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Immune responses induced by *Pelargonium sidoides* extract in serum and nasal mucosa of athletes after exhaustive exercise: Modulation of secretory IgA, IL-6 and IL-15

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ABSTRACT

The evidence that exhaustive exercise may compromise the immune response is mainly confirmed by upper respiratory tract infections which are probably related to the decrease in secretory immunoglobulin A in the upper airway mucosa and/or profile changes of systemic cytokines as well as local cytokines of the upper respiratory tract. An extract from *Pelargonium sidoides* roots is currently used to treat infections in the upper airways. The aim of the present study was to evaluate the action of this herbal medicine on the immune response of athletes submitted to an intense running session by analyzing the production of immunoglobulin A in their saliva and of cytokines both locally and systemically, using a placebo as control. The results show that *Pelargonium sidoides* extract modulates the production of secretory immunoglobulin A in saliva, both interleukin-15 and interleukin-6 in serum, and interleukin-15 in the nasal mucosa. Secretory immunoglobulin A levels were increased, while levels of IL-15 and IL-6 were decreased. Based on this evidence, we suggest that this herbal medicine can exert a strong modulating influence on the immune response associated with the upper airway mucosa in athletes submitted to intense physical activity.

Introduction

The main evidence that exhaustive exercise can impair immune response comes from the increased risk for upper respiratory tract infections (URTIs) in athletes performing this level of exercise (Nieman 2000). Decreased concentrations of secretory immunoglobulin A (sIgA) have been observed as a result of reduced synthesis and accelerated degradation of this immunoglobulin (Mackinnon and Hooper 1994). In addition, altered systemic cytokine profiles and local epithelial alterations caused by cold dry air can lead to changes in athletes' respiratory tracts (Gleeson 2000).

The "open window" and "J curve" theories (Nieman 2000) explain the increased incidence for upper respiratory tract infections in athletes after exhaustive exercise. However, in human studies, the lack of control over variables such as previous infections, exposure to pathogens and stress factors, probably accounts for the fact that the mechanisms by which URTIs develop in endurance athletes have not yet been clarified (Malm 2004). Recent studies by Cox et al. (2008) propose that allergy, asthma and respiratory tract inflammation might also be responsible for such alterations, which are sometimes shorter than cold or flu episodes (Bermon 2007). Furthermore, several studies have shown an increase in the number of airway inflammatory cells in athletes performing different types of sports, either at rest, as well as after exercise (Bonsignore et al. 2003).

The role played by cytokines in athletes with URTIs is still unknown. Cox et al. (2007) showed that URTI-prone athletes had low absolute plasma concentrations of interleukin (IL)-10, IL-1ra and IL-8 at rest and increased IL-6 concentrations post-exercise, suggesting an inadequate control of inflammation.

An extract from *Pelagonium sidoides* roots, a plant species with anti-infective properties used in South African folk medicine to treat different diseases, has also been administered for the treatment of acute or chronic upper respiratory tract infections. Clinical studies have shown that this herbal medicine possesses antibacterial, antiviral and immunomodulatory properties and that it acts

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on the respiratory tract mucosa (Bachert et al. 2009; Conrad et al. 2007).

The objective of the present study was to evaluate the possible immunomodulatory effect of *Pelargonium sidoides* after a high-intensity running session. The results obtained show that this herbal medicine modulates the production of serum IL-15 and IL-6, intracellular IL-15 in nasal mucosa and salivary sIgA in athletes and it is potentially able to prevent upper respiratory illness in athletes.

Materials and methods

Pelargonium sidoides extract and placebo composition

Each 1 ml of the herbal medicine preparation contained 825 mg of a fluid ethanolic extract from root of *Pelargonium sidoides* [(1:9–11), standardized for a content of 0.08–0.32% of total phenols] in a solution with glycerol 85% and 11% (w/w) ethanol. The placebo was prepared with ethyl alcohol, glycerin, methylparaben, propylbaraben, caramel dying and purified water. It was similar to the verum with respect to color, smell, taste and consistence.

Chemical characterization of Pelargonium sidoides extract and placebo by LC–MS

For chemical characterization, 1.2 ml of the herbal medicine or placebo was dried in a vacuum at a pressure of 10^{-3} mbar and at a temperature of 65 °C (SC110 SpeedVac® Concentrator, Savant, NY, USA), until reaching the volume of 150 μl. To this sample 100 μl of aqueous methanol (80/20, MeOH/H_2O, v/v) was added and 60 μl of this solution was used for LC/ESI-MS analysis.

