

## Wrestlers' immune cells produce higher interleukin-6 and lower interleukin-12 and interleukin-13 in response to *in vitro* mitogen activation

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### ABSTRACT

**Objective(s):** Although recent investigations have shown chronic inflammation and inflammation-associated diseases might be ameliorated by exercise; little is known about the relation between exercise training with anti/pro-inflammatory cytokines.

**Materials and Methods:** This cross sectional study was conducted to compare interleukin-4 (IL-4), IL-6, IL-10, IL-12, IL-13, interferon gamma (IFN- $\gamma$ ) levels in serum, and their *in vitro* production by whole blood (WB) cells and by peripheral blood mononuclear cells (PBMCs) in response to mitogens lipopolysaccharide and phytohemagglutinin. Twelve elite wrestlers with history of three times per week exercise training for about 9.5 years, and thirteen healthy silent controls were recruited. To analysis the cytokines by enzyme linked immunosorbent assay (ELISA), the blood samples were taken 24 hr after the last training session from the wrestlers.

**Results:** Serum analysis for IL-4, IL-6, IL-10, IL-12, IL-13 and IFN- $\gamma$  indicated no statistical difference between the two groups. Meanwhile, 48 hr *in vitro* activation of WB and PBMCs by the mitogens revealed that IL-6 production was elevated in both WB and PBMCs. Whereas, IL-12 and IL-13 were decreased in supernatant of PBMCs and WB cells cultures, respectively.

**Conclusion:** It seems that wrestling cause immune system cells to produce anti-inflammatory myokine IL-6 and decrease production of pro-inflammatory cytokine IL-12 and IL-13.

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## Introduction

It is well known that chronic inflammation is involved in pathogenesis of many chronic diseases such as insulin-resistance diabetes, vascular diseases, atherosclerosis, neurodegeneration and cancers (1, 2). As well as, recent investigations suggest that these chronic inflammation-related diseases may be postponed or ameliorated with regular exercise. Exercise contributes to decline in the fatty tissues which cause a decrease in the release of pro-inflammatory adipokines (3, 4). In addition, exercise and muscles contraction cause to release some myokines, cytokines produced by muscle fibers, which have anti-inflammatory effects (5, 6).

Therefore, the anti-inflammatory effect of regular exercise may be achieved by several underlying mechanisms, including reduction of pro-inflammatory adipokines production from fatty tissues, increase of anti-inflammatory myokines release from the muscles

and stimulation of immune cells to produce anti-inflammatory cytokines. Due to some controversies about the nature of cytokines released from immune cells in exercise training, it seems need to be more investigated (3, 7, 8).

Recent data also indicate that interleukin-4, IL-6, IL-10, IL-12, IL-13 and gamma interferon (IFN- $\gamma$ ) may play an important role in inflammation (9-11). According to the literature although most researches have been dealing with the effect of acute exercise on the cytokine production in the serum, little is known about the relationship between chronic or professional exercise and capacity of the immune cells to produce anti/pro-inflammatory cytokines during rest state especially when they were stimulated by mitogens (12-15).

Thus, releases of these cytokines in the sera of the elite wrestler in comparison with silent controls were assayed. In addition, we focused on the

capability of the whole blood (WB) culture, peripheral blood mononuclear cells (PBMCs) for the production of IL-4, 6, 10, 12, 13 and IFN- $\gamma$  when activated by mitogens.

## Materials and Methods

### Study groups

This cross sectional study was conducted in Hamadan University of Medical Sciences, Iran. Ethical approve was achieved from Ethics Committee of Hamadan University of Medical Sciences and all the participants signed informed written consents. Fifteen elite free style wrestlers, who were studying in Azad University of Hamadan, were requested to fill in a questionnaire for identifying their demographic characteristics such as age, sex, past medical history and details related. Twelve out of fifteen wrestlers were chosen with a history of three times per week exercise training (one hr and half weight lifting) for more than 9.5 years. Also simultaneously, 13 age-sexes matched apparently healthy unrelated controls that were in the sedentary state for more than six months were recruited from the same university. The wrestlers and the controls didn't consume any drugs within the past two weeks before the experiments.

### Blood collection

Ten ml sterile blood sample was taken from each wrestler at 24 hr after their last training session, and the controls. The bloods were collected in two bottles, one containing ethylenediamine tetraacetic acid (EDTA) as an anticoagulant and other without anticoagulant. The sera were isolated and frozen at -80°C for ELISA analysis and anticoagulant blood used to culture whole blood and PBMCs.

