

Concurrent high-intensity aerobic and resistance exercise modulates systemic release of alarmins (HMGB1, S100A8/A9, HSP70) and inflammatory biomarkers in healthy young men: a pilot study

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Abstract

Background: Intense exercise is a systemic stressor associated with the release of “danger” molecules – alarmins, by damaged or dying cells into systemic circulation to evoke a sterile inflammatory response. Compared with research in clinical diseases, physiological responses of alarmins to exercise and training are not well studied. Short-term responses to exercise and training using a panel of alarmins – HMGB1, S100A8/A9, HSP70 and sRAGE may reveal unique aspects of stress responses to strenuous exercise with important ramifications when prescribing exercise to generally healthy adults. **Methods:** A 3-week, high-intensity training program was performed by healthy young men (N = 7). Concurrent aerobic and resistance exercises were performed on 3 consecutive days each week. Blood and saliva were collected before (*Pre*), immediately after (*Post*), and 30 min (*30 min*) after exercise each week, and 24 h after the final exercise session in week 3 (*24 h*). **Results:** Plasma HMGB1, S100A8/A9 and HSP70 increased from *Pre* to *Post* ($P < 0.05$), although at different timepoints during the study, and displayed different kinetics from IL-10, IL-8 and IFN- γ , suggesting unique mechanisms involved in modulating their release and clearance. CD14⁺CD16⁻ monocytes increased from *Pre* to *Post* across 3 weeks; CD14⁺CD16⁺ monocytes increased from *Pre* to *Post* in week 2 and 3 ($P < 0.05$). Δ HMGB1 and Δ HSP70 correlated positively with Δ MCP-1 during 3 weeks of training, while Δ HMGB1 correlated positively with CD14⁺CD16⁻ monocytes, suggesting higher alarmin release after strenuous exercise may involve increase in circulating monocytes. **Conclusions:** Perturbations in systemic alarmins are novel biological signatures for assessing the inflammatory milieu of healthy adults during high-intensity exercise.

Background

Intense acute exercise has been compared to a state of mild trauma, with the systemic inflammatory response after a bout of heavy endurance exercise mimicking that of clinical sepsis (1). During intense exercise, multiple organ systems experience increased physiological stress, including cyclic bouts of ischemia-reperfusion and hypoxia in contracting skeletal muscles, increased oxidative stress, changes in body temperature and metabolic substrate flux as well as shunting of blood flow from visceral organs to contracting skeletal muscles. Taking reference from Matzinger’s “danger” model (2), such disturbances in systemic homeostasis can be sensed by the immune system and result in the activation of the inflammatory response. The immune system plays a role in modulating physiological adaptation to exercise stimuli - in essence, its response entails the recruitment of specific leukocytes for the mitigation of cellular damage, especially in skeletal muscle. It is plausible that alarmins, as “danger” signals, may play a role in the adaptation to exercise training.

Alarmins are endogenous molecules that activate the immune system when encountering “danger” to the host, such as in the case of infection or cellular stress/damage (2–4). During homeostasis, alarmins are sequestered within the cytosol or nucleus and perform regulatory functions (5). Stressed or damaged cells can quickly release alarmins into systemic circulation, where they bind pattern recognition receptors (PRRs) on antigen presenting cells to activate the immune system (6). Since the first study on alarmins was published, the list of putative alarmins has grown and currently include high mobility group box

protein-1 (HMGB1), high-mobility group nucleosome-binding protein 1 (HMGN1), S100 proteins, heat shock proteins (HSPs), cathelicidin, eosinophil-derived neurotoxin (EDN), defensins, granulysin and mitochondrial DNA, mitochondrial peptides and ATP (3).

The concept of alarmins as “danger” signals has been studied in various clinical diseases, such as polytrauma (7), heat stroke (8, 9), cardiovascular disease (10) and cancer (11). Although the phenomenon of exercise-induced inflammatory cytokine responses is well-established (11), the responses of alarmins to exercise are poorly understood and represent an exciting research vista. In brief, investigators have mostly described the effects of acute exercise (one bout of exercise) on individual alarmins, with only one research group (12) having studied them as a part of a panel of biomarkers. Furthermore, alarmins as a panel of biomarkers have never been studied within the context of exercise training. Nevertheless, separate findings from these studies show that acute exercise increases circulating alarmins, such as HMGB1, and supports our working hypothesis that strenuous/exhaustive exercise is a “danger” or stressor to the physiological system (13). Systemic HMGB1 increased after strenuous aerobic exercise, including exhaustive treadmill running (14), a half- or full-marathon race (12) and a 5-min step test in a hypoxic chamber (15). Likewise, serum HSP70 increased after a single bout of treadmill running in an ambient environment (20 °C) at an intensity of 70% VO_2 peak for 60 min (16) and also after 2 h of semi-recumbent cycling at $\sim 60\% \text{VO}_{2\text{max}}$ (17). Finally, systemic S100A8/A9 increased after high-intensity interval or continuous cycling (18), 3 h of dynamic knee extension (19), as well as treadmill running (75% $\text{VO}_{2\text{max}}$, 30 min) (20), but not to one-legged calf-raise exercise (10 sets x 40 repetitions) (21). While most of the studies confirm the systemic elevation of alarmins after acute exercise, it is unclear how an acute bout of exercise affects the responses of different alarmins, as this has not been studied. Furthermore, the impact on systemic release of alarmins after a period of exercise training is unclear since no studies have investigated training effects on alarmins.

The “repeated bout effect” is a phenomenon describing physiological adaptations to unaccustomed high-intensity exercise, wherein the initial increase in inflammatory cytokines and proteins associated with muscle damage and soreness are attenuated after repeated bouts of exercise (22). The release of cytokines and chemokines from tissue macrophages may mediate this beneficial adaptation (22). Furthermore, exercise-induced release of prototypical alarmins such as HMGB1, HSP70 and S100A8/A9 represent “danger” signals that can also orchestrate the migration of monocytes and macrophages to skeletal muscle for tissue repair (23–25) or to prime them for activation (26). For instance, the resultant protein complex formed from the binding of HMGB1 and receptor for advanced glycation end products (RAGE) could activate downstream inflammatory responses, such as the chemotaxis and adhesion of monocytes at sites of injury (27), modulating the production of inflammatory cytokines such as TNF- α (28) and activation of other immune cells, such as natural killer cells (28).

Applying concepts from the “danger” model (2, 29), we speculate that the physiological and mechanical stress imposed by unaccustomed, strenuous exercise across repeated, multiple sessions, can activate danger signaling and result in increased alarmins in blood circulation. This study is novel as it is the first to investigate the cumulative effects of multiple days of intense exercise training on different alarmins

that are normally sequestered in various cellular compartments (nucleus and cytosol) during homeostasis. The effects of this exercise program are also investigated for multiple aspects of immune function, including cytokines, chemokines and monocyte subsets. Understanding the responses of alarmins to intense exercise training, a form of physiological stress, can reveal novel mechanistic links between immune activation and subsequent systemic responses (e.g. cytokine release, increased body temperature, muscle damage).

Materials & Methods

Subjects

Healthy men (N = 7; means \pm SD: 24.9 \pm 2 yrs) without cardiovascular, metabolic or respiratory disease were recruited from the general public and completed all exercise trials. All subjects were recreationally active and participated in sports/exercise for a minimum of 30 min, at least three times a week. None of the subjects were taking nutritional supplementation for weight gain or weight loss, nor taking supplemental vitamins or minerals.

Physical activity monitoring

All subjects recorded their habitual physical activity on the short version of the international physical activity questionnaire (I-PAQ). Further, subjects were asked to refrain from any form of recreational and competitive sports or exercise training during the entirety of this study, except for the exercises prescribed in the study. Weekly expended metabolic equivalents (METs) were calculated based on formulas designed by Craig et al. (30), and included energy expenditure for walking, moderate physical activity and vigorous physical activity.

Diet monitoring

Subjects were asked to adhere to a self-selected weekly diet, which was kept the same for three weeks. Each subject recorded his daily food intake in a food diary throughout the entire study. Finally, subjects fasted overnight (allowed plain water) prior to each trial visit. The energy and nutrient composition of the diets were assessed using an interactive nutrient analysis tool designed by the Singapore Health Promotion Board (<http://focos.hpb.gov.sg/eservices/ENCF/>).

Baseline cardiorespiratory and strength tests

A VO_2 peak test and 10-repetition maximum (10-RM) test were administered at baseline, a week prior to the first exercise training session, in order to determine cardio-respiratory fitness and muscle strength, respectively.

VO_2 peak Test

Each subject warmed up on a treadmill (4Front, Woodway, Waukesha, WI, USA) by jogging at a self-selected speed (5–9 km/h), at 1% incline for 10 min. This was followed by 5 min rest before commencing

the maximal aerobic test. The starting speed was 10 km/h and increased every 3 min by 1 km/h. Metabolic gas exchange during exercise was monitored continuously with a metabolic cart (Parvomedics TrueOne 2400, Sandy, UT, USA). Heart rate was monitored continuously with a heart rate monitor (Polar RS800CX, City, Finland) and ratings of perceived exertion (RPE) was determined during the last 15 s of each stage. The test was terminated upon volitional fatigue. The subjects were considered to have achieved their maximal aerobic capacity if any three of the following criteria were met:

- 1) Age-predicted maximal heart rate ($220 - \text{age}$)
- 2) Respiratory exchange ratio (RER) of > 1.1
- 3) Volitional fatigue
- 4) Rating of perceived exertion (RPE) > 17 (6–20 point scale)

Muscular endurance test

Each subject rested for 20 min after the VO_2 peak test. Thereafter, he performed a 10-repetition maximum (10-RM) test of the following exercises in sequential order: bench press, shoulder row and squat. The bench press and squats were performed with an Olympic barbell, and the shoulder row was performed using dumbbells. When the subject was able to complete more than 10 repetitions of each exercise, he was given a 5-min break before performing another round of 10 repetitions with a heavier weight. The final 10-RM for each subject would be the heaviest weight lifted with proper form, for 10 repetitions.

Exercise training protocol

One week after baseline testing, subjects were prescribed an exercise training protocol spanning 3 consecutive weeks, with 3 consecutive days of training per week (Fig. 1). Days 1, 4, and 7 represent the first day of each week of exercise training, whereas Days 3, 6, and 9 represent the third and last day of each week's exercise session. Subjects also arrived 24 h after the last exercise training session in week 3 (Day 10), where a final blood and saliva sample were obtained with the subjects in a seated position (24 H).

