

Interleukin-6 in human plasma during ultra endurance exercise

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Abstract

IL-6 was investigated in plasma during ultra endurance exercise in well-trained endurance athletes (n= 26 men and 10 women). The intention was also to investigate if there were any sex differences in the IL-6 response to exercise. The setups were one laboratory controlled study, 24 h (setup 1) and two competitions of differing durations, 3 days (setup 2) and 6 days (setup 3). The food intake in setup 1 was controlled, in setup 2 and 3 food intake was *ad libitum*. The intensity was set to 60% of individual $\text{VO}_{2\text{ peak}}$ in setup 1 and in the other setups the intensity was race pace. Blood samples were drawn before, during and after the exercises. IL-6 in plasma was analyzed using a high-sensitive ELISA-method. In setup 1 the participants had nearly identical IL-6 levels from 12 h (14 fold elevation from baseline), the levels remained steady during the test. In setup 2 the IL-6 levels was elevated 15 fold from baseline to the end. In setup 3 the levels were elevated 18 fold from baseline to end. Women had significantly lower baseline and end values after setup 3 but not after setup 2. The conclusions based on the present study are that intensity and not duration is the main determinant of the IL-6 response in plasma in endurance exercise lasting >12 h, and that women display lower IL-6 levels, both at baseline and post-exercise.

Introduction

Ultra endurance exercise is characterised by duration of more than 6 h where intensity is moderate to high (e.g. triathlon, ultra marathon and adventure racing). Adventure racing (AR) may last for several consecutive days and involves multiple disciplines, e.g. running, kayaking, swimming, climbing and cycling, performed through a predetermined course. The participants usually compete in mixed teams consisting of three men and one woman. The teams decide how to allot the time between exercising, eating and sleeping. The competitors usually sleep approximately 1-2 h/night. It is not unusual that participants fail to complete competitions due to illness and fatigue. During the 2005 World Adventure Racing Championships 28.8% of the participants reported illness during the race (1). Pathogenesis is likely a major factor concerning uncompletion.

Endurance exercise has been shown to cause alterations of immune functions (2), one mechanism suggested is an increased release of cytokines into the circulation resulting in perturbation of the immune system. Cytokines are glycoproteins that are produced by and mediate communication between and within immune- and non immune cells, organs and organ systems. The cytokine production is modulated by various stimuli including trauma, infection and physical activity. Interleukin-6 (IL-6) is a pleiotropic cytokine that is ubiquitously expressed. It has diverse activities such as modulation of proliferation, differentiation and regulation of immune responses. IL-6 inhibits T helper class 1 (TH_1) cells, thereby shifting the balance towards TH_2 class cytokines (3). IL-6 also mediates fever and is main inducer of the acute phase response to infection (4).

During conditions like sepsis and trauma, a vigorous IL-6 response can be detected (5). Also strenuous, prolonged physical activity has been shown to increase concentrations of several cytokines in the circulation and among those IL-6 is the most prominent (6). Athletes suffering from underperformance syndrome, a state including various symptoms such as fatigue and a decrement in performance capacity also often exhibit elevated levels of cytokines (7). Exogenous administration of IL-6 has been shown to increase the sense of fatigue at rest (8), and administration to male runners significantly decreased performance during a 10-km time trial (9).

Steensberg et al. showed that it is not immune cells but the contracting muscle that is the main origin of IL-6 during exercise (10), it was proposed that IL-6 was released in response to muscle damage due to its role as an inflammatory responsive cytokine (11) but further research showed that IL-6 is released independent of muscle damage (12). It is suggested that IL-6 could have a metabolic role during exercise by acting as an energy sensor to increase hepatic glucose output and induce lipolysis and fat oxidation (13).

Intensity (14), duration, and the muscle mass involved in the exercise are factors known to influence the quantity of IL-6 found in plasma. Duration has been shown to account for more than 50% of the measured variation in IL-6 levels after exercise (12). The exercise-induced elevation of IL-6 is not linear, rather there is an accelerating enhancement in an almost exponential manner, with levels peaking at the end of exercise or into recovery (6).

Several previous studies have described IL-6 kinetics during exercise, yet the majority have focused on changes with exercise of shorter duration, e.g. marathons, where the levels of IL-6 can rise up to 100 fold (15). Inflammatory response to triathlon competitions has also been investigated (16), however triathlon and AR differ in intensity and duration.