LC/ESI-MS data was obtained on a Micromass instrument, model ZMD (Waters Corporations, Milford, MA) coupled to a Waters Alliance model 2690 LC system using a Waters Nova-Pak C_{18} column (2.2 mm × 150 mm, 3.5 μm particle size, 60 Å pore size) at a flow rate of 0.3 ml/min and constant temperature of 25 °C. The liquid chromatographic step was done as described by Schnitzler et al. (2008). We used as eluent A: 0.1% aqueous TFA solution and as eluent B: 0.1% TFA in CH_3CN/H_2O (90:10, v:v). Starting at 100% A for 5 min, a gradient was followed to 85% A at 60 min, 80% A at 75 min and 70% at 105 min, and 0% A at 110 min before re-equilibration to the starting conditions. Absorbency was measured between 191 and 400 nm using a Waters photodiode array model 996. Mass measurements were performed in a positive mode in the following conditions: mass range between 50 and 1000 *m/z*; nitrogen gas flow: 4.1 l/h; capillary: 2.5 kV; cone voltage: 47 V; extractor: 8 V; source heater: 100 °C; solvent heater: 400 °C; ion energy: 1.0 V and multiplier: 996 V.

Subjects

Twenty-five volunteer male marathon runners (mean age 40.4 ± 7.9 years; range 26–56) living in the city of São Paulo were recruited into this double-blind study. All the subjects were informed of the risks involved in the study and provided their written consent for the procedure before participation. Both the protocol and the informed consent forms were approved by the Federal University of São Paulo Research Ethics Committee. Subjects presenting chronic allergic conditions, or undergoing any pharmacological treatment were excluded. The athletes were randomly separated into two groups: placebo (14 subjects) and herbal medicine (11 subjects).

Treatment

The protocol was designed to evaluate the effect of *Pelargonium sidoides* extract on the modulation of the immune response in marathon runners, using a placebo for comparison. The treatment schedule consisted in the ingestion of herbal medicine or placebo (3 × 30 drops/day of a solution of 80 g of extract/100 ml in solution), medicine or placebo (3 × 30 drops/day), for 28 consecutive days. The treatment was withdrawn immediately if any adverse effects were observed.

Physical activity and running

All subjects maintained their routine levels of physical activity pre- and post-training. Maximal aerobic capacity (VO_2max) tests were performed 30 days before the intense running session on a digital computer-based exercise system (CardioO_2 System: Medical Graphics Corporation) breath-by-breath analysis of metabolic, ventilator and cardiovascular variables (Department of Pneumology, Federal University of São Paulo, Brazil). The rate of power increment was selected to provide exercise duration of >8 but <12 min. All subjects were submitted to a high-intensity running session (approximately to 85% of VO_2max) after 28 days of treatment.

Sample collection

Fasting blood, saliva and upper respiratory tract cell samples were collected in the morning before the treatment and 48 h after the run. Blood samples were collected from a peripheral vein and placed in appropriate tubes for serum separation. After blood clotting, tubes were centrifuged at 2500 rpm for 10 min to obtain 500 μl of serum, which was stored at −80 °C for later use to determine cytokine concentration.

Saliva samples were collected directly into sterile 15 ml Falcon® tubes (without either previous stimulation or use of any collection material), refrigerated at 4 °C for 15 min and then centrifuged at 3000 rpm for 5 min. A total of 400 μl of the supernatant was stored at −80 °C without any buffer or preserving compound in 1.5 ml Eppendorf tubes for later use to determine secretory immunoglobulin A concentrations. Samples containing blood were discarded and collected again.

Nasal cell samples (representative of the upper respiratory tract) were collected by a swab, placed in sterile 15 ml Falcon® tubes containing 4 ml of sterile RPMI 1640 medium (pH 7.3) and kept at 4 °C. After 30–45 s of intense agitation on a Vortex mixer to detach them from the swab, the cells were centrifuged at 1400 rpm for 4 min. The supernatant was discarded, and 300 μl of a lysing solution [25 μl Tris–HCl 1 M, 10 μl NaCl 0.5 M, 50 μl NaF 0.5 M, 5 μl $NaVo_4$ 0.1 M, 25 μl NP40 10%, 5 μl PMSF 0.1 M, 0.5 μl aprotinin (10 mg/ml), 0.5 μl leupeptin (5 mg/ml) and 379 μl H_2O] was added to the pellet. The reaction was maintained at 4 °C for 15 min and then centrifuged at 1400 rpm for 15 min. A total of 200 μl of the supernatant was collected and stored at −80 °C for later analysis of intracellular cytokines.