—Insert Fig. 1—

In week 1, each subject warmed up by jogging on a treadmill for 5 min. Thereafter, he ran on the treadmill at a speed corresponding to 80% of his maximal heart rate (pre-determined during VO_2 peak testing) for 5 min. This training intensity corresponded with the first ventilatory threshold (VT_1) and reflects an intensity of “somewhat hard” to “hard” on the RPE scale (31). A 2 min rest was given after the treadmill run. After the rest, a series of resistance exercises (bench press, shoulder row, squats) was performed. Each resistance exercise was performed for 10 repetitions at 70% of 10-RM, with a 2 min rest between each exercise. This series of exercises (i.e. run + resistance exercise) constituted one set of the exercise protocol. Each subject completed a total of 4 sets of exercises. Water was consumed ad libitum for all sessions.

The treadmill speed and number of repetitions for the resistance exercises were increased by 10% each week. In addition, the rest interval between each exercise set was shortened from 2 min to 1 min in weeks 2 and 3. We increased the training load weekly to prevent any adaptation to the prior week's exercise.

All exercises were performed under ambient conditions (22–25 °C) in the human performance laboratory at the Singapore Sports Institute.

Heart rate and metabolic gas monitoring

Heart rate was monitored continuously (Polar RS800CX, Polar Electro, City, Finland) from rest (seated for 5 min) until each subject completed his daily exercise session. Metabolic gases were monitored during the first and fourth set of treadmill running at each session. Heart rate (HR), VO_2 and RER were measured every minute for 5 min during the 1st and 4th sets of treadmill running. We report only the 1st and 4th set of metabolic data to track changes in exercise intensity during each training session from weeks 1–3.

Blood sampling and bioassays

Blood was obtained from the antecubital vein on day 1 (first training day of week 1), day 6 (last training day of week 2), day 9 (last training day of week 3) and day 10 (24 h recovery). On days 1, 6 and 9, blood was drawn after 5 min of seated rest (Pre), immediately after exercise (Post) and after 30 min of recovery (30 min). Blood was collected into K_3EDTA vacutainer tubes (Vacuette, Greiner Bio-One, Austria). Tubes were centrifuged at 2000 rpm for 10 min at 25 °C and plasma samples were extracted immediately and stored on ice, before samples were transported back to DSO National Laboratories within 6 h. On day 10 (24 h), blood was drawn from the antecubital vein after the subject had been seated quietly for at least 5 min.

All plasma samples were stored at -80 °C until subsequent analyses. EDTA blood for flow cytometry experiments were kept at room temperature for a maximum 6 h before analysis. We were unable to obtain blood from one subject at Post on day 9, hence all biomarker results were reported for six subjects.

Alarmin assays

Plasma samples were quantified using commercial ELISA kits for the following alarmins: HMGB1 (IBL, Germany), HSP70 (Cloud-Clone Corp, USA), S100A8/A9 (BioVendor, Czech Republic) and soluble receptor advanced glycation end product (sRAGE) (Abcam, U.K). Assays were performed according to manufacturers' instructions. Experiments for each alarmin were repeated at least once, except for HSP70, which was performed once. Subject samples were randomly assayed in duplicates, with intra-assay coefficients of variation (CV) as follows: HMGB1 (9.6%, 8.6%), S100A8/A9 (9.7%, 3.5%), sRAGE (8.7%, 6.5%), HSP70 (6.3%). The assay sensitivity of each kit was: 0.2 ng/mL (HMGB1), 0.22 ng/mL (S100A8/A9), < 3 pg/mL (sRAGE), < 1.25 ng/mL (HSP70). Plasma concentrations of the alarmins were quantified with a microplate reader (Spectrostar Nano, BMG Lab Tech, City, Country).

Multiplex cytokine and chemokine assays

Cytokines (IL-10, IFN- γ) and chemokines (IL-8, MCP-1) were quantified using custom-made ProcartaPlex® kits (Affymetrix Inc, City and/or State, U.S.A). The magnetic beads were quantified using a Luminex® 200 reader (Bio Rad, U.S.A) with the MasterPlex® CT software (v1.0) with the following settings: i) sample size: 50 μ L, ii) DD gate: 5,000–25,000, iii) timeout: 45 s, iv) bead event: 100. Data analysis was performed with the MasterPlex® QT software (v2.0) with logistic 4-point weighting for standard curve fitting. Endogenous concentrations of biomarkers in the sample were determined from this standard curve. The assay sensitivity of each biomarker of interest was: 2.71 pg/mL (IL-8), 2.49 pg/mL (IL-10), 11 pg/mL (IFN- γ), and 1.29 pg/mL (MCP-1).

Quantification of monocyte subsets

Absolute cell counts were assessed using a single-platform lyse-no-wash and flow count bead procedure (32). Briefly, 100 μ L of EDTA whole blood were added by reverse pipetting to BD TruCOUNT™ tubes (BD Biosciences, City and/or State, USA) and incubated for 15 min at room temperature (RT) with the following monoclonal antibodies: CD14, CD16 and CD45, conjugated to anti-Allophycocyanin (APC), Phycoerythrin (PE) and Fluorescein isothiocyanate (FITC), respectively. Erythrocytes were lysed with 450 μ L of 1x FACS lysing solution per tube, vortexed and incubated in the dark at RT for 15 min before acquisition with the FACS Canto II flow cytometer (Becton Dickinson, City and/or State, USA). Compensation was adjusted using three single-stain on a control sample. A control sample was obtained from a healthy male volunteer who was not part of the study, under fasted conditions. TruCount beads, CD45⁺, CD14⁺CD16⁻ and CD14⁺CD16⁺ monocyte cell populations were gated with the FACS diva software (version 6.1.3). Acquisition was set at 10,000 counts in the CD14⁺16⁻ monocytes gate (Fig. 2) and the number of events recorded was used to calculate the absolute cell number according to the equation: (number of events in region containing cell/number of events in absolute count bead region) x (number of beads per test/test volume).

—Insert Fig. 2—

Plasma creatine kinase activity

The enzymatic activity of creatine kinase in plasma was tested with a commercial kit (ABNOVA, City, Taiwan) and plasma concentrations were quantified with a microplate reader (Spectrostar Nano, BMG Lab Tech, City, Country) according to the manufacturer's instructions.

Salivary cortisol assays

Saliva samples were collected by passive drooling into cryovials (Salimetrics®, Carlsbad, CA, USA) on the same days as phlebotomy: days 1, 6, 9 and 10. Samples were stored at 4 °C and transported to DSO National Laboratories within 6 h and stored in aliquots at -80 °C until further analyses. Salivary cortisol concentrations were detected with a cortisol ELISA kit (IBL, City, Germany) and quantified with a microplate reader (Spectrostar Nano, BMG Lab Tech, City, Country) according to the manufacturer's instructions.

Statistics

Two sets of metabolic data (HR and VO_2) were collected daily from the 1st and 4th set of treadmill running. A one-way repeated measures ANOVA was used to determine significant differences in mean HR and VO_2 during 3 weeks of training. A 2-way repeated measures ANOVA was used to determine the effects of: i) increasing exercise intensity (weeks 1, 2 and 3) and ii) time (Pre, Post, 30 min) on plasma concentration of biomarkers. When main effects for either intensity, time or interaction were statistically significant ($P < 0.05$), post hoc Tukey's multiple correction testing was used to assess mean differences within factors. A paired t-test was used to determine mean differences in biomarker concentrations between Pre (Day 1, week 1) and 24 H (Day 10, week 3). To determine the effect sizes (η^2) of exercise training on alarmin response, sum of squares was divided over the total sum of squares. Effect sizes of $\eta^2 \sim 0.2$, $\eta^2 \sim 0.5$ and $\eta^2 \sim 0.8$ were considered "small", "medium" and "large", respectively (33). Linear regression was used to assess the relationship between absolute or relative changes (Δ) in exercise-induced alarmins with i) MCP-1 and ii) monocyte subsets. Statistical significance was set at $P < 0.05$. Analyses were performed with Graphpad Prism (version 6.0.5) with data presented as means \pm SD.

Results

The descriptive profiles of the subjects are shown in Table 1. The mean age of the subjects was 24.9 ± 2 yrs, with a range between 23 and 29 yrs. The mean BMI was 22.6 ± 1.6 , and the mean VO_2 peak was 48.7 ± 4.4 mL/kg/min.

Table 1
Descriptive data of subjects

Parameters	Mean \pm SD
Age (yrs)	24.9 \pm 2
Body mass (kg)	70.9 \pm 4.0
Height (cm)	177.1 \pm 4.7
BMI	22.6 \pm 1.6
Habitual physical activity (METS-min/wk)	503.4 \pm 237.5
VO ₂ peak (mL/kg/min)	48.7 \pm 4.4
10-RM (kg) Bench press	44.3 \pm 18.2
10-RM (kg) Squat	55.0 \pm 46.9
10-RM (kg) Row	24.6 \pm 7
Daily Caloric Intake (kcal)	1798.0 \pm 236.2
Baseline characteristics of the subjects (mean \pm SD) for anthropometric profiles, habitual physical activity patterns, cardiovascular fitness, strength and caloric intake.	

Physiological responses to exercise training

The subjects completed all training sessions and the parameters are summarized in Table 2. The treadmill speed in week 1 was 9.7 ± 1.0 km/h (71.2% of maximal speed; V_{\max}) and the resistance training load was set at 27.2 ± 15.9 kg (52.6% of 1-RM) for Bench Press, 34.7 ± 32.5 kg (52.1% of 1-RM) for Squat and 17.7 ± 4.9 kg (53.1% of 1-RM) for Row with 10 repetitions per set, for a total of 4 sets. Running speed was increased to 10.7 ± 1.0 km/h (78.5% of V_{\max}) in week 2 and to 11.7 ± 1.0 km/h (85.9% of V_{\max}) in week 3. The number of repetitions for the resistance exercises were increased to 11 in week 2 and 12 in week 3 without changing the total weight lifted. This increase in repetitions reduced the %1-RM to 50.8% and 49.1% for Chest, 50.3% and 48.6% for Squat, 51.3% and 49.6% for Row in week 2 and 3, respectively.

Table 2
Training parameters of the subjects.