Athletes participating in AR are potentially exposed to several stressors such as strenuous exercise, hypothermia, endotoxaemia and sleep deprivation. These factors have all been shown to affect the IL-6 response (16, 17-19).

To our knowledge, no study has investigated the cytokine response to multi-day ultra endurance exercise. However, several studies have investigated the cytokine response to intense military training (20-23). They all found elevations of IL-6 except Bøyum *et al.* (22) who showed a decline of the levels after the training programme. Military practices share many resemblances with AR. Common denominators are energy deficit and sleep deprivation, yet intensity and energy expenditure are reportedly lower in military practices and often include more rest (24).

Gender-based differences in the immune system are well recognized (25), yet a limited number of studies have investigated a potential sex-dependent cytokine response to exercise. A study by Edwards *et al.* (26) showed that the IL-6 response was greater in women than in men at 60 min after maximal, but not after submaximal exercise. Steptoe *et al.* and Prather *et al.* investigated IL-6 response to psychological stress and found higher IL-6 response in women (27,28). It seems conceivable that the women would experience higher levels since men and women compete together in AR hence the relative intensity will probably be higher for the women.

We investigated the kinetics of IL-6 in men and women during ultra endurance exercise of differing durations and intensities. Due to the earlier findings that IL-6 can rise 100 fold after marathon and triathlon we hypothesized that the levels after a multi-day exercise would be elevated to those seen in pathological conditions. We further hypothesized that women would exhibit higher levels than men.

Materials and methods

Subjects

Subject characteristics are described in table 1.

Subjects were fully informed about the procedure, possible discomfort involved, and their right to terminate the experiment at any point. All subjects were acquainted with the test methods used.

Written informed consent was obtained from all subjects.

Experimental protocol

Setup 1

Nine male well-trained Swedish ultra endurance athletes with several years of experience from endurance training and competition at international elite level took part in this laboratory setting. The subjects had been practicing the last 3-9 years for ultra endurance competitions, and had completed several Adventure Races with duration of at least 48 h.

Two to four weeks before the test the athletes $VO_{2\text{peaks}}$ for cycling (Monark Ergomedic 839E, Monark Exercise AB, Varberg, Sweden), kayaking (Dansprint aps, Hovide, Denmark) and treadmill running (Rodby Electronics, Vansbro, Sweden) were measured by incremental all-out tests with raised work rate every minute. The subjects also performed incremental steady state tests (5 steps à 3 min) in each discipline in order to establish a relation between HR and VO_2 . VO_2 was measured with an online ergospirometry system (AMIS 2001, Innovision A/S, Odense, Denmark) based on mixed expired method with an inspiratory flowmeter. HR was continuously recorded with HR-monitor 610S (Polar Electro Oy, Kempele, Finland).

The test included 24 h of nearly continuous mixed exercise under controlled conditions. The athletes arrived in the laboratory after one night of fasting, following three days of standardized food intake (4250 kcal, 52 % carbohydrates, 31 % fat and 18 % protein). The subjects performed 12 blocks of exercise (4 x kayaking, 4 x running and 4 x cycling) in groups of three. Each block included 110 min of exercise and 10 min for food intake and change of equipment and clothes. The energy intake during the exercise was (59 % carbohydrates, 29 % fat and 12 % protein) intended to give each person 50 % of their estimated energy expenditure. Intake of water was *ad libitum*. Control of food intake ceased after 24 h. All tests took place in-doors with a temperature ranging between 18 and 22 °C. Running and cycling, except for the last 20 and 30 min of every exercise block, respectively, were performed outdoor. The temperature outdoor ranged between 2 °C at night and 22 °C during daytime. Work rate aimed at 60 % of the individual $VO_{2\text{peak}}$ in respective exercise mode, using corresponding HR from characterization tests (five stage incremental steady-states) to control intensity.

