of peptides with eleven different potential biological activities, such as antihypertensive, protease inhibition, opioid, immuno-modulation, antithrombotic, antioxidant, and antitumor, amongst others [11,12].

However, there is evidence that oxidative stress injury of the skin caused by UVB irradiation is mediated predominantly by reactive oxygen species (ROS) immediately after irradiation. UVB light stimulates the production of reactive species, which are the primary cause of the resulting skin lesions, and results in accelerated aging and the development of malignant skin diseases [13-15].

This study aims to evaluate the protective effect of peptides (albumin and hydrolysates) isolated from amaranthus (pA) against UVB irradiation and lipid peroxidation (malondialdehyde, MDA) and endogenous antioxidant defense enzymes such as catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX). Supernatant levels of proinflammatory and anti-inflammatory cytokines were also investigated and correlated.

Results

pA prevents UVB-induced depletion of endogenous antioxidant defense systems

Our results demonstrated that the exposure of human skin samples to UVB (G2) resulted in a reduction of CAT (20%, $p<0.01$) compared with non UVB-exposed human skin (C), whereas treatment of skin samples (G3) with pA (albumin or hydrolysates 24, 36 and 48 hr) restored the activities of CAT (Figure 1). Similar to other enzyme levels, the UVB exposition of human (G2) skin samples depleted the level of SOD by 10% compared with non-UVB exposed skin (C), and the pretreatment of human skin samples with pA (G3) restored the activity of the SOD enzyme (Figure 2). The exposure of human skin samples

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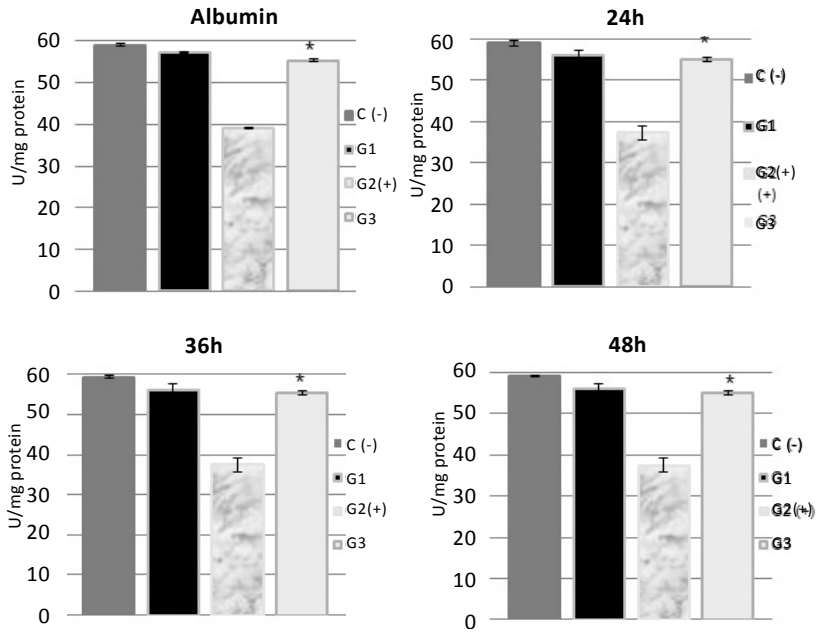

Figure 1: *In vitro* treatment of human skin samples with pA inhibits UVB-induced depletion of catalase (CAT) activity. Skin samples were exposed thirty minutes to UVB with or without pretreatment with pA (12%). Non-UVB-exposed skin "n-UVB-" (negative control, C); non- UVB- exposed skin plus pA "n-UVB+" (Group G1); UVB-exposed skin "UVB-"(positive control, Group G2); and UVB-exposed skin plus pA "UVB+" (Group G3). Results are expressed as U/mg protein and reported as the means ± SD from six independent experiments. * p<0.01.