Parameters	Week 1	Week 2	Week 3
Speed (km/h)	9.7 ± 1.0 (71.2%)	10.7 ± 1.0 (78.5%)	11.7 ± 1.0 (85.9%)
Bench press (kg)	27.2 ± 15.9 (52.6%)	27.2 ± 1 (50.8%)	27.2 ± 15.9 (49.1%)
Squat (kg)	34.7 ± 32.5 (52.1%)	34.7 ± 32.5 (50.3%)	34.7 ± 32.5 (48.6%)
Row (kg)	17.7 ± 4.9 (53.1%)	17.7 ± 4.9 (51.3%)	17.7 ± 4.9 (49.6%)
HR set 1 (bpm) *	149.9 ± 6.7 (77.7%) ^{1,2}	158.3 ± 6.4 (82.0%) ¹	163.8 ± 5.6 (84.9%) ²
HR set 4 (bpm) **	160.3 ± 5.2 (82.0%) ^{3,4}	171.5 ± 6.9 (88.6%) ³	177.0 ± 8.4 (91.9%) ⁴
VO ₂ set 1 (mL/kg/min) §	33.2 ± 2.3 (68.1%) ⁵	37.3 ± 4.7 (76.6%)	40.6 ± 3.6 (82.5%) ⁵
VO ₂ set 4 (mL/kg/min) [†]	34.1 ± 3.9 (70.1%) ⁶	39.2 ± 5.1 (80.6%)	42.1 ± 4.7 (85.4%) ⁶
RER set 1	0.96 ± 0.04	0.99 ± 0.05	1.03 ± 0.06
RER set 4	0.90 ± 0.04	0.94 ± 0.05	0.97 ± 0.05
RPE set 1	11.4 ± 1.7	12.7 ± 1.9	13.8 ± 1.8
RPE set 4	12.7 ± 2.0	14.5 ± 1.9	15.7 ± 1.8
Running speed (% of maximal aerobic speed), weight (% of 1-RM) and physiological responses during three weeks of increasing workload (HR – heart rate bpm (% of HR max); VO ₂ – oxygen uptake (mL/kg/min, %VO ₂ peak*); RER – respiratory exchange ratio; RPE – rating of perceived exertion). The metabolic parameters were recorded every minute and averaged for the 5 min of treadmill running for the 1st and 4th exercise set. RPE was recorded during the last 15 s per minute of each running bout.			
* Statistical significance of main effects between weeks: P < 0.0001.			
¹ Tukey's multiple comparison between week 1 and 2: P = 0.0038.			
² Tukey's multiple comparison between week 1 and 3: P < 0.0001.			
** Statistical significance of main effects between weeks: P = 0.0002.			
³ Tukey's multiple comparison between week 1 and 2: P = 0.0054.			
⁴ Tukey's multiple comparison between week 1 and 3: P = 0.0002.			
§ Statistical significance of main effects between weeks: P = 0.0002			
⁵ Tukey's multiple comparison between week 1 and 3: P = 0.0011			

Parameters	Week 1	Week 2	Week 3
† Statistical significance of main effects between weeks: P = 0.0028			
⁶ Tukey's multiple comparison between week 1 and 3: P = 0.0043			

Increases in running speed and resistance load from week 1 to week 3 resulted in an increase in physiological strain, with HR, VO₂ significantly increased between week 1 and week 3. The subjects attained 82.0% of HR_{max} and 70.1% of VO_{2 peak} in week 1 and 88.6% of HR_{max} and 80.6% of VO_{2peak} in week 2, respectively. These parameters were between the first and second ventilatory thresholds (VT₁ and VT₂) during weeks 1 and 2, as indicated by RER < 1.0. In week 3, the subjects ran at 91.9% of HR_{max} and 85.4% of VO_{2 peak} which indicated a workload above VT₂ (RER > 1.0). RPE scores were elevated by 24%, increasing from 12.7 ± 2.0 at week 1 to 15.7 ± 1.8 at week 3.

Effects of exercise on plasma alarmins

Significant main effects of time were found for HMGB1 (P = 0.03, η² = 0.12) (Fig. 3), S100A8/A9 (P < 0.0001, η² = 0.42) (Fig. 4) and HSP70 (P = 0.0003, η² = 0.19) (Fig. 5) across the three weeks of exercise training, but not for sRAGE (P = 0.91, η² = 0.0025) (not shown). There was no significant main effect of intensity on any of the alarmin response to exercise. Post hoc analyses did not detect significant differences of time for HMGB1 within each week.

— Insert Fig. 3 —

— Insert Fig. 4 —

Post hoc Tukey's multiple testing demonstrated a 5.8- (P < 0.0001), 2.5- (P < 0.05) and 4.7-fold (P < 0.01) increase in plasma S100A8/A9 at Post compared with Pre in week 1 (3031 ± 1964 ng/mL vs. 529.4 ± 234.3 ng/mL), week 2 (1728 ± 793 ng/mL vs. 572.8 ± 321.6 ng/mL) and week 3 (2061.0 ± 776.5 ng/mL vs. 434.4 ± 391.7 ng/mL), respectively (Fig. 4). In week 3, plasma S100A8/A9 concentrations was 3.7-fold higher at 30 min compared with Pre (P < 0.05; 1609.0 ± 737.3 ng/mL vs. 434.4 ± 391.7 ng/mL).

— Insert Fig. 5 —

Plasma HSP70 increased 1.9- (P < 0.01) and 1.5-folds (P < 0.05) at Post, compared with Pre, in week 1 (9.17 ± 3.06 ng/mL vs. 4.96 ± 1.46 ng/mL) and week 3 (9.30 ± 4.14 ng/mL vs. 6.07 ± 1.51 ng/mL) respectively, as depicted in Fig. 3. Plasma HSP70 remained elevated by 1.4-fold at 30 min, compared with Pre, in week 1 (P < 0.01; 6.75 ± 1.74 ng/mL vs. 4.96 ± 1.46 ng/mL).

Effects of exercise on plasma cytokines and chemokines

A significant main effect of time was observed for IL-10 (Fig. 6, P = 0.01, η² = 0.053). Post hoc Tukey's multiple testing showed a 1.5-fold increase in IL-10 from Pre to Post in week 1. There was also a

significant effect of time and interaction for MCP-1 (Fig. 7, $P = 0.0003$; $\eta^2 = 0.073$, $P = 0.05$). Post hoc Tukey's multiple testing demonstrated a 1.2-fold increase in MCP-1 from Pre to Post, and from Pre to 30 min, both in week 3 ($P < 0.01$). There was no significant effect of intensity for either IL-10 or MCP-1. As well, there were no significant main effects of either intensity or time for IFN- γ ($P > 0.05$, not shown) or IL-8 ($P = 0.06$). However, there was a significant main effect of interaction for IL-8 ($P = 0.04$), with data from week 3 showing elevated concentrations of this chemokine from Pre to Post ($P = 0.0054$), and Pre to 30 min (Fig. 8; $P = 0.0076$).

—Insert Fig. 6—

—Insert Fig. 7—

—Insert Fig. 8—

Correlations between exercise-induced alarmins and MCP-1

Δ HMGB1 and Δ HSP70 correlated positively with Δ MCP-1 at all timepoints, as depicted in Fig. 9. A stronger linear relationship was observed for Δ MCP-1 with Δ HSP70 (slope = 0.9711) than with Δ HMGB1 (slope = 0.6574).

—Insert Fig. 9—

Effects of exercise on total leukocytes and monocyte subsets

A significant main effect of time was observed for mean cell counts of all leukocytes ($CD45^+$; $P < 0.0001$, $\eta^2 = 0.30$) (Fig. 10). The effect of exercise in enhancing leukocyte trafficking immediately after a bout of exercise was evident during the 3-week study. Post hoc Tukey's multiple testing demonstrated a 1.2- and 1.6-fold increase at Post relative to Pre for weeks 1 (9124 ± 1826 cells/ μ L vs. 7377 ± 1597 cells/ μ L) and 3 (10511 ± 2444 cells/ μ L vs. 6492 ± 1158 cells/ μ L), respectively. Exercise-induced leukocytosis was relatively mild in week 2 (6179 ± 2071 cells/ μ L vs. 5925 ± 1210 cells/ μ L), with a 4% increase at Post relative to Pre.

—Insert Fig. 10—

—Insert Fig. 11—

There was a significant effect of time on the response of $CD14^+CD16^-$ monocytes ($P < 0.0001$, $\eta^2 = 0.17$) (Fig. 11) and $CD14^+CD16^+$ monocytes ($P < 0.0001$, $\eta^2 = 0.20$) (Fig. 12). Furthermore, a significant interaction (time x intensity) was observed for $CD14^+CD16^+$ monocytes ($P = 0.02$). Post hoc Tukey's multiple testing demonstrated that an exercise-induced monocytosis was evident for both subsets. $CD14^+CD16^-$ monocytes were increased by 1.2-, 1.3- and 1.5-fold from Pre to Post in week 1 (486 ± 109

cells/ μL vs. 587 ± 152 cells/ μL , $P < 0.05$), 2 (381 ± 76 cells/ μL vs. 491 ± 130 cells/ μL , $P < 0.05$) and 3 (419 ± 131 cells/ μL vs. 610 ± 174 cells/ μL , $P < 0.0001$), respectively.

CD14⁺CD16⁺ monocytes also showed a similar response to exercise, increasing by 1.7-fold from Pre to Post in week 2 (25 ± 16 cells/ μL vs. 41 ± 23 cells/ μL , $P < 0.005$) and 1.5-fold from Pre to Post in week 3 (31 ± 21 cells/ μL vs. 47 ± 19 cells/ μL , $P < 0.01$). In addition, there was a 50% reduction in CD14⁺CD16⁺ monocytes at 30 min from Pre (31 ± 21 cells/ μL vs. 16 ± 9 cells/ μL , $P < 0.01$) in week 3.

—Insert Fig. 12—

Correlations between exercise-induced alarmins and monocytes

Of all the alarmins tested, only HMGB1 correlated significantly with CD14⁺CD16⁺ monocytes across 3 weeks of training (Fig. 13).

—Insert Fig. 13—

Effects of exercise on plasma creatine kinase and salivary cortisol

Mean plasma creatine kinase activity did not differ significantly with respect to time, intensity or interaction during the study ($P > 0.05$, Week 1: 31.51 ± 1.62 U/L; Week 2: 35.73 ± 3.61 U/L; Week 3: 44.93 ± 13.38 U/L). Whereas main effects were not significant, a significant interaction effect (time x intensity) was observed for salivary cortisol ($P = 0.01$, Week 1: 0.88 ± 0.51 $\mu\text{g/dL}$; Week 2: 1.14 ± 0.15 $\mu\text{g/dL}$; Week 3: 1.36 ± 0.34 $\mu\text{g/dL}$), with post hoc Tukey's testing showing decreased concentrations of cortisol at Post and 30 min, relative to Pre. Main effects of time or intensity for salivary cortisol were not statistically significant ($P > 0.05$).