Setup 2

Eight men and four women participated in the competition Explore Sweden, which lasted three days and took place in the High Coast area, Sweden, in the autumn of 2008. The subjects were all well-trained endurance athletes who had several years of experience from ultra endurance competitions. In the morning the day before the race and immediately after the end of the race height and weight were determined. The participants performed mixed ultra endurance exercise (running, trekking, kayaking, cycling, climbing and swimming). The race was held on a predetermined course of about 400 km, and the subjects competed in teams consisting of three men and one woman. Intensity was not measured during the present competition, but other ultra-endurance investigations have reported a mean intensity of about 40% of $VO_{2\text{max}}$ (29). The temperature was approximately 6 °C at night and ranged between 11 °C and 13 °C during daytime. Sleeping, resting, eating and drinking were *ad libitum*.

Setup 3

The participants were twelve men and six women who participated in the AR World Championship (ARWC) in Hemavan, Sweden, 2006. The subjects were all highly trained endurance elite athletes, with experience from international elite level competitions. Anthropometric measurements; height, weight and total body fat (handheld bio-impedance; Body Fat monitor BF 360, Omron Healthcare Co., Ltd., Kyoto, Japan) were measured the morning the day before the race and immediately after finishing.

During the competition the subjects performed mixed ultra endurance exercise (trekking, kayaking, cycling and climbing). The teams, consisting of three men and one woman, navigated through a course of more than 800 km. Sleeping, resting, eating and drinking were *ad libitum*.

Blood sampling

In setup 1 a polyethylene catheter was introduced into an antecubital vein in order to facilitate repeated blood sampling during exercise. (Due to technical problems, two subjects underwent blood sampling without inserting of a catheter). Blood samples were drawn at rest before exercise and during the periods of steady state cycling (0, 6, 12, 24 h).

Blood samples were drawn in the morning the day before the race and at the end of the race in setup 2 and 3. Additional blood samples were collected after approximately 24 h and after 72 h of competing in setup 3 (n = 7).

Samples were put on ice for one hour to allow for clotting. Plasma samples were stored at -70°C and later analysed for IL-6.

Analyses

Total IL-6 in plasma was determined using Quantikine[®], ELISA, a high-sensitive enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA).

Procedure

All samples and reagents are prepared in room temperature.

1. 100 μl of assay diluent is added to each well (A microplate precoated with a primary antibody, monoclonal anti-IL-6).
2. 100 μl of sample is added to each well and incubated for 2 h at room temperature.
3. Washing
 - a) Liquid is removed from the wells by inverting the plate and decanting the contents.
 - b) Each well is filled with 400 μl of wash buffer with an autowasher.
 - c) Liquid is removed from the wells with an autowasher.
 - d) Washing procedure is repeated 6 times. Excess wash buffer is removed by paper towel.
4. 200 μl of conjugate (an enzyme-linked polyclonal antibody) is added to each well and incubated for 2 h at room temperature on the shaker.
5. The wash procedure in step 4 is repeated 6 times.
6. 50 μl of substrate solution is added to each well and incubated for 60 min at room temperature.
7. 50 μl of amplifier solution is added to each well and incubated at room temperature (colour develops in proportion to the IL-6 bound in the initial step).
8. 50 μl of stop solution (sulphuric acid) is added to each well.
9. Optical density of each well is determined by using a microplate reader set to 490 nm.

A standard curve was created by using standards provided by the manufacturer. The reported sensitivity was 0.039 pg/mL with an intra-assay variation of 7.8% and an inter-assay variation of 7.2%. All samples were determined within the same assay to avoid inter-assay variation.

Statistical analysis

All values are presented as means \pm SD. Student's paired t-test was used for making statistical validation. The level of significance was set at $\alpha=0.05$ and trends considered at $0.05 < P < 0.1$.

When multiple comparisons were made the p-value was adjusted with Bonferroni correction. Accepted p-value = $< 0.05/n$ (n=number of tests). Graphs were made using Microsoft Excel.

Results

Setup 1

All nine men completed the exercise and provided blood samples at 0, 6, 12, 24, and 52 h (i.e. after 28 h into recovery), except one subject which not provided blood at 6 h.

IL-6 increased from 0.76 ± 0.48 pg/mL at baseline to 7.16 ± 2.70 at 6 h (9.4 x). It increased to 10.58 ± 1.04 pg/mL at 12 h (13.9 x from baseline), and to 10.89 ± 0.36 pg/mL at 24 h. All changes had $p < 0.001$ compared to baseline. At 28 h into recovery IL-6 was 3.10 pg/mL (4.1 x from baseline). For individual IL-6 kinetics see fig. 1.