Determination of secretory immunoglobulin A (sIgA) in saliva

Salivary sIgA concentration was determined by enzyme-linked immunosorbent assay (ELISA) (Salimetrics LLC, State College, Pennsylvania, USA) following the manufacturer's instructions. Standards, negative and positive controls were prepared following the manufacturer's instructions.

Determination of cytokine concentrations

The concentrations of TNF-α, IL-15, IL-10, IL-6, IL-8, IL-4 and IL-1β in serum and nasal cell extracts that had previously been kept at −80 °C were determined using the Human Luminex bead-based kit with the LINCOplex simultaneous multianalyte detection system (Linco Research, Inc., St. Charles, MO, USA) and standards, negative

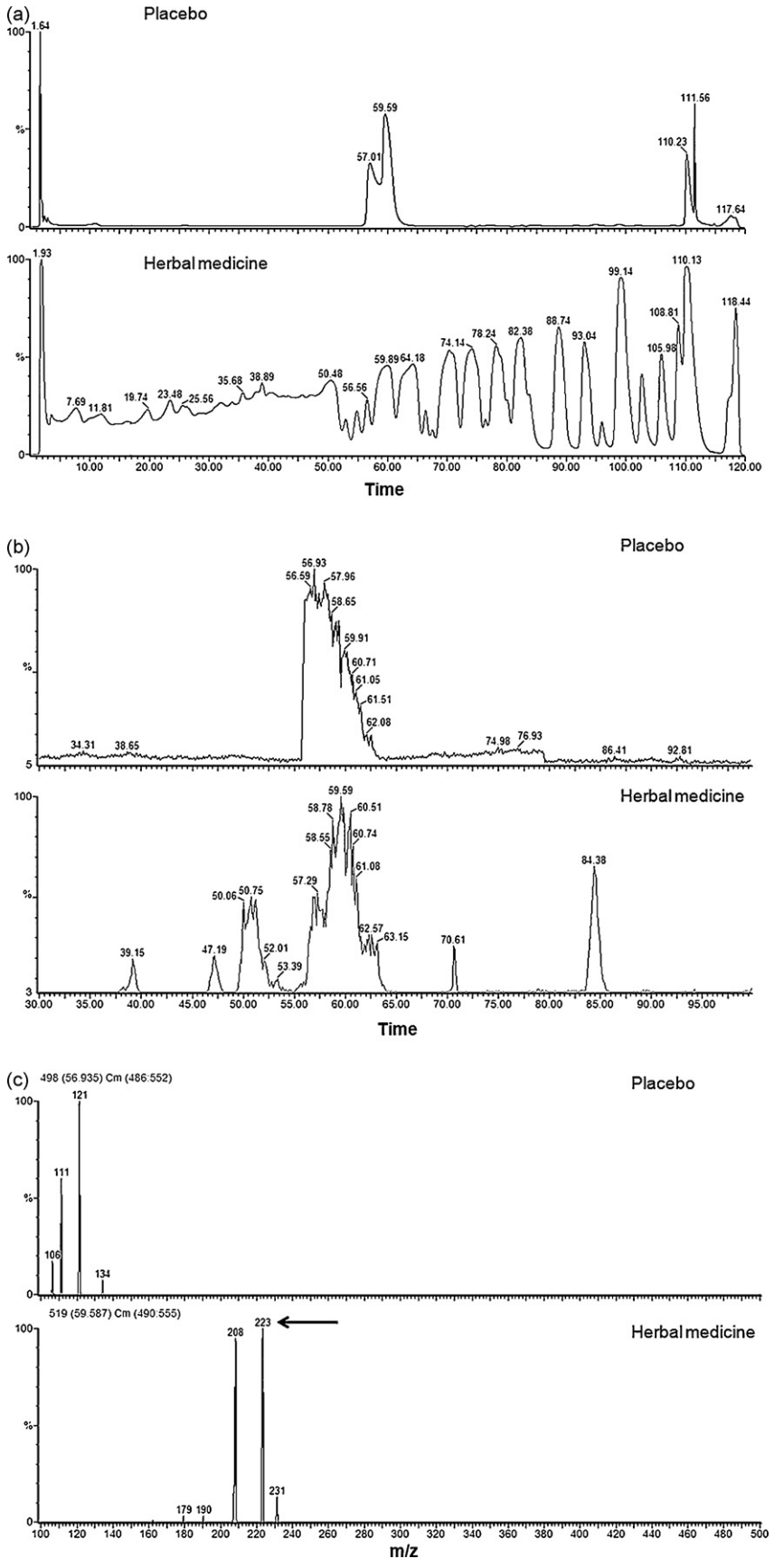

Fig. 1. HPLC chromatogram (A) and electrospray ionization-mass spectrometry (B and C) of herbal medicine and placebo.

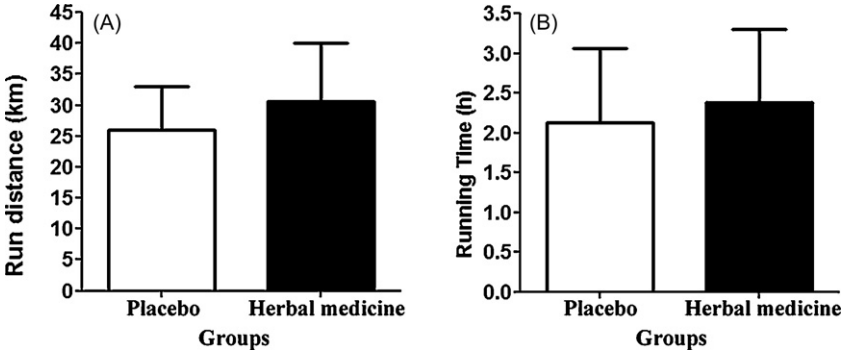

Fig. 2. Means ± SD of run distance (km) and running time (h) of the athletes (placebo and herbal medicine) in running session. Values do not differ significantly between placebo and herbal medicine groups.

Table 1
Values and significance level (p) of concentrations (expressed in medians and interquartile range) of secretory salivary immunoglobulin A (sIgA), serum IL-6 and IL-15, relation of nasal/serum IL-15 and the relation between Pre- and Post-treatment concentration in the placebo and herbal medicine groups.