Discussion

The novelty of our study is that this is the first study to measure multiple alarmins after repeated bouts of exercise with increasing intensity across 3 consecutive weeks. Furthermore, the increase in circulating alarmins (HMGB1, S100A8/A9, HSP70) varied temporally with one another and occurred in the absence of, or minimal corresponding changes in traditional cytokine markers of exercise-induced stress, such as IFN- γ , IL-8 and IL-10. As well, concentrations of plasma creatine kinase, a commonly used marker of skeletal muscle damage, did not show significant differences across time. We discuss these findings in detail in the subsequent sections.

Concurrent exercise training model

The combined training paradigm comprised multiple sets of short bouts of treadmill running at an initial 80% of maximal heart rate, as well as resistance exercises at approximately 50% of 1-RM, targeting major muscle groups and increasing workload by 10% and 20% in weeks 2 and 3 respectively. During treadmill running, subjects used mainly energetic systems between VT_1 and VT_2 (31) for the first two weeks and switched to slightly above VT_2 in week 3, suggesting the subjects were more reliant on anaerobic energetic systems during week 3. The exercise model in the present study closely resembles high-intensity interval exercises such as Crossfit® programs (34) that are gaining popularity with the general public,

Intense aerobic and resistance exercise training increases systemic alarmins without perturbing systemic creatine kinase

An interesting outcome in the present study is the dynamic responses of alarmins in the apparent absence of creatine kinase perturbation. Increased circulating creatine kinase as a consequence of skeletal muscle damage elicited by eccentric exercise typically occurs several hours after an exercise bout, with a peak around 5–7 days (35). The recovery period in this study was ~ 24 h on training days, with biological samples collected on the 3rd (72 h) day in week 2 and 3. Despite this, no significant changes in creatine kinase activity were observed across the study period, suggesting that skeletal muscle damage may be minimal. Hence, the fact that alarmins were induced with exercise in the absence of muscle damage may indicate either a subtler response to cellular stress or a systemic response to muscle contraction, increased body temperature and tissue hypoxia, and increased metabolic stress.

Exercise-induced alarmin release is modulated by exercise duration, intensity and type

The increase in systemic HMGB1 during 3 weeks of exercise training ranged from 1.2- to 1.4-fold in the present study, and is much lower, compared with reports by Beiter et al. (14) and Bekos et al. (12). Beiter et al. reported a 3-fold increase in plasma HMGB1 immediately after an exhaustive run (5.0 ng/mL), compared with resting concentrations (1.5 ng/mL). In the Bekos study, serum concentrations increased ~ 1.5-fold (half-marathon; 3.13 ± 1.63 ng/mL to 4.78 ± 2.1 ng/mL) and ~ 2.3-fold (marathon; 2.58 ± 1.58 ng/mL to 6.02 ± 2.18 ng/mL) after the respective races. These are fascinating comparisons, because they suggest that HMGB1 responds in a dose-dependent manner to the intensity of the exercise.

The outcomes in the present study for S100A8/A9 are similar with previous studies (18–20, 36). Fico et al. (18) demonstrated S100A8/A9 to be upregulated by ~ 2-fold with high-intensity interval cycling, whereas continuous cycle ergometry increased S100A8/A9 by ~ 5-fold (19). As well, Maharaj et al. (20) showed that a 30-minute treadmill running at 75% VO_2 max elicited an ~ 3-fold increase in S100A8/A9. In our study, S100A8/A9 appeared to be most responsive to increases in weekly training load, compared with other alarmins, with a 5-fold increase in plasma concentrations at Post, compared with Pre ($P \leq 0.01$) in the third week (Fig. 4). The magnitude of the increase in circulating S100A8/A9 may depend on

exercise intensity and duration, or the number of muscle fibers recruited, given that the concentrations of this alarmin were not increased with calf-raise exercise (21), whereas our subjects were performing whole body exercises with the strength exercises and treadmill runs. Alternatively, it cannot be ruled out that the exercise-induced release of the alarmins could be tissue-specific. Contracting skeletal muscle can secrete S100A8/A9 per se (19), with transcript expressions increasing by 5-fold in skeletal muscle after cycle ergometry (19).

Systemic elevation of HSP70 was observed during 3 weeks of exercise training, suggesting that increased concentrations of this alarmin may be necessary to facilitate protein degradation and folding, prevent cellular apoptosis and also to up-regulate innate and adaptive immunity (37). Although it is possible that contracting skeletal muscle can secrete HSP70 (22) per se, the exercise-induced release of HSP72 into circulation precedes the increase in mRNA transcription in skeletal muscle (16), suggesting that the acute release of HSP70 into peripheral blood may have originated from other organs, such as the liver (17) or alternatively, HSP70 may be released from skeletal muscles and packaged within extracellular vesicles, such as exosomes (38), given their rapid appearance in plasma. However, since exosomal HSP70 was not isolated in the present study, this point remains speculative.

sRAGE was of interest in this study, as it binds extracellular HMGB1 and S100 proteins, and has been shown to be present in soluble form (35). Exercise training in the present study appeared not to modulate changes in sRAGE concentration. This contrasted with previous reports, where high-intensity aerobic exercise training for 8 weeks increased plasma concentrations of sRAGE in sedentary men (34), and 3 months of aerobic exercise also enhanced plasma sRAGE in diabetic patients (5). One explanation may be that 3 weeks of exercise with increasing workload was too short to induce similar effects as reported by others. Another possibility is that the subjects in this study were young, active men with no co-morbidities, which was different from the subjects recruited by Santilli et al. (34) and Choi et al. (5). Another possibility for the discrepancy could be due to the combined energetic pathways (aerobic, anaerobic) of the exercise training program, which may be insufficient to modulate increased sRAGE release.

Concurrent exercise training increased circulating monocyte subsets

The role of CD14⁺CD16⁻ and CD14⁺CD16⁺ monocyte subsets in modulating alarmin and immune responses (11), as well as in tissue remodeling (3), was of interest, particularly in the context of intense exercise training. The results are generally consistent with the findings of others (20) in that acute exercise of moderate-to-high intensity mediates an increase in circulating CD14⁺CD16⁻ and CD14⁺CD16⁺ monocytes. Exercise also increased CD14⁺CD16⁻ monocytes from resting levels in all three weeks, whereas the elevation in CD14⁺CD16⁺ monocytes was seen only in week 2 and 3 (Fig. 12). The present findings differed from Leicht and co-authors (39) in that increases in both monocyte subset numbers occurred immediately after exercise, whereas Leicht et al. reported only a significant increase in CD14⁺CD16⁻ monocytes but not CD14⁺CD16⁺ monocytes, and only during a two hour recovery from

exercise. Significant changes were not detected immediately after exercise in their study. The exercise program in the present study involved both upper and lower body muscle groups, and thus the subjects could have experienced a higher oxygen cost of activity, which may coincide with greater recruitment of monocytes via increased secretion of epinephrine. This could then explain for the faster increase in appearance of the monocyte subsets.

The magnitude of CD14⁺CD16⁺ monocyte responses to exercise and after recovery appears to be dependent on the intensity and duration of the exercise, with exercise-induced catecholamines mobilizing CD14⁺CD16⁺ monocytes (40). The absence of a significant increase in CD14⁺CD16⁺ monocytes in week 1 suggested that the prescribed exercise intensity was below the threshold needed to mobilize them from the marginal pool into the peripheral blood circulation, whereas the increase in this monocyte subset in weeks 2 and 3 suggested that an augmented stress response may have been responsible. Reduced numbers of circulating CD14⁺CD16⁺ monocytes were observed below baseline levels during recovery (30 min) in week 3, suggesting that some demargination of monocytes into tissues may have occurred post-exercise (40).

Exercise-induced alarmin release is associated with increased circulating monocyte subsets

Whether exercise training-induced changes in alarmins were associated with that in different monocyte subsets was of interest. To this extent, both HMGB1 and HSP70 demonstrated a positive correlation with MCP-1, with the case for HSP70 and monocyte recruitment appearing to be more sensitive to exercise intensity per se. Throughout the 3 weeks of exercise training, the regression slopes of Δ HSP70 relative to Δ MCP-1 decreased in week 2 and increased again in week 3 (Fig. 9B). Conversely, HMGB1 appears to be a generic modulator of exercise stress (Fig. 9A; all 3 linear regressions have similar slopes), although it appears to be a more specific inducer of CD14⁺CD16⁻ monocytes than the other alarmins (Fig. 13).

While cautious interpretation of these results is warranted, the present data suggest that the subjects experiencing greater metabolic stress or inflammation at various timepoints during the study may have increased circulating monocytes, particularly the CD14⁺CD16⁻ subset, and this may be related to the level of circulating alarmins. The temporal responses of each of these alarmins in the current study are complicated. It is known that HSP70 can inhibit both nuclear translocation of HMGB1 as well as HMGB1-induced release of pro-inflammatory cytokines in RAW264.7 macrophages (41, 42). While speculative, we suggest that exercise-induced HMGB1 resulted in a corresponding response in increased HSP70 to dampen the inflammatory response (43). The increased appearance of the CD14⁺CD16⁻ subsets may be driven by the need to resolve tissue inflammation.

Conclusion

In this short-term training study, alarmins are released into systemic circulation by combined aerobic and resistance exercise, with their release and clearance differing temporally. Especially, increased levels of

circulating alarmins (HMGB1, S100A8/A9, HSP70) occurred in the absence of, or minimal corresponding changes in traditional cytokine markers of exercise-induced stress and inflammation, such as IFN- γ , IL-8 and IL-10 as well as in muscle damage markers (e.g. creatine kinase). Further investigations are necessary to elucidate the alarmin responses to long-term exercise training, as well as to different duration, types and intensities of exercise. As well, future studies will need to demonstrate downstream signaling pathways in relevant tissues (e.g. skeletal muscle) or immune cells to delineate how exercise stress modulates alarmin and inflammatory signaling (44).

Declarations

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Author Contributions

J.G conceptualized and designed the study. J.G., N.H.A, L.S.H.G and P.L.T conducted the trials. J.G., N.H.A. L.S.H.G and P.L.T performed the experiments. J.G, N.H.A., P.H, L.S.H.G, F.T., A.M, G.T and S.C.W analyzed and interpreted the data. J.G. and L.S.H.G drafted and revised the manuscript. All authors reviewed, commented and approved the submitted manuscript.

Competing Interests

The authors have no competing interests to declare.

Availability of Data and Materials

The datasets used and/or analyzed in this study are available upon request from the corresponding author.