Setup 2

Mean race time was for the men 75.2 ± 8.1 h and for the women 77.2 ± 8.8 h. All participants provided blood samples at baseline and at the end. IL-6 was elevated from 0.35 ± 0.22 pg/mL to 5.16 ± 3.32 pg/mL at the end. The elevation was 14.7 x compared to baseline ($p < 0.001$).

For women IL-6 was 0.18 ± 0.07 pg/mL at baseline and 5.53 ± 3.04 pg/mL at the end. For men IL-6 was 0.43 ± 0.23 pg/mL at baseline and 4.97 ± 3.64 pg/mL at the end. Women had significantly lower IL-6 concentration at baseline ($p = < 0.05$), but there was no significant difference between men and women at the end.

Five of the participants had 10 h of mandatory rest (due to prohibition to be on the water at night time) approximately 4 h before the finish while the others did not. They are displayed as “rest” respectively “no rest” in fig. 2. Their IL-6 levels at the end were 2.36 ± 0.67 and 7.16 ± 2.95 respectively.

Setup 3

Mean race time was for the men 145.9 ± 10.0 h and for the women 139.9 ± 23.4 h.

All fifteen participants provided blood samples at baseline and at the end. IL-6 was elevated from 0.53 ± 0.47 pg/mL at baseline to 9.87 ± 5.91 pg/mL at the end (18.7 x).

IL-6 was 7.87 ± 3.37 pg/mL at 24 h and 10.11 ± 4.78 pg/mL at 72 h. (Only seven subjects were analyzed for IL-6 at 24 and 72 h). The elevation was significant from baseline at all time points ($p > 0.001$). There were no significant elevation from 24 h to 72 or 144 h.

For women IL-6 was 0.29 ± 0.06 pg/mL at baseline and 6.40 ± 2.64 pg/mL at the end. For men IL-6 was 0.69 ± 0.56 pg/mL at baseline and 12.19 ± 6.45 pg/mL at the end. The end value was significantly lower for women ($p = < 0.05$), and the baseline value showed a trend towards lower value for women ($p = 0.071$).

One of the recruited teams dropped out after 24 h and are therefore not included in the analyses with the other participants. They (n=3) are displayed separately and had IL-6 concentrations at baseline of 2.76 ± 2.85 pg/mL, and when they dropped out 26.54 ± 14.49 pg/mL. Both values were significantly higher than of the participants that completed the setup.

Discussion

To our knowledge this is the first study to investigate IL-6 kinetics during multi-day ultra endurance exercise. Our findings of an elevation of IL-6 levels after multi-day exercise are in

accordance with Gundersen *et al.* and Gomez-Merino *et al.* (20-21,23) who found modest elevations of IL-6 after military training. The higher IL-6 values obtained in our studies seem not surprising since energy expenditure and exercise intensity is higher in AR (29) than in military training and less time is spent on resting/sleeping (23).

We expected to see levels comparable to those in pathological conditions due to earlier data which points out duration as an important factor for IL-6 release during exercise (12). The levels are lower than we hypothesized.

Intensity

Intensity has earlier been reported to correlate with IL-6 levels (14). The intensity in setup 1 was predetermined and set to 60% of individual VO_{2peak} . The levels of IL-6 in plasma reach its peak already after 12 h and remain steady during the test (see fig 1). This is in contrast with earlier studies that have shown that IL-6 levels in plasma peak at the end of exercise or into recovery (6). Subjects exhibit striking similarities at all time-points except at 6 h. Of note is that we have not measured time points between 6 and 12 h, hence it is possible that IL-6 values reach a peak earlier than at 12 h. The results from setup 1 shows evidence for that individual differences in the IL-6 response when performing equal modes of ultra endurance exercise of more than 12 h at the same relative intensity and are small. The differences in the data from 6 h implies that the results are not due to methodological error.

In the competitions (setup 2 and 3) the intensity is self-selected, hence the participants will compete with differing intensities (due to individual differences in training status, effort during the race, etc.) The competitors display large individual differences in the IL-6 response. In setup 2, there were concrete differences among the participants. Five of the participants had more rest close to the finish (they were prohibited to go out on the water at night) and the other participants finished the race without the extra rest. By looking at the individual level it is evident that the subjects which rested had lower levels than the others ($p < 0.01$, see fig 2).