Parameters analyzed	Groups	Pre-treatment[a]	Post-exhaustive running session[a]	Relation of Pre- and Post-treatment concentration[a]	Significant difference (p)
Salivary sIgA (μg/ml)	Placebo	241.1 (151.5–592.9)	96.55 (52.33–371.1)	41.16 (16.06–85.28)	<0.001
	Herbal medicine	132.3 (67.01–229.5)	389.9 (203.6–648.7)	213 (29.2–1436)	
Serum IL-6 (pg/ml)	Placebo	12.85 (3.2–106)	17.88 (3.2–194.4)	103.6 (100–122.2)	<0.05
	Herbal medicine	34.45 (5.44–154)	29.02 (5.35–116.5)	82.47 (63.67–100)	
Serum IL-15 (pg/ml)	Placebo	3.2 (3.2–23.2)	6.88 (3.2–35.23)	104.9 (100–130.5)	<0.02
	Herbal medicine	9.95 (3.2–110.5)	9.92 (3.2–115.1)	94.62 (78.59–100)	
Relation of Nasal/Serum IL-15 (pg/ml)	Placebo	3.39 (3.2–13.25)	3.78 (3.2–8.35)	122.8 (91.85–231.8)	<0.05
	Herbal medicine	5.70 (3.2–16.9)	3.4 (3.2–12.87)	90.18 (69.64–178.1)	

[a] Data are expressed as median (interquartile range).

and positive controls were prepared following the manufacturer's instructions.

Statistical analysis

Distance run and running times are shown as mean ± SD. Data regarding secretory immunoglobulin A in saliva and cytokines concentrations are shown as medians (interquartile range) and represents the relation between Pre- and Post-treatment concentration [(Post/Pre) × 100]. The Mann–Whitney test was used to determine if the differences between the two groups were significant. The significance level was set to 5% ($p < 0.05$).