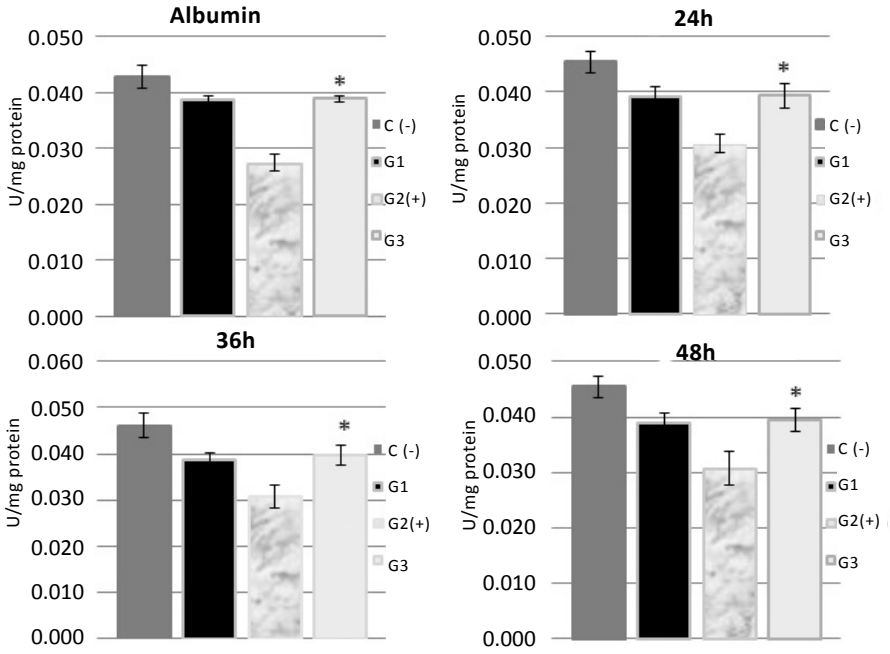

Figure 2: *In vitro* treatment of human skin samples with pA inhibits UVB-induced depletion of superoxide dismutase (SOD) activity. Skin samples were exposed thirty minutes to UVB with or without pretreatment with pA (12%). Non-UVB-exposed skin "n-UVB-"(negative control, C); non- UVB- exposed skin plus pA "n-UVB+"(Group G1); UVB-exposed skin "UVB-"(positive control, Group G2); and UVB-exposed skin plus pA "UVB+" (Group G3). Results are expressed as U/mg protein and reported as the means ± SD from six independent experiments. * p<0.01.

(G2) to UVB decreased GPx ($p<0.05$) compared with non-UVB-exposed skin (C). Treatment (G3) with pA (albumin or hydrolyzated 24, 36 and 48 hr) prevented the UVB-induced depletion of endogenous antioxidant GPx in human skin (Figure 3). These results indicated a significant protective effect of pA against the UVB-induced depletion of antioxidant defense in an *in vitro* model. Moreover, the treatment of human skin samples with pA (G1) alone did not significantly affect the original levels of antioxidant enzymes.