The fact that individuals exercising at the same relative intensity display stunningly similar levels, while subjects performing exercise at different relative intensities exhibit large differences in IL-6 levels points out intensity and not duration as main determinant of IL-6 in multi-day ultra endurance exercise. What further supports this is that the athletes do not show significantly higher levels after 144 h of exercise than after 24 h.

Individual differences

Individual differences might contribute to the disparities in IL-6 levels seen among the athletes during the competitions. The food intake was not controlled during setup 2 and 3, and there is a possibility that individual differences in glycogen stores and energy intake could reflect the IL-6 release. Low glycogen levels during exercise is associated with greater increases in IL-6 (30) and carbohydrate ingestion has been shown to decrease IL-6 in plasma during exercise (31). Since we have no food records for the competitors it is impossible to establish an association between energy intake and IL-6 response during setup 2 and 3. According to our own observations there are great differences in food intake among the individuals. In setup 1 food intake was controlled three days prior to and during the test, yet the subjects' energy intake in proportion to energy requirements was not equal (data not shown). Differences in food intake could not account for the individual disparities seen at 6 h since there were no correlation between energy intake from 0 to 6 h in percent of energy requirements and the IL-6 response (data not shown). The subjects display almost similar IL-6 levels at 12 and 24 h despite the differences in energy intake.

With the decrement in intensity that occur during AR (from 60 to 40% of VO_{2max} from 24 h) (29) it seems conceivable that it is easier for the participants to ingest food and keep it.

Zimberg *et al.* (32) have investigated the nutritional intake during a simulated adventure race lasting three days. This study showed the opposite; energy intake goes down during the race, with the lowest energy and carbohydrate intakes seen on day 3. According to this it seems that the stable levels of IL-6 from 24 h to 144 h during setup 3 cannot be explained by an increase in food intake during the later stages of the competition.

There are differences in fat mass among the participants (see table 1), and adipose tissue may contribute to 35% of the IL-6 response in the circulation at rest, but Lyngsø *et al.* showed with arteriovenous measurements that fat tissue does not contribute to the exercise-induced IL-6 in the circulation until recovery (33). This indicates that differences in fat mass have no impact on IL-6 levels during exercise, whether this is also valid for exercise of more prolonged nature is not clear. However, there was no correlation between fat mass and IL-6 levels after setup 3.

Interestingly, the three persons who chose to terminate setup 3 (due to fatigue), showed significantly higher levels of IL-6 at baseline and at 24 h ($p > 0.01$ for both), see fig 2. They were also the ones considered less trained (according to their lower $\text{VO}_{2\text{peak}}$ values, see table 1). This observation might be an indication of that it is detrimental to performance to start exercising with already elevated IL-6 levels.

Sex differences

Several studies have shown gender differences in substrate utilization during exercise, with women oxidizing proportionally more fat than men (34,35). A study attempting to investigate potential sex differences in substrate utilization during military training showed that the relative contribution of fat mass to total energy expenditure among the cadets was nearly 90% in the women compared with 74% in the men (23). IL-6 have been shown to induce lipolysis and fat oxidation in humans (13). Since women seems to better be able to use fat and spare glycogen during exercise they might be less reliable on the effect of IL-6 on fat oxidation and hence display smaller increase in IL-6 levels.

Disparities exist between men and women regarding inflammatory response after trauma and burns. This has been attributed to estrogens ability to influence the immune response after injury (36). It has been shown that physiological concentrations of estrogen directly inhibits IL-6 production (37). Regarding sex differences in the cytokine response to exercise, there is a lack of information. Studies have investigated a sex-dependent cytokine response to psychological stress and found higher levels in women (27,28) Edwards *et al.* (26) showed no gender difference after 45 min of bicycle exercise, yet women had significantly higher levels 60 min into recovery after a maximal but not after a submaximal exercise task. In contrary to our hypothesis we found lower IL-6 levels in the women, this is in spite that women probably compete at a higher relative intensity than the men. Whether our finding of a smaller IL-6 response in women is due to estrogen or some other factor is not clear.

Conclusion

This investigation shows that intensity and not duration is the main determinant of IL-6 in plasma during ultra endurance exercise of more than 12 h. Women have a smaller IL-6 response than men, both at rest and during exercise.