### In vitro production of cytokines by the whole blood culture activated with mitogens

To determine *in vitro* production of cytokines, one ml of fresh blood containing anticoagulant was suspended in one ml complete RPMI1640 medium (Gibco-BRL, Australia) containing 100 U/ml penicillin G (Hayan, Iran), 10% fetal calf serum (FCS) (Gibco-BRL, Australia), 100  $\mu$ g/ml streptomycin (Hayan, Iran) and 5  $\mu$ g/ml phytohemagglutinin (PHA) (Sigma, Germany), 25  $\mu$ g/ml lipopolysaccharides (LPS) (Sigma, Germany), and incubated for 48 hr in a CO<sub>2</sub> incubator at 37°C. Thereafter, the supernatants were collected and frozen at -80°C until cytokine measurements (11, 16).

### In vitro production of cytokines by PBMCs culture activated with mitogens

Briefly, four ml of fresh blood containing anticoagulant was diluted with 8 ml Hank's solution. PBMCs were isolated by Ficoll-Paque and washed twice with Hank's solution.  $7 \times 10^5$  cells were cultured as monolayer culture in 1ml

RPMI1640 medium, supplemented with materials like for the whole blood culture and incubated for 48 hr in a CO<sub>2</sub> incubator at 37°C. The supernatants were collected and frozen at -80°C until cytokine measurements (16).

### Cytokine measurement by ELISA

Serum levels of Cytokines as well as supernatant levels of these cytokines were determined by sandwich enzyme-linked immunosorbent assays according to the manufacturer's instructions (ID Lab, London, Canada). All assays were carried out in duplicate.

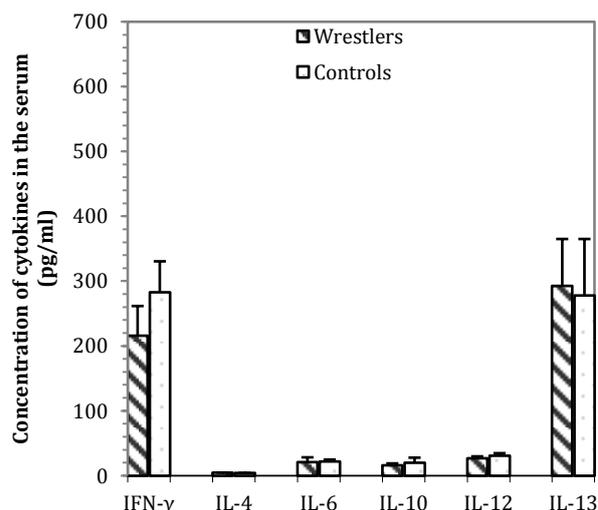
### Statistical analysis

Results were expressed as mean  $\pm$  standard error. Unpaired Student's t-test was used to compare the means of the examined groups. All comparisons were two-sided with *P*-values that are noted for each group to show statistical significance.

## Results

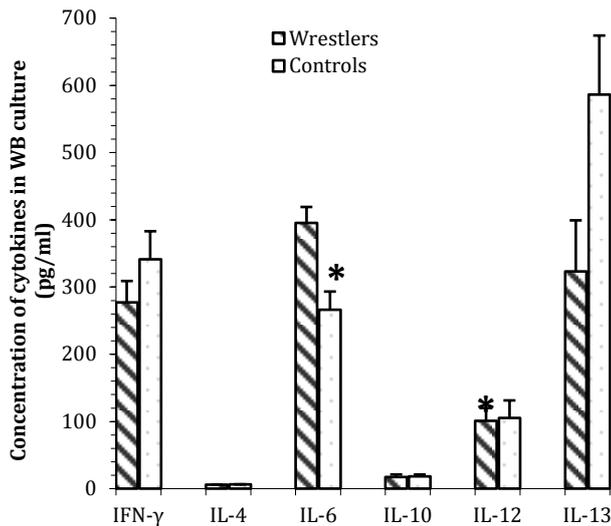
Twelve professional wrestlers and thirteen silent controls were enrolled in this study and the data in Table 1 show the main demographic characteristics of the study populations. There were no differences between ages and weights of the two groups.

Analysis of the sera for cytokines, IL-4, IL-6, IL-10, IL-12, IL-13 and IFN- $\gamma$  levels showed there were no significant differences between IL-4 ( $4.98 \pm 0.6$ ,  $4.3 \pm 0.03$ ,  $P=0.325$ ), IL-6 ( $21 \pm 7.5$ ,  $22 \pm 2.8$ ,  $P=0.905$ ), IL-10 ( $16 \pm 3$ ,  $20 \pm 8$ ,  $P=0.586$ ), IL-12 ( $27 \pm 3$ ,  $31 \pm 4$ ,  $P=0.348$ ), IL-13 ( $293 \pm 72$ ,  $278 \pm 87$ ,  $P=0.892$ ) and IFN- $\gamma$  ( $216 \pm 46$ ,  $283 \pm 48$ ,  $P=0.334$ ) in the wrestlers and the controls respectively (Figure 1).