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Ethics Approval

All subjects gave written informed consent and underwent comprehensive medical screening. Medically cleared subjects were then enrolled in the study. Baseline testing and all exercise sessions were performed in the Human Performance Laboratory at the Singapore Sports Institute. This study was approved by both Institutional Review Boards of DSO National Laboratories/ Singapore Armed Forces (ethics no. 0023/2014) and the Singapore Sports Institute (ethics no. PH/EXP/008). All procedures performed on our subjects were in accordance with the ethical standards of the institution and with the 1964 Helsinki declaration and its later amendments.

Consent for Publication

All authors have reviewed the manuscript and give consent for its publication.

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Figures

* Treadmill speed increased by 10%
 *Reps increased by 10%

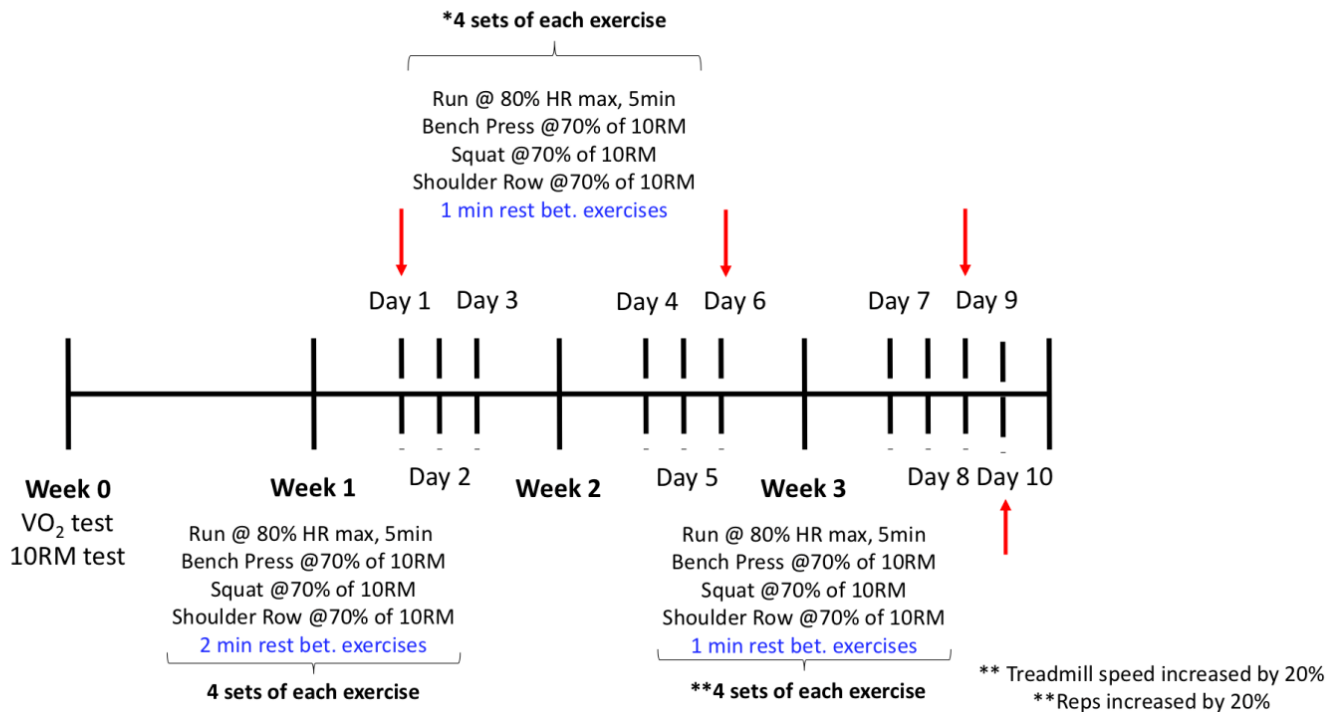


Figure 1

Schematic overview of the study design. Days 1 - 3 represent the 3 consecutive days of training in week 1, days 4 - 6 in week 2, and days 7 - 9 in week 3 correspondingly. Day 10 represents the 24 h timepoint where participants return to the laboratory for a resting biospecimen collection. All biological sampling timepoints are indicated by red arrows.

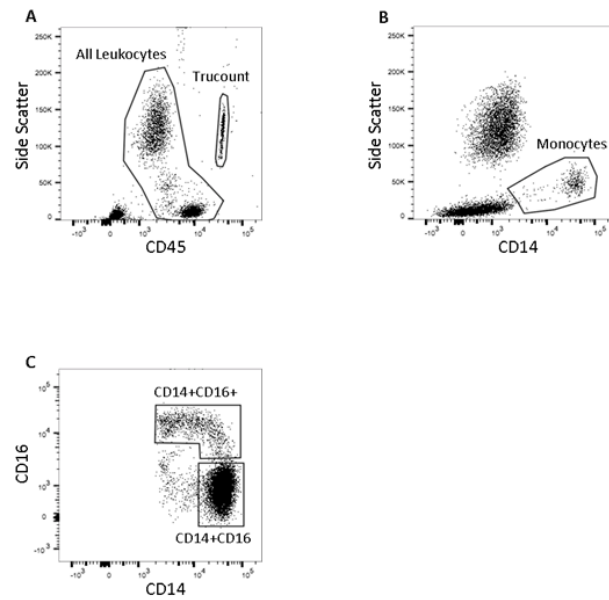


Figure 2

Multicolor flow cytometric analyses of absolute cell count in whole blood. All Trucount beads and all leukocytes represented as CD45+ were gated in (A). Total monocytes were gated on CD45+ leukocytes as shown in (B). The CD14+CD16- and CD14+CD16+ monocyte subsets were further gated from the monocyte population as shown in (C).

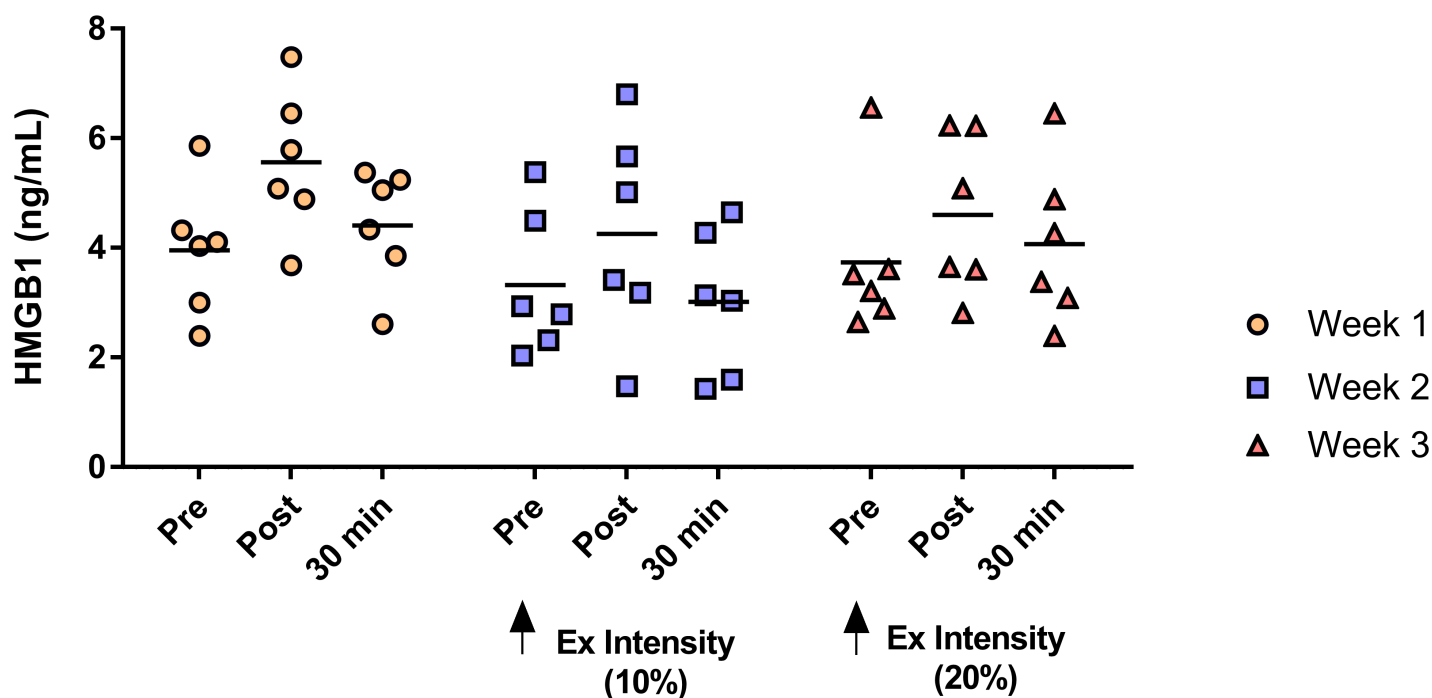


Figure 3

Plasma [HMGB1] during 3 weeks of exercise training. Data showed a significant main effect of time ($P = 0.028$) but Post hoc Tukey's multiple testing did not show statistical significance in mean HMGB1 within each week. No significant main effect of intensity or interaction was detected ($P > 0.05$). Pre, Post and 30 min are timepoints of blood sampling before, immediately after, and 30 min after the exercise bout respectively.

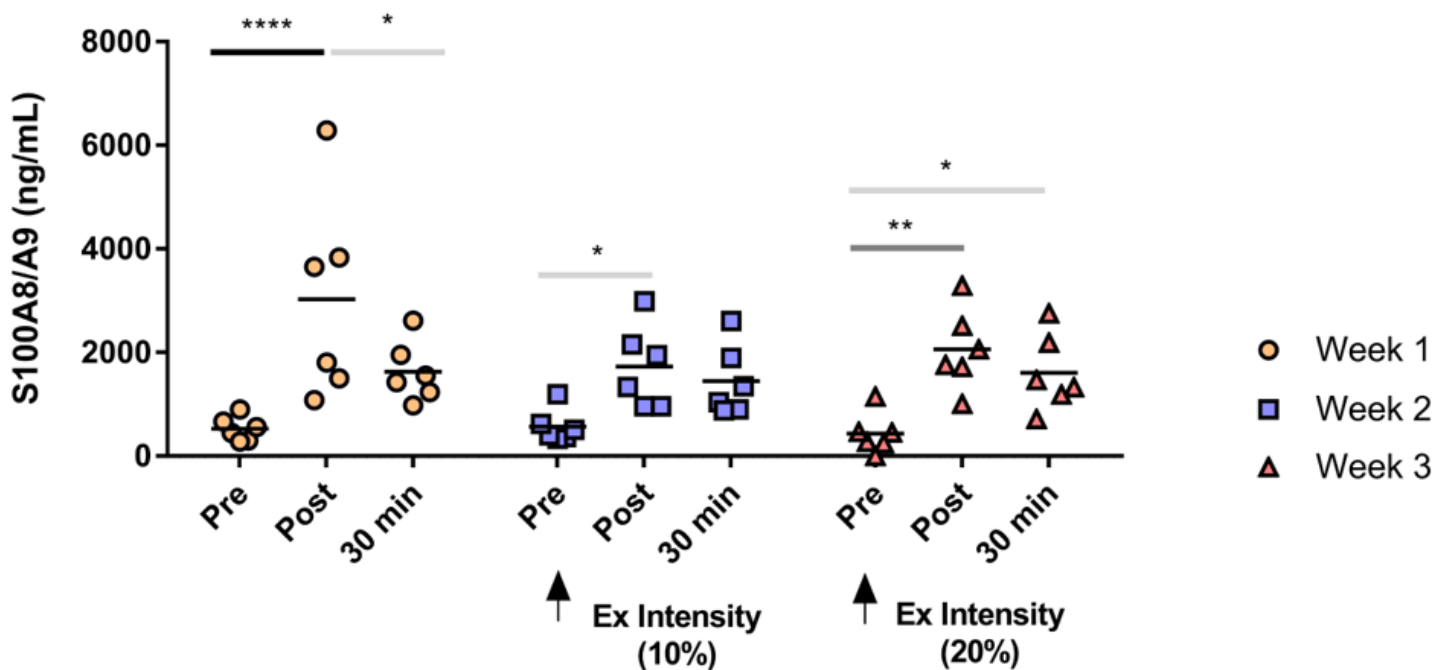


Figure 4

Plasma [S100A8/A9] from individual participants during 3 weeks of exercise training. A main effect of time was observed ($P < 0.0001$). Post hoc Tukey's multiple comparison test within each week: * $P < 0.05$, ** $P < 0.001$, **** $P < 0.0001$.

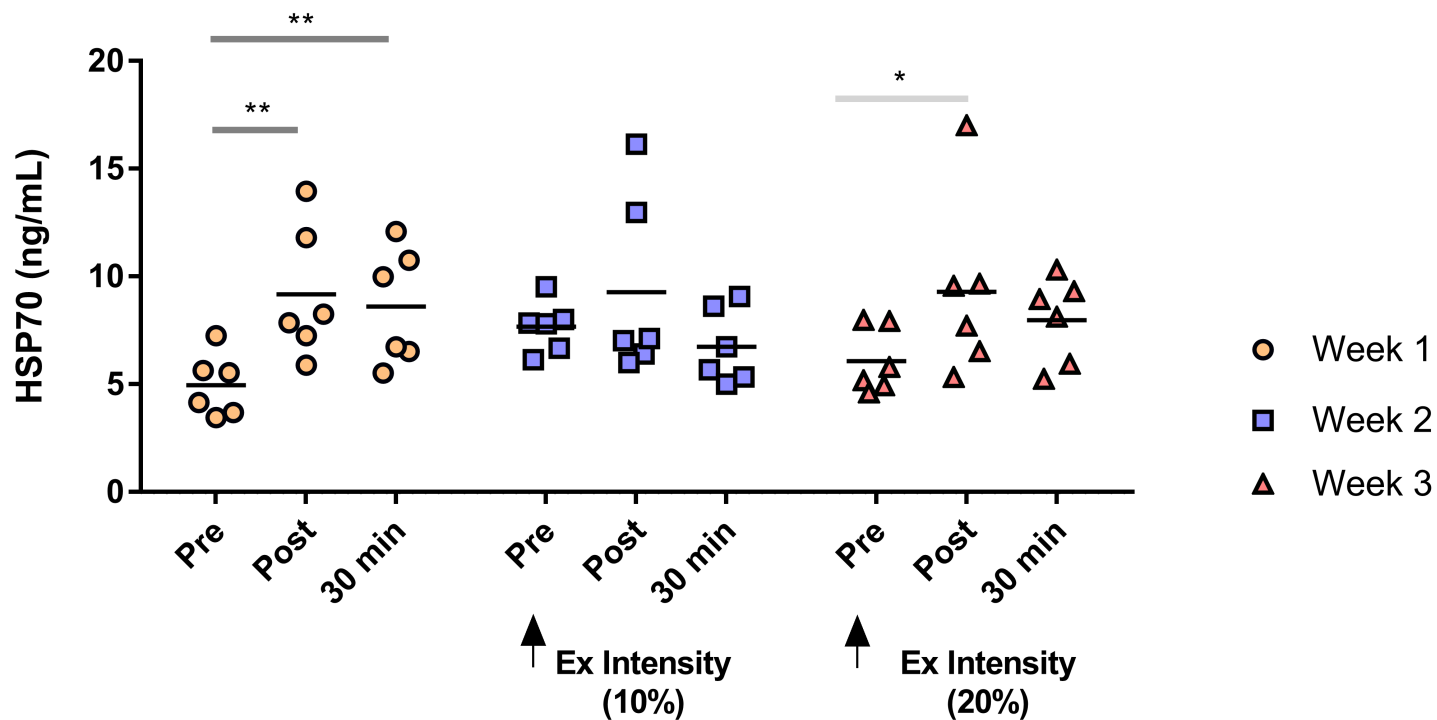


Figure 5

Plasma [HSP70] during 3 weeks of exercise training ($N = 6$). A significant main effect of time was observed ($P = 0.0003$). Post hoc Tukey's test was used to compare mean HSP70 between timepoints within each week. * $P < 0.05$ and ** $P < 0.01$.

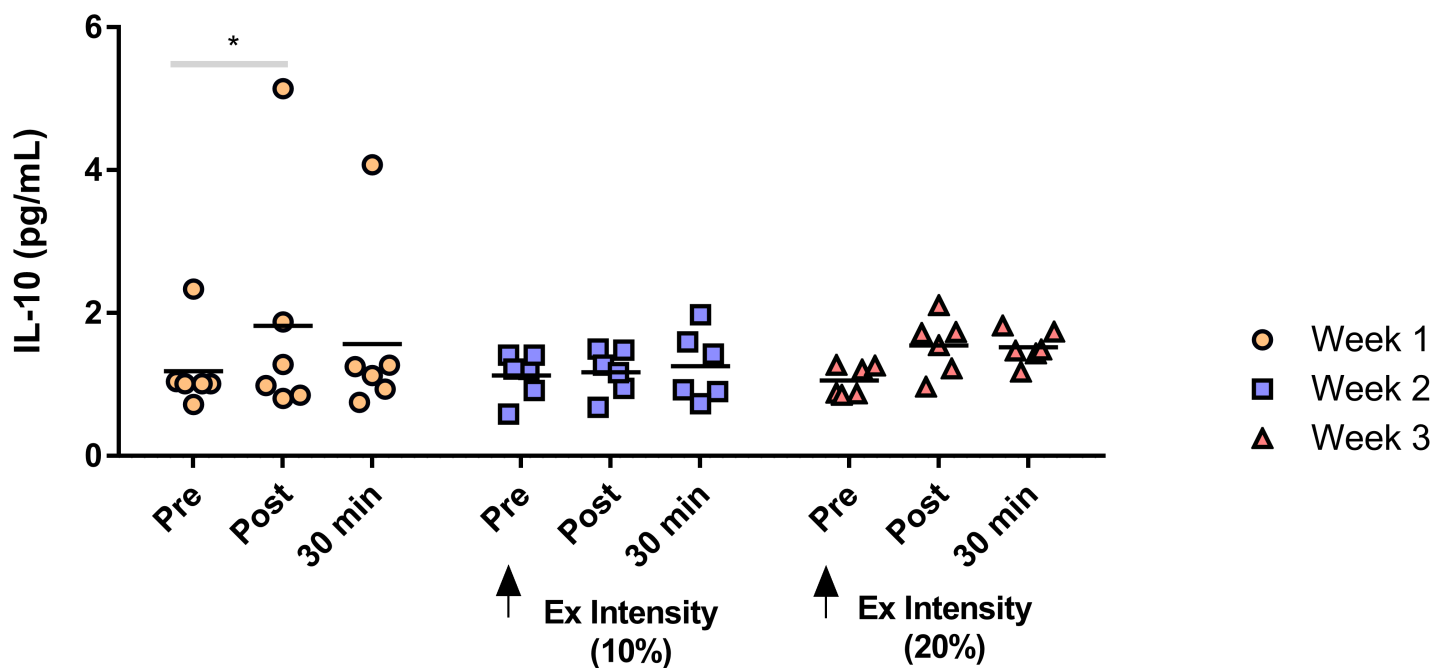


Figure 6

Plasma [IL-10] during 3 weeks of exercise training (N = 6). A main effect of time was detected (P = 0.01). Post hoc Tukey's multiple testing further showed significant differences in plasma IL-10 at Pre vs. Post in week 1. *P < 0.05.