Further studies are required to evaluate a broader spectrum of immune alterations during AR and elucidate how parts of this multifactorial stress situation affects components of immune system. By examining dietary components' impact on immune responses and illness, perhaps there is a greater possibility to avoid pathogenesis and fatigue during the races.

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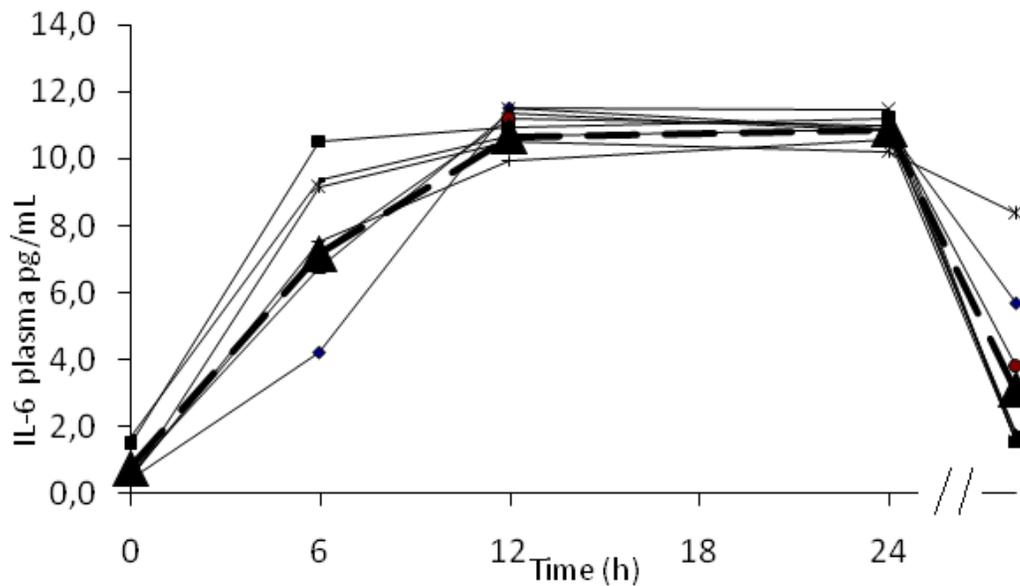
Table 1. Subject characteristics of the participants.

Setup	Sex	n	Age(years)	Height(cm)	Weight(kg)	VO _{2peak} run (l/kg/min)	Bodyfat(%)	VO _{2peak} run (l/min)
1	M	9	27.1±0.9	182.0±1	80.4±2.4	62.5±5,3	17.1±4	5.01±0.43
2	M	8	33.9±4.8	185.5±7	80.9±9.6	‡	‡	‡
3	M	9	30.3±3.5	179.9±5	79.3±2.6	61.5±2.5, n=8	16.8±3.7	4.85±0.20, n=8
2	F	4	31.4±2.6	166.0±6	57.3±4.0	‡	‡	‡
3	F	6	32.2±6.6	166.2±8	60.7±7.9	55.8±1.8, n=3	22.4±2.7	3.11±0.59, n=3
3(fail)†	M	3	33.0±3.6	176.2±1	77.3±6.8	51.8±2.2, n=2	18.0±5,6	4.18±0.47, n=2

Values are given as mean±SD. †=Represents the participants that dropped out after 24 h.