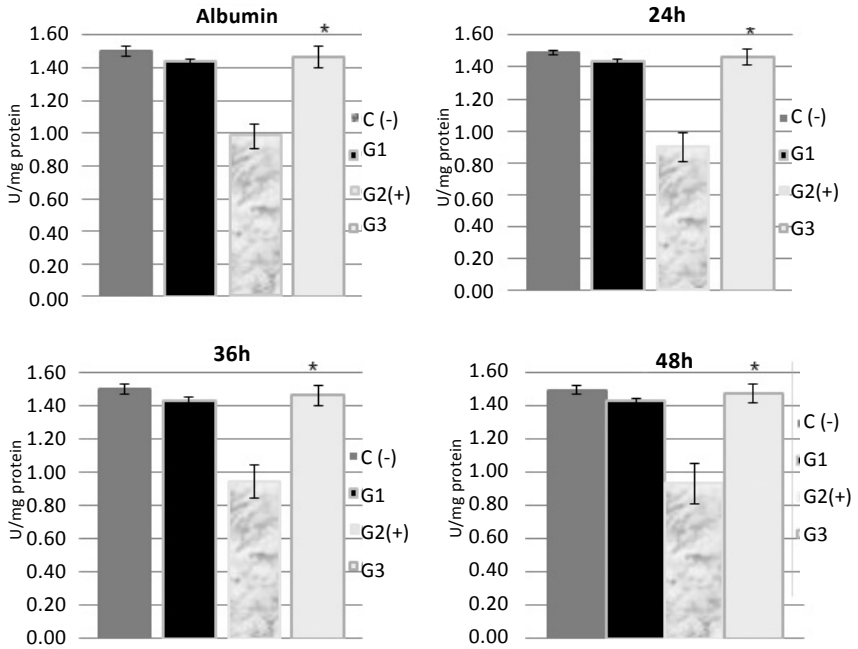

Figure 3: *In vitro* treatment of human skin samples with pA inhibits UVB-induced depletion of glutathione peroxidase (GPX) activity. Skin samples were exposed thirty minutes to UVB with or without pretreatment with pA (12%). Non-UVB-exposed skin "n-UVB-"(negative control, C); non- UVB- exposed skin plus pA "n-UVB+"(Group G1); UVB-exposed skin "UVB-"(positive control, Group G2); and UVB-exposed skin plus pA "UVB+" (Group G3). Results are expressed as U/mg protein and reported as the means ± SD from six independent experiments. * p<0.01.

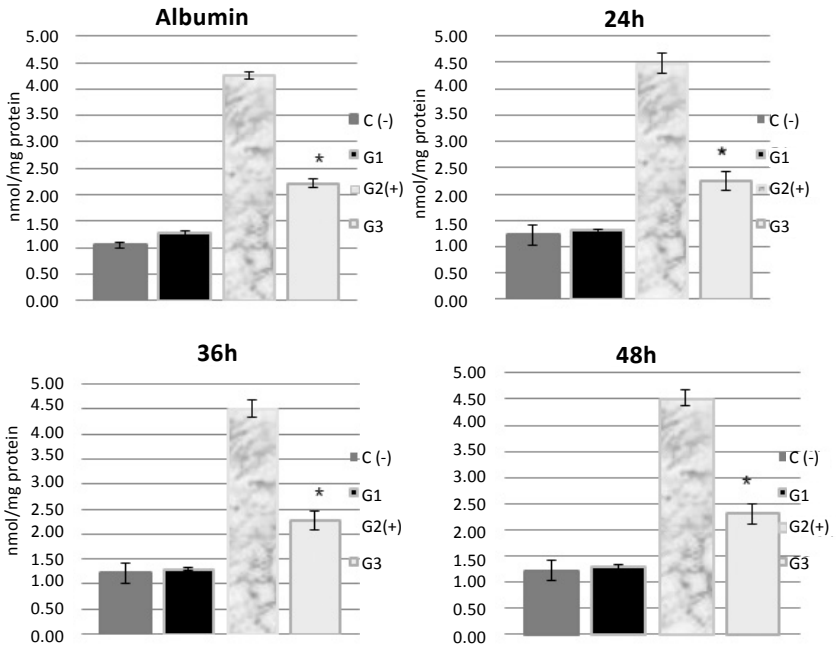

Figure 4: *In vitro* treatment of human skin samples with pA decreased UVB-induced MDA. Skin samples were exposed thirty minutes to UVB with or without pretreatment with pA (12%). Non-UVB-exposed skin "n-UVB-"(negative control, C); non- UVB- exposed skin plus pA "n-UVB+"(Group G1); UVB-exposed skin "UVB-"(positive control, Group G2); and UVB-exposed skin plus pA "UVB+" (Group G3). Results are expressed as nmol MDA/mg protein. Data are presented as the means ± SD from six independent experiments. * p<0.001.