Results and discussion

The chemical characterization of *Pelargonium sidoides* extract and placebo used in this study was performed by HPLC chromatography (Fig. 1A) and electrospray ionization-mass spectrometry (EIS-MS) (Fig. 1B and C) in order to identify their major compounds. Schnitzler et al. (2008) found in a analysis by LC/MS the mono-hydroxy-dimethoxy-coumarin (most probably Umckalin = 7-hydroxy-5,6-dimethoxycumarin) which is a coumarin compound presenting a mass of 223 (m/z). In our analysis the same compound was obtained only for the herbal medicine at 59.6 min and not for the placebo (Fig. 1C).

As respiratory tract symptomatology is associated not only with infectious processes but also with inflammatory (Bermon 2007) and allergic diseases (Gleeson et al. 2008), the need to understand the causes of upper respiratory illness in athletes (Spence et al. 2007) and to find ways of protecting them from this condition reached a new significance. It is clear that the effective protection of the athlete must consider allergic/inflammatory and infectious processes. Our results support the use of *Pelargonium sidoides* extract as a treatment of these conditions with no adverse effect displayed by the athletes during the study no participant withdrawn from the study for any reason. The mean distance run and mean running time did not differ significantly between athletes in the placebo group (25.92 ± 7.18 km and 2.13 ± 0.93 h, respectively) and those in the herbal medicine group (30.64 ± 9.41 km and 2.38 ± 0.93 h) (Fig. 2).

The relative increase in salivary sIgA levels after an exhaustive running session in the group of athletes who were treated with the herbal medicine [213 (129.2–1436) μg/ml] compared with those in the placebo group [41.16 (16.06–85.28) μg/ml] was remarkable (Table 1 and Fig. 3). Even though reduced sIgA concentration

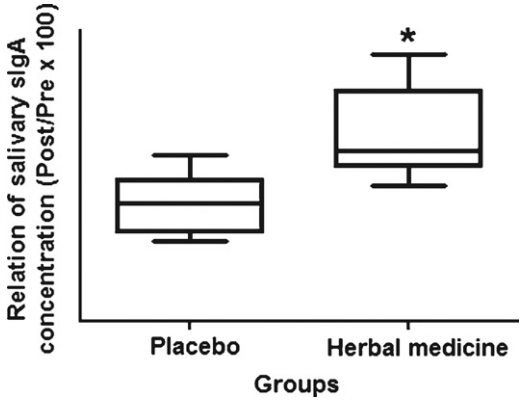

Fig. 3. Relation between Pre- and Post-treatment concentration of salivary sIgA [(Post/Pre) × 100] in placebo or herbal medicine groups (μg/ml). Pre = before treatment and Post = 48 h after high-intensity running session. The box represent the medians (interquartile range) and whiskers represent the minimum and maximum of all data with a significance level of $^{*}p < 0.001$.

in athletes submitted to exercise has been reported for almost two decades (Mackinnon and Hooper 1994), its cause and the role played in triggering respiratory tract symptoms are still not clear.

Secretory immunoglobulin A (sIgA) is secreted at mucosa, where it plays an important defensive role against invading microorganisms. This is achieved by different mechanisms, such as sIgA binding to the pathogen to prevent the mucosa invasion, neutralization of viruses by complexing with viral proteins, and deposition of immunocomplexes in the submucosa, which are excreted through the epithelium into the lumen and then eliminated. Exercise influences sIgA levels significantly, with lower concentration of this immunoglobulin in runners after heavy training. Also, it has been shown that periods of intense training and competitions alter the mucosal immune response (Gleeson 2000; Mackinnon and Hooper 1994).

Recent studies suggest that activation of an allergic/inflammatory response by mechanisms other than infection is responsible for upper respiratory illness in athletes. After a 5-month surveillance of athletes presenting two or more defined symptoms of URTI, Spence et al. reported that only 30% of the episodes had been caused by a respiratory pathogen (Spence et al. 2007). In agreement with these findings, clinical data showed that 55% of athletes followed up presented URTI, but only 27% of them had infections. In another clinical study, only 30% of URTI symptoms in athletes were related to some infectious agent (Gleeson et al. 2008).

Spence et al. (2007) also observed that the mean duration of infectious URTI episodes during the 5-month surveillance was 50% higher than the mean duration of episodes of upper respiratory illness with no infectious agent identified. These findings suggest that alterations in the respiratory tract mucosa might be caused by inflammatory or allergic reactions, inhalation of dry, cold air, and/or vasomotor phenomena.

