Modulation of macrophage polarization by level-1 Yo-Yo intermittent recovery test in young football players

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Abstract
The aim of this study was to examine the effect of the level-1 Yo-Yo intermittent recovery test (YYIRT1) on polarization of macrophages in young football players. Fourteen male football players (19.9 ± 1.4 years old) were enrolled in this study. YYIRT1 was performed with 20-meter shuttle runs at increasing speeds and 10-second active recovery in a 5-meter distance between runs till exhaustion. Fasting blood