Effect of pA on UVB-induced MDA formation

Figure 4 show that UVB induced the formation of MDA in human skin. The levels of MDA were increased four-fold in the skin exposed to UVB (G2) compared with non-UVB-exposed skin (C). The treatment of skin (G3) with pA (albumin or hydrolyzated) significantly decreased UVB-induced MDA formation. And the treatment of skin samples with pA alone (G1) did not significantly affect the original levels of MDA.

The assessment of supernatant levels of pro-inflammatory cytokines and anti-inflammatory cytokines

The values of the supernatants of pro-inflammatory cytokines IL-1 beta, TNF-alpha, INF-gamma and IL-12 were significantly higher in skin exposed to UVB (G2) than in controls (C) (p=0.006), G1 (skin samples with pA) and G3 (skin samples with pA and more skin exposed to UVB) (Figure 5). Regarding anti-inflammatory cytokines, IL-2, IL-4 and IL-10 in group G1 (p = 0.01) had higher values compared with the control group (C), G2 and G3, (Figure 6).

Discussion

When oxidative stress overwhelms skin antioxidant capacity, the subsequent modification of cellular redox apparatus leads to an alteration of cell homeostasis and to the generation of a degenerative process. These reactive oxygen species cause extensive damage to DNA, proteins and lipids, and it is argued that this damage is a major contributor to aging and to degenerative aging diseases, such as cancer [16,17]. A wide variety of oxidizing molecules, such as ROS, and/or depleting agents can alter the glutathione redox state, a key compound in the regulation of body redox homeostasis. The glutathione redox state is normally maintained by the activity of GSH-depleting (GPx) and -replenishing enzymes (GR). The importance of glutathione and related enzymes and their variation in skin exposed to UVB has thus far been poorly investigated [18]. In this paper, we attempted to further define the protective effect as well as the antioxidant properties of pA using human skin organ culture as an *in vitro* model.

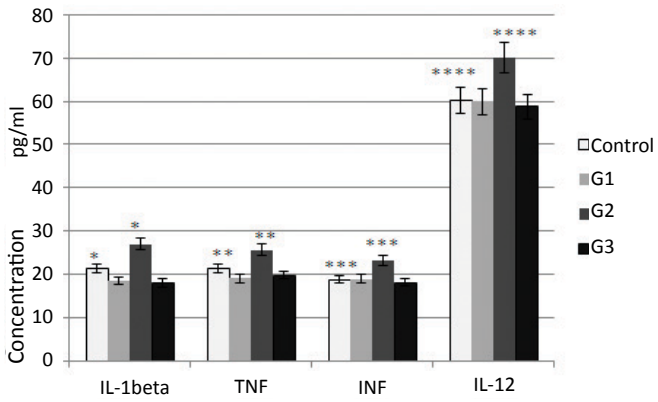

Figure 5: Supernatant levels of cytokines measured by Luminex 100. The values of pro-inflammatory cytokines IL-1 beta, TNF-alpha, INF-gamma and IL-12 were significantly higher in skin exposed to UVB (G2) than in controls (C). Non-UVB-exposed skin "n-UVB-"(negative control, C); non- UVB- exposed skin plus pA "n-UVB+"(Group G1); UVB-exposed skin "UVB-"(positive control, Group G2); and UVB-exposed skin plus pA "UVB+" (Group G3).* p<0.01.