Allergic diseases are aberrant inflammatory reactions to innocuous environmental antigens elicited in susceptible individuals as a result of an imbalance between allergen activation of regulatory T cells and effector T helper 2 cells. Dendritic cells are key players in this process and are influenced by cytokines such as IL-6, IL-21 and IL-25 (Montero Vega 2006). Interleukin-15 has also been shown to play an important role in the induction of the allergic process. Blocking of IL-15 suppresses the induction of differentiation in antigen-specific Th2 T cells, thus preventing the trigger of allergic response (Ruckert et al. 2005).

In our study the extract of *Pelargonium sidoides* altered the synthesis in serum and nasal epithelium of IL-6 and IL-15 which are essential for triggering the allergic process. The production of serum IL-6 and IL-15 at rest and after exercise in both groups is presented in Table 1. The analysis of relative IL-6 concentration in the group treated with the herbal extract [82.47 (63.67–100) pg/ml] was significantly lower when compared to the placebo group [103.6 (100–122.2) pg/ml] (Table 1 and Fig. 4A). Likewise, relative sera concentration of IL-15 after exercise in the *Pelargonium sidoides* group [94.62 (78.59–100) pg/ml] was significantly lower than those in placebo group [104.9 (100–130.5) pg/ml)] (Table 1 and Fig. 4B). Reduced production of IL-6 and IL-15 may attenuate allergic/inflammatory response by interfering with the regulation and differentiation of effector Th2 cells. In addition to the modulation caused by *Pelargonium sidoides* in serum cytokine production (Table 1), we also observed a significant reduction in the relative concentration of IL-15 obtained from nasal epithelium cells in the group treated with the herbal medicine [90.18 (69.64–178.1) pg/ml] in comparison with the placebo group [122.8 (91.85–231.8) pg/ml] (Table 1 and Fig. 4C). The relative concentration of the cytokine represents the difference between serum and intracellular cytokine concentrations evaluated after lysis of

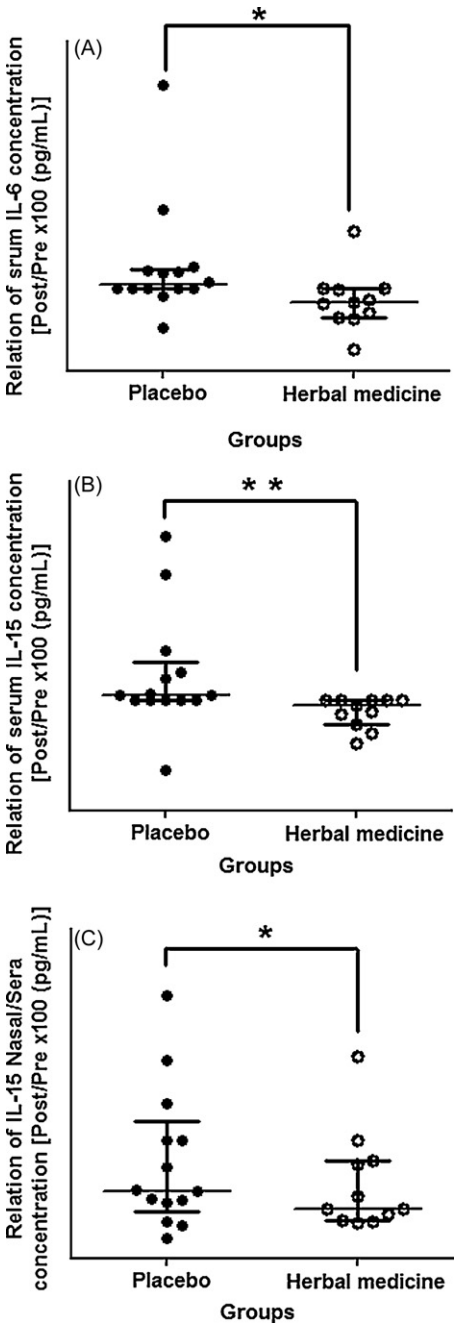

Fig. 4. Relation between Pre- and Post-treatment concentration of serum IL-6 (A) and IL-15 (B) [(Post/Pre) × 100] in placebo or herbal medicine groups (pg/ml). (C) IL-15 ratio of nasal cell-derived cytokine concentration [(Post/Pre) × 100] to serum cytokine concentration [(Post/Pre) × 100] in placebo or herbal medicine groups (pg/ml). Pre = before treatment and Post = 48 h after high-intensity running session. Data are presented as medians (interquartile range) with a significance level of $^{*}p < 0.05$ or $^{**}p < 0.02$.