**Figure 1.** Production of IL-4, IL-6, IL-10, IL-12, IL-13 and IFN- $\gamma$  in serum of the wrestler and control groups

The blood collections were performed 24 hr after the last training session in the wrestlers and simultaneously in the controls. The results of ELISA were compared by unpaired student's *t*-test in the two groups. Productions of IL-4, IL-6, IL-10, IL-12, IL-13 and IFN- $\gamma$  in the serum were not significantly difference



**Figure 2.** Production of IL-4, IL-6, IL-10, IL-12, IL-13 and IFN- $\gamma$  in WB culture of the wrestler and control groups

One ml whole blood from each participant was cultured in one ml RPMI in the presence of mitogens PHA (5  $\mu$ g/ml) and LPS 25  $\mu$ g/ml for 48 hr. The supernatant of each whole blood culture was assayed for IL-4, IL-6, IL-10, IL-12, IL-13 and IFN- $\gamma$ . Final results for each cytokine level in the wrestlers and controls showed that *in vitro* production of IL-6 increased in the wrestlers (395±24 pg/ml) in comparison with IL-6 in the controls (266±27 pg/ml,  $P=0.001$ ). Whereas IL-13 in the wrestlers was lower (323±76 pg/ml) than IL-13 in the controls (586±88 pg/ml,  $P=0.004$ ). All ELISA tests were performed duplicated

*In vitro* activation of WB cells by PHA and LPS increased production of IL-6 to 395±24 pg/ml and decreased the release of IL-13 to 323±76 pg/ml in the wrestlers compared with IL-6 (266±27,  $P=0.001$ ) and IL-13 (586±88 pg/ml,  $P=0.004$ ) levels in the controls, respectively. Meanwhile, the levels of IL-4 (6±0.2, 6.3±0.3,  $P=0.505$ ), IL-10 (17±4, 18±3,  $P=0.749$ ), IL-12 (101±19, 105±26,  $P=0.900$ ) and IFN- $\gamma$  (277±32, 341±42,  $P=0.226$ ) were not different in the wrestlers and the controls (Figure 2).

According to the results in Figure 3, the level of IL-6 (494±77) in the culture supernatant of mitogens activated PBMCs of the wrestlers significantly ( $P=0.028$ ) increased compared with the controls (353±89). In contrast, the level of IL-12 (85±14) significantly ( $P=0.038$ ) decreased in the supernatant of mitogens activated PBMCs of wrestlers in comparison with the level of IL-12 (266±81) in the controls. The levels of other cytokines IL-4 (7.9±0.8),

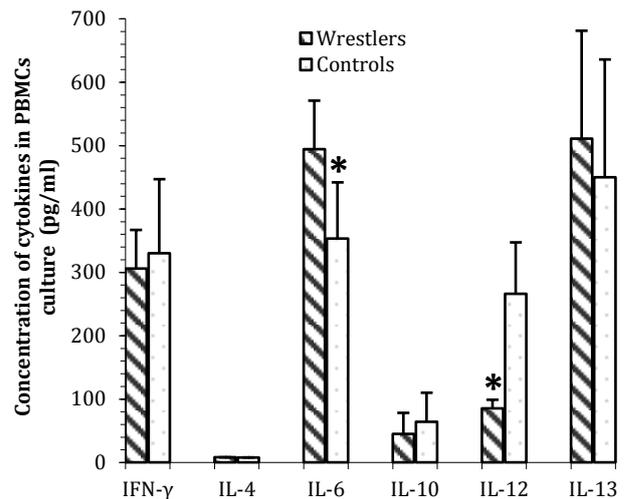
**Table 1.** Main demographic characteristics of the study populations

Group	No.	Sex	Age (years)	Weight (Kg)
Wrestlers	12	Male	24±1.2*	76±5
Controls	13	Male	24±0.8	85±4
<i>P</i> - Value**	----	----	0.864	0.154

The wrestlers had a history of exercise training for more than 9.5 years. The controls were also in silent state during the last six months. All comparisons were two-sided with *P*- values that are noted for each group to show statistical significance.

\*Results are presented as mean±SEM

\*\*Independent t-test



**Figure 3.** Production of IL-4, IL-6, IL-10, IL-12, and IL-13 and in PBMCs culture of the wrestler and control groups

7×10<sup>5</sup> PBMCs of each participant were isolated from blood by Ficoll-Paque and cultured for 48 hr in the presence of mitogens PHA (5  $\mu$ g/ml) and LPS 25  $\mu$ g/ml. The supernatant of PBMCs cultures were assayed by ELISA. The statistical analysis of two groups by unpaired student's t-test showed that IL-6 level (494±77 pg/ml) in the wrestlers was significantly higher than IL-6 level in the controls (353±89 pg/ml,  $P=0.028$ ). Whereas, IL-12 level decreased in the wrestlers (85±14 pg/ml) compared with controls (266±81 pg/ml,  $P=0.038$ )

IL-10 (45±33), IL-13 (511±170) and IFN- $\gamma$  (306±61.) were not different in the wrestlers compared with the levels of IL-4 (7.8±0.7,  $P=0.959$ ), IL-10 (64±46,  $P=0.752$ ), IL-13 (450±186,  $P=0.818$ ) and IFN- $\gamma$  (330±117,  $P=0.867$ ) in the controls.