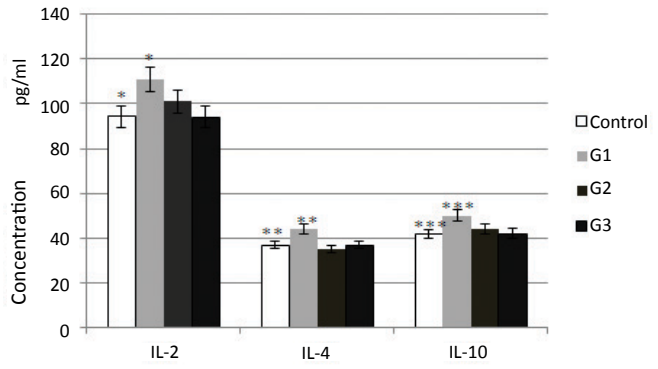

Figure 6: Supernatant levels of cytokines measured by Luminex. The values of anti-inflammatory cytokines, IL-2, IL-4 and IL-10 in group G1 ((p = 0.01) had higher values compared with the control group (C), (G2 and G3). Non-UVB-exposed skin "n-UVB-"(negative control, C); non- UVB- exposed skin plus pA "n-UVB+"(Group G1); UVB-exposed skin "UVB-"(positive control, Group G2); and UVB-exposed skin plus pA "UVB+" (Group G3).* p<0.01.

We postulated that the antioxidants localized in the peptides of Amaranthus could assist in preventing tissue damage and stimulating wound healing. The topical application of antioxidants has been recently suggested as a preventive therapy for skin damage because it protects skin against oxidative injury [19]. These antioxidants protect the UVB-irradiated skin from membrane destabilization and lipid peroxide chain reaction. Glutathione is an intracellular mediator that is critical for cellular defense against reactive intermediates. In addition to its role as a substrate for glutathione-dependent antioxidant enzymes, this thiol tripeptide participates in the regeneration of ascorbate and α-tocopherol and directly detoxifies reactive species via its ability to conjugate with pro-oxidants [20,21].

Muramatsu et al. [22] reported the role of normal skin catalase as a marker of epidermal differentiation and its role in redox damage [22]. The same study verified that catalase expression in the upper epidermal layers decreased 24 hr after exposure to UVB. These studies suggest that UVB impairs endogenous antioxidants and that catalase is one of the most sensitive components of these mechanisms. For this reason, the use of amaranth peptides could help protect the skin. In this study, the *in vitro* treatment of human skin with pA resulted in the prevention of the UVB-induced depletion of antioxidant defense enzymes such as GPX, CAT and SOD, thus providing a possible mechanism for the protection of pA via the reduction of free radical generation.

Our interest was also focused on the ability of antioxidant agents to down regulate the supernatant levels of pro-inflammatory cytokines, which are well known to be involved in the onset of damage to the skin caused by UVB [13]. Regarding this topic, we showed that the levels of pro inflammatory cytokines and particularly IL-1beta, IL-12, INF and TNFα were higher in UVB-exposed skin compared with controls. We also found that the levels of anti-inflammatory cytokines in an *in vitro* model of skin are higher in a skin control with pA, suggesting the regulation of immune factors.

In conclusion, our results indicated that skin irradiation with UVB leads to immediate oxidative stress damage that can be arrested with amaranth grain. Thus, amaranth is a potential food to decrease the risk of human diseases.

Material and Methods

Organ culture

All skin samples (n=6) were obtained from healthy adults (25 to 35 years of age) undergoing abdominal plastic surgery. Immediately after being excised, the explants were cleaned in 70% ethanol for 30 seconds and directly immersed in cold DMEM for transport to the laboratory (maximum 30 minutes). The subcutaneous fat was removed, and samples were cut into fragments of 2 mm^3. Skin specimens were placed with the dermal side down in 2 cm^2-plates with 2.5 ml of serum-free medium DMEM supplemented 200 ug/mL glutamine, 100 U/mL penicillin, and 100 µg /ml streptomycin and stored in a humidified incubator containing 5% CO^2 at 37°C for 72 h. The medium was changed after 24 h. To dissolve the various peptide compounds of *Amaranthus*

in the medium, part of the medium was dissolved in dimethyl sulfide to obtain a concentration of 100 ug/ml (stock solution).