nasal epithelium cells. This result suggests that the extract could also reduce IL-15 production by epithelial cells from the nasal mucosa and thus contribute to the blockade of the allergic process. No significant differences between the two groups were observed in terms of relative serum and nasal concentrations for the other cytokines evaluated (TNF-α, IL-1β, IL-4, IL-10 and IL-8) (Fig. 5).

Interleukin-15 modulates both T and NK cell-mediated inflammatory responses, probably by sharing many functional activities of IL-2, whose production and secretion is induced by several pro-inflammatory cytokines such as IL-1β, TNF-α and IFN-γ. IL-15

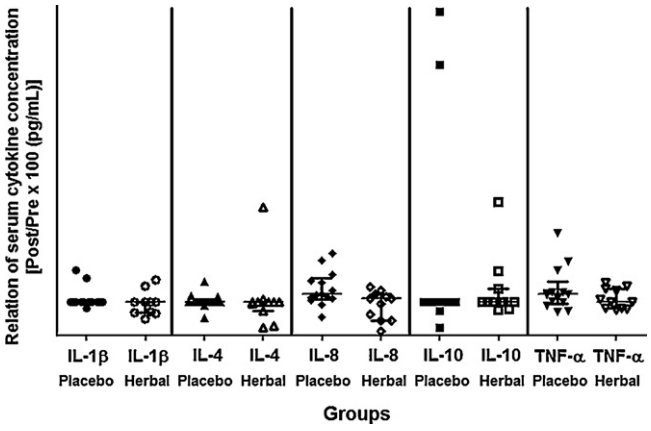

Fig. 5. Relation between Pre- and Post-treatment concentration of IL-1β, IL-4, IL-8, IL-10 and TNF-α in serum [(Post/Pre) × 100] in placebo or herbal medicine groups (pg/ml). Pre = before treatment and Post = 48 h after high-intensity running session. Values do not differ significantly between placebo and herbal medicine groups.

mRNA is constitutively expressed by respiratory tract epithelial cells, in which IFN-γ, but not other cytokines, induces synthesis and secretion of IL-15 protein. Interleukin-15 receptors IL-15Rα and IL-2Rβ are also constitutively expressed, so these cells may contribute to T and NK cell-mediated respiratory tract inflammation by producing IL-15 (Ge et al. 2004).

It has also been shown that IFN-γ can induce expression of IL-15 and its receptor on dendritic cells. Ohteki et al. (2006) showed that dendritic cell-derived IL-15 plays an important role as a mediator of inflammatory responses *in vivo*.

Saikh et al. (2001) showed that IL-15 induces differentiation of monocytes into functional dendritic cells in response to inflammatory stimuli. In agreement, Regamey et al. (2007) observed that IL-15 derived from airway epithelial cells induces maturation of monocytes and differentiation of these cells into dendritic cells, whereas a neutralizing monoclonal antibody to IL-15 blocked the differentiation. This information is remarkably important given the essential role of dendritic cells in the regulation of allergic processes.

Min and Lee (2000) and Message and Johnston (2004) showed the importance of cytokines, particularly IL-6 in recruiting inflammatory cells to the airway mucosa during acute infectious processes or allergy-related rhinosinusitis. Tavian et al. (2008) showed that IL-15, as well as IL-6, play an important role in the activation of neutrophils. Verri et al. (2007) demonstrated a fundamental role for IL-15 in neutrophil migration to inflammatory sites. Blocking this cytokine can hinder inflammatory processes, especially in the respiratory tract.

We therefore propose that *Pelargonium sidoides* extract acts as a modulator of upper respiratory tract-associated immunological responses in athletes submitted to heavy exercise by increasing production of salivary sIgA and reducing production of cytokines such as IL-6 and IL-15. This effect could modulate allergic and/or inflammatory responses, as well as activation and migration of neutrophils, monocytes and dendritic cells. However, studies with a higher number of athletes practicing different modalities of sports are needed to confirm the laboratory findings obtained in this study using *Pelargonium sidoides* extract.

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