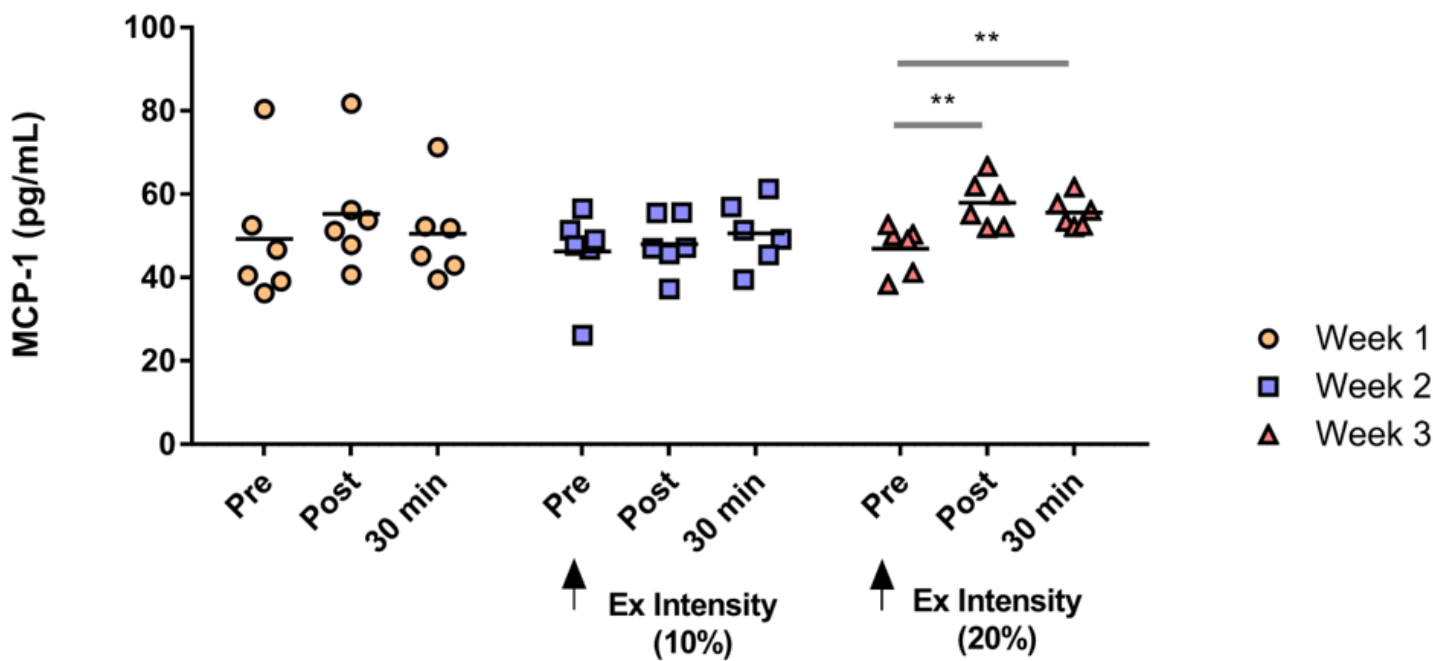


Figure 7

Plasma [MCP-1] during 3 weeks of exercise training (N = 6). Significant main effects of time ($P = 0.0003$) and interaction ($P = 0.05$) were detected. Post hoc Tukey's multiple testing showed significant differences in week 3, between Pre and Post ($P < 0.01$) and between Pre and 30 min ($P < 0.01$). ** $P < 0.01$. No significant main effect of intensity was observed ($P > 0.05$).

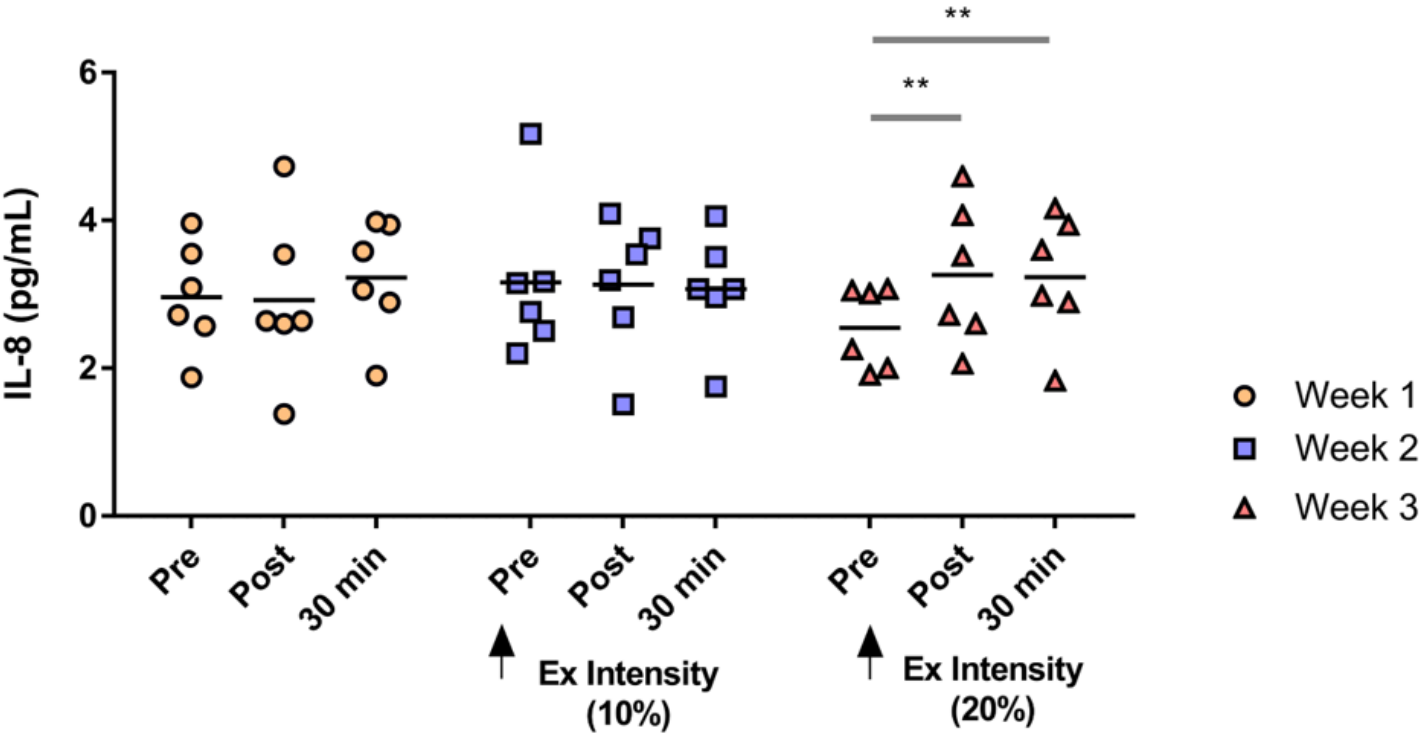


Figure 8

Plasma [IL-8] during 3 weeks of exercise training (N = 6). A significant main effect of interaction (time x intensity) was observed ($P = 0.04$). Post hoc Tukey's multiple testing showed significant differences in week 3, between Pre and Post ($P < 0.01$), and between Pre and 30 min ($P < 0.01$). ** $P < 0.01$.

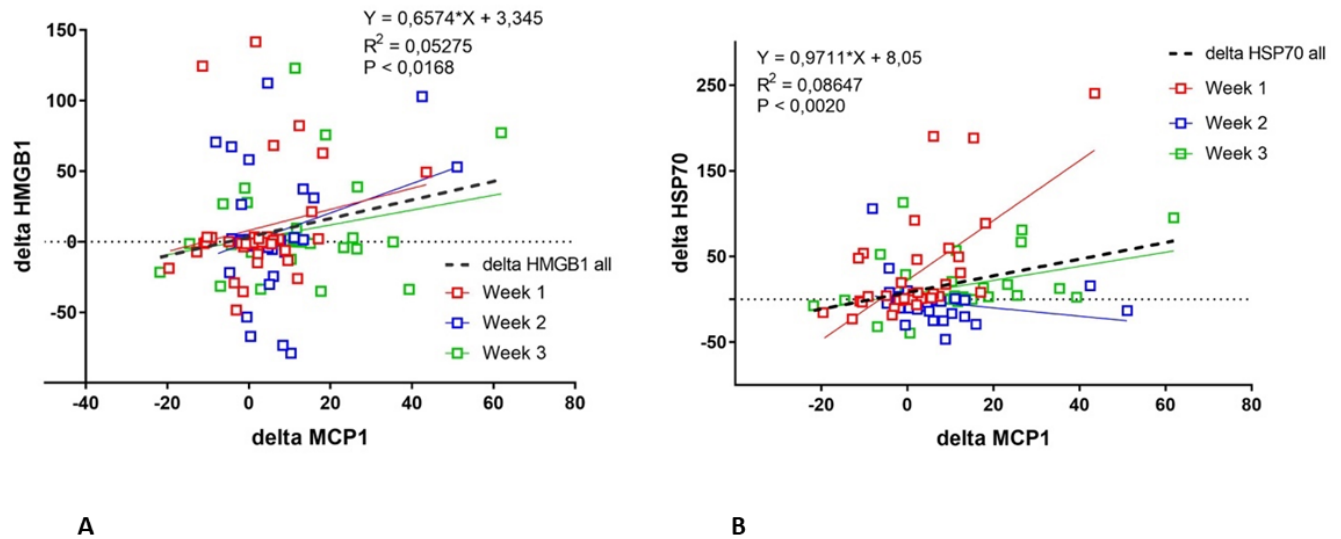


Figure 9

Relationship of Δ HMGB1 and Δ HSP70 with Δ MCP-1 during 3 weeks of exercise training. Linear regression plots demonstrating Δ HMGB1 (A) and Δ HSP70 (B) relative to Δ MCP1 during 3 weeks of exercise training.

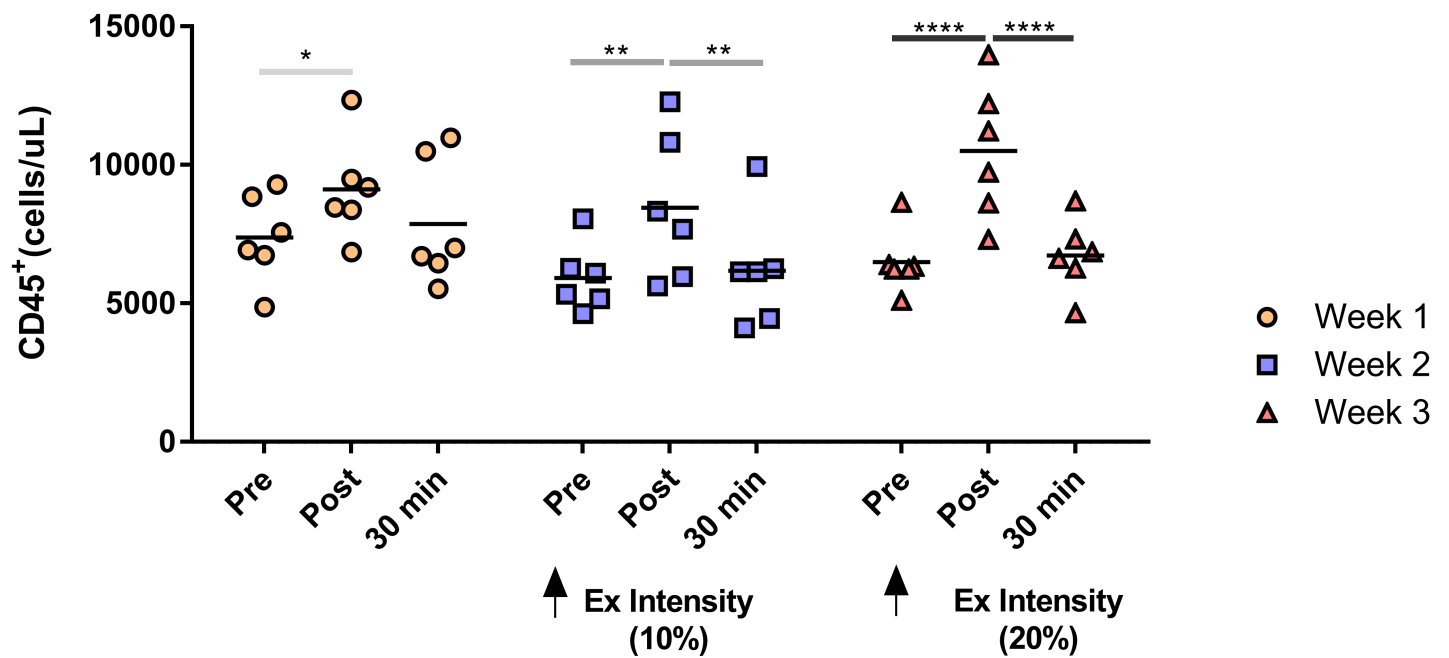


Figure 10

Circulating numbers of CD45+ leukocytes during 3 weeks of exercise training (N = 6). Significant effect of time was detected ($P < 0.0001$). Post hoc Tukey's adjusted significance: * $P < 0.05$, ** $P < 0.01$, **** $P < 0.0001$. No significant main effect of intensity or interaction was observed ($P > 0.05$).

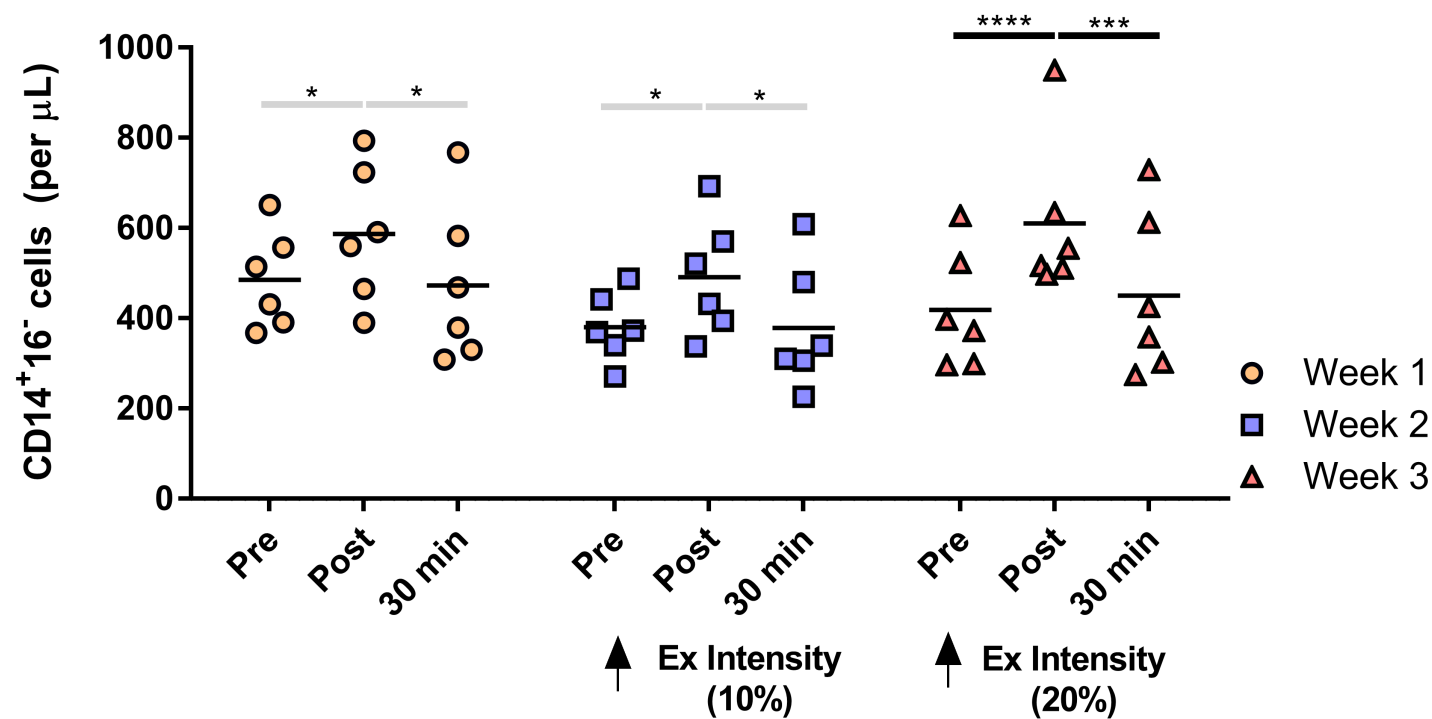


Figure 11

Circulating numbers of CD14+CD16- monocytes during 3 weeks of exercise training (N = 6). Significant effect of time was detected ($P < 0.0001$). Post hoc significance using Tukey's multiple test: * $P < 0.05$, *** $P < 0.001$, **** $P < 0.0001$. No significant main effect of intensity or interaction was observed ($P > 0.05$).

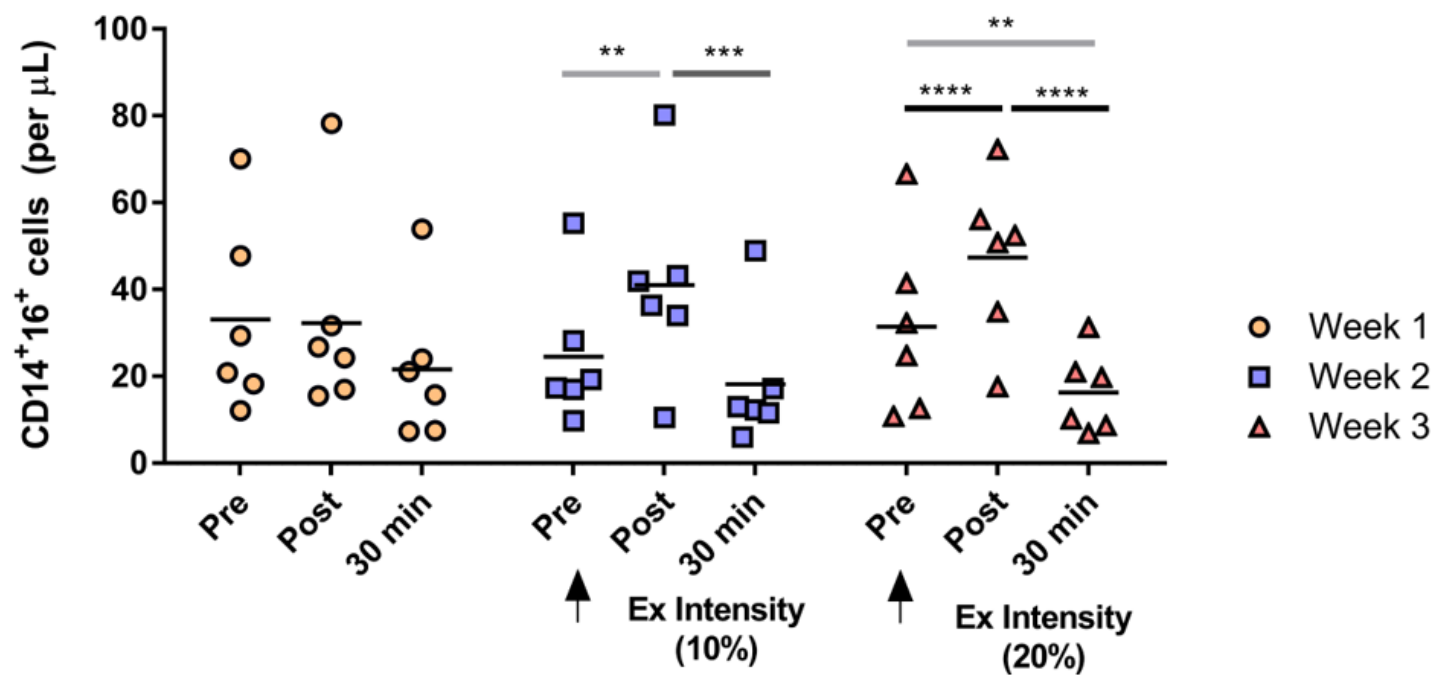


Figure 12

Circulating numbers of CD14+CD16+ monocytes during 3 weeks of exercise training (N = 6). Significant effect of time ($P < 0.0001$) and interaction (time x intensity; $P = 0.02$) were detected. Post hoc significance using Tukey's multiple test: ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$. No significant main effect of intensity was observed ($P > 0.05$).

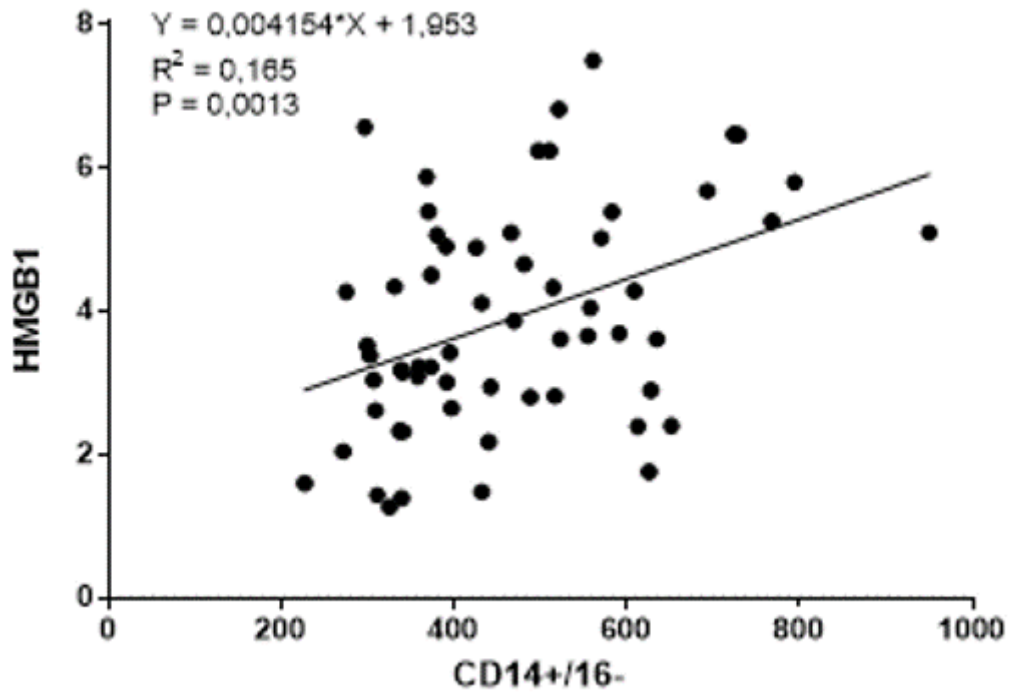


Figure 13

Correlation between plasma HMGB1 with circulating CD14+CD16- monocytes during 3 weeks of exercise. HMGB1 showing a positive correlation with CD14+CD16- cell counts.