In vitro application of peptides from *Amaranthus* and UVB

We prepared four groups for each sample (the albumin and hydrolyzates at 24, 36 and 48 hr): non-UVB-exposed skin n-UVB-(negative control, C); non- UVB- exposed skin plus pA n-UVB+(Group G1); UVB-exposed skin UVB-(positive control, Group G2); and UVB-exposed skin plus pA UVB+(Group G3). A concentration of 12% peptides (albumin and hydrolyzates) was found to be optimal in previous studies (data not shown). The explants have exposed of 300 J/m^2, thirty minutes before of incubation. After incubation with UV light, skin samples were incubated, 24, 36 and 48 hr. Finally have to obtained, homogenized and subsequently subjected to biochemical evaluation.

Antioxidant systems: catalase, superoxide dismutase and glutathione peroxidase

Skin specimens were homogenized (5% W/v) in 50 mM phosphatase buffer containing 0.1 M EDTA and centrifuged at 250 g for 10 min at 4°C. The supernatant was used for the determination of the enzymatic activity of catalase (CAT, Cayman 707002, and Ann Arbor, Michigan 48108, USA), superoxide dismutase (SOD, Cayman 706002, Ann Arbor, Michigan 48108, USA) and glutathione peroxidase (GPX, Cayman 703102, Ann Arbor, Michigan 48108, USA). The data are expressed as U/mg proteins. The total protein content was quantified as described in the Bradford method. The experiments for antioxidant enzymes were repeated at least three times.

Determination of MDA

Samples prepared for the GPx, CAT and SOD assays were used for the quantification of lipid peroxidation, which measures the amount of MDA obtained from reactions of lipid peroxides with thiobarbituric acid (TBARS, Cayman 10009055, and Ann Arbor, Michigan 48108, USA). The result was expressed as nmol MDA/ mg protein. The experiments for the determination of MDA were repeated as least three times.

Supernatant levels of cytokines

Using a multiplex cytokines kit (IL-1β, IL-2, IL-4, IL-10, IL-12, and IFN-γ), tumor necrosis factor [TNF]-α) was obtained, and the assay was performed in accordance with the manufacturer's instructions (Bio-Rad, Hercules, CA, USA). Briefly, the appropriate cytokine standards and samples (50 µl) were diluted in plasma dilution buffer and were added to wells of a filtered plate. The samples were incubated with 50 µl of the antibody-coupled microsphere set at room temperature for 30 min on a plate shaker (set to 300 rpm) in the dark and filter-washed three times with 100 µl wash buffer. Freshly diluted secondary/detection antibody (25 µl/well) was added to the wells and then incubated at room temperature on a plate shaker for 30 min in the dark and filter-washed three times with 100 µl of wash buffer. Fifty microliters of streptavidin-PE (16 µg/ml in assay buffer) was added to the wells, and incubation at room temperature continued for the first 10 min on a plate shaker. Unbound analytes were filtered through the wells using the vacuum manifold, and the bound beads were washed three times with 100 µl of wash buffer. After the last wash step, 125 µl of assay buffer was added to each well, and the plate was placed for 1 min on a plate shaker set at 500 rpm and then for 3 min at a reduced speed of 300 rpm. Fifty microliters of the sample was analyzed on the Bio-Plex system (Bio-Rad) in accordance with the manufacturer's instructions. Cytokine detection using multiplex bead array assays exhibits high degrees of intra-assay (<10% variation) and inter-assay (10% to 20% variation) precision [15,23]. Cytokine detection via Luminex xMAP technology is comparable to that with an enzyme-linked immunosorbent assay (ELISA; correlation coefficient **r** ranges from 0.75 to 0.99) [24].

Statistics

Statistical analysis was performed using the t-test, ANOVA and Tukey test. The protective effect of pA was considered significant if $p<0.05$. The results are presented as the means ± SD.

Conflict of Interest

The authors state no conflict of interest.

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