## Discussion

Analysis of the sera for IL-4, IL-6, IL-10, IL-10, IL-12, IL-13 and IFN- $\gamma$  showed that the levels of these cytokines were not statistically different in wrestlers 24 hr following from the last training activity and the sedentary controls. Our results are consistent with a report by Kara, *et al* who demonstrated that IL-2, IFN- $\gamma$ , and TNF- $\alpha$  in the sera of young wrestlers who actively involved in wrestling for about 6 to 7 years were not different when compared with the ones not involved in sports (13). Considering that normal resting values of cytokines usually could be restored within 24 hr and regarding the results of this study and Kara's study; we can conclude that wrestling does not have measurable effect on the studied cytokine 24 hr after last training session (17).

In this study, we also sought to determine if *in vitro* mitogens activation of the whole blood of wrestlers was able to change the cytokine production levels compared with the whole blood cells activated in the controls. Although there were no significant differences between IL-4, IL-10, IL-12 and IFN- $\gamma$  in two study groups, IL-6 production was elevated and IL-13 production was lower in the wrestlers' activated whole blood cells when compared with the controls.

IL-13 is mostly produced by CD4 T cells. However, other cells such as eosinophils, basophils, mast cells and natural killer cells have some capacity to produce IL-13 (18). Recently IL-13 has been known as a key cytokine in allergy, in mucosal inflammation and other inflammatory diseases (19). Currently, scientists try to block IL-13 production in some diseases like asthma (10). It seems that wrestling can inhibit *in vitro* or maybe *in vivo* capacity of whole blood cells to produce IL-13 when activated by mitogen or antigen. Although, it is difficult to extrapolate from *in vitro* stimulated response of isolated cells to how these same cells would respond in the very complex *in vivo* environment.

*In vitro* activation of PBMCs by PHA and LPS also increased production of IL-6 and decreased release of IL-12 in the wrestlers compared with the controls. In contrast, the levels of other cytokines IL-4, IL-10, IL-13 and IFN- $\gamma$  were not different. This finding is somewhat consistent to the existing literature in which the effect of strenuous exercise on the mRNA concentrations of IL-4, interleukin-12p35 subunit (IL-12p35) and IFN-gamma in equine peripheral blood mononuclear cells was not changed when stimulated with mitogen (20).

In a study by Capomaccio *et al* pointed out that in humans and horses, IL-6 mRNA levels in PBMCs determined by quantitative reverse transcription-polymerase chain reaction were significantly higher in highly trained subjects (21).

IL-12 is mainly produced by activated monocytes, macrophages, dendritic cells, B-cells and plays critical roles in the regulation of helper T (Th) cell differentiation and inflammation. In a pro-inflammatory milieu, resistin as an adipokine will be increased and will result in enhanced secretion of pro-inflammatory cytokines, TNF-alpha and IL-12, similar to that obtained using 5 microgram/ml lipopolysaccharide (22). On the other hand, there are some evidences that inhibition of IL-12 production increases the production of IL-23. IL-23 plays major role in the release of very important inflammatory cytokine IL-17 by Th17 cells (9, 23). It seems that this controversy in the effect of IL-12 on the inflammation can be explained by the fact that the features attributed to IL-12 in inflammation actually depend on IL-23 and not on IL-12 (24).

The pleiotropic cytokine, interleukin-6 (IL-6), which modulates the function of immune cells in response to exercise and training, thereby playing a major role in the exercise-induced inflammatory process. In this context, it is widely presumed that IL-6 could mediate the protective, long-term anti-inflammatory effects of exercise by orchestrating an anti-inflammatory reaction involving macrophages, lymphocytes and monocytes (21).

Unlike IL-1 and TNF- $\alpha$ , IL-6 does not up-regulates inflammatory mediators. It seems IL-6 release alone

during exercise has anti-inflammatory effects. On the other hand in a sepsis condition, IL-6 in combination with TNF- $\alpha$  stimulates inflammatory process (3). Another important action of IL-6 is that it suppresses production of TNF- $\alpha$  which is a potent activator of inflammation (25).

## Conclusion

It can be concluded that exercise training would elevate the capacity of immune system cells in production of some anti-inflammatory cytokines such as IL-6 and decrease this capacity by the production of some pro-inflammatory cytokines like IL-12 and IL-13.

## Perspective

It is proposed that the exercise seems to be used as an anti-inflammatory agent and would be useful in the cure of the chronic inflammatory related diseases.

## Acknowledgment

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