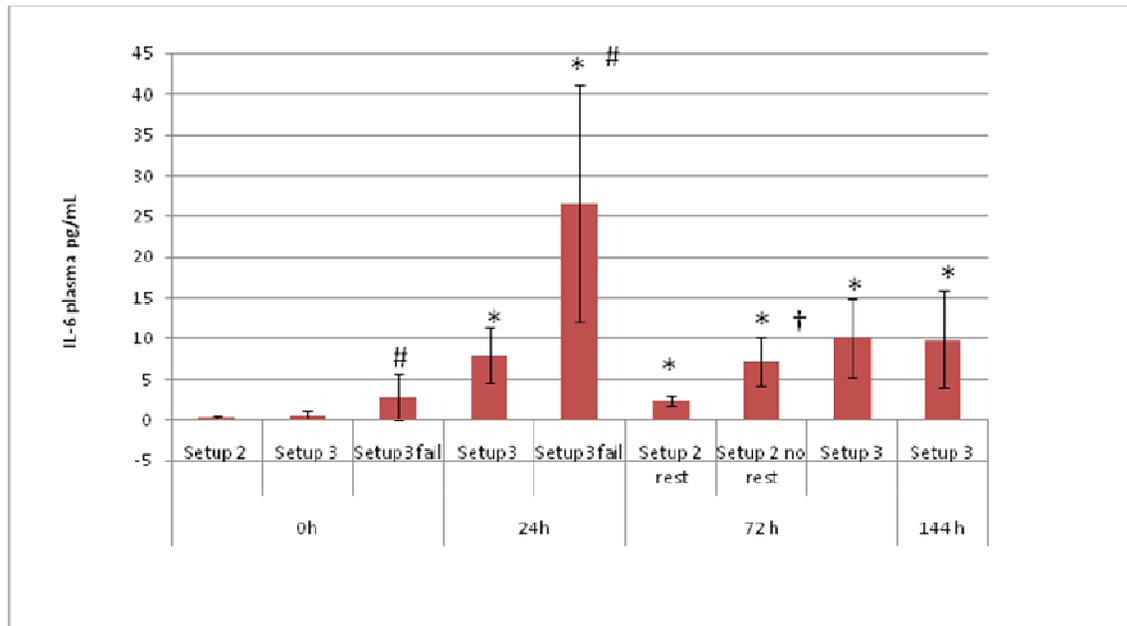
‡= Not measured in the present study. Subjects are considered equal to the participants in setup 1 and 3.

Fig 1. Individual IL-6 kinetics for setup 1.



----- = mean value

Fig 2. IL-6 in plasma before, during and after setup 2 and 3.



Values are presented as mean±SD.

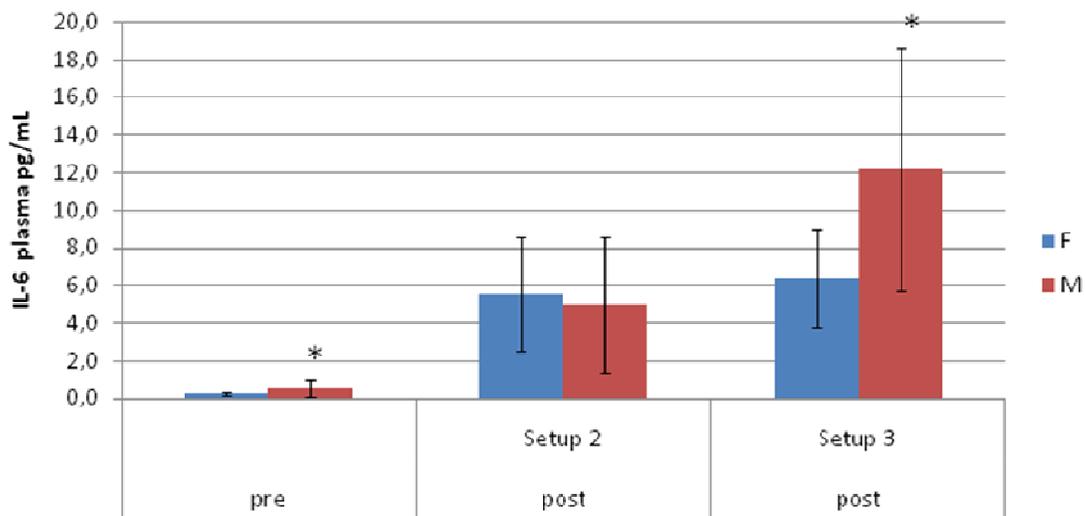
Range= **0h**, setup 2: 0.10-0.85, setup 3: 0.08-1.73, setup 3 fail: 0.89-6.04. **24h**, setup 3(n=7): 2.21-12.04, setup 3 fail: 12.12-41.11. **72h**, setup 2 “no rest”: 3.61-11.85, setup 2 “rest”: 1.41-3.24, setup 3(n=7): 3.53-16.17. **144h**, setup 3: 4.28-22.30.

* Significantly different ($p < 0.05$) from pre-exercise.

Significantly different ($p < 0.05$) from the rest of the participants in setup 3.

† Significantly different from ($p < 0.05$) “rest” in setup 2.

Fig 3. IL-6 in plasma in men and women before and after setup 2 and after setup 3.



Values are presented as mean±SD. * Significantly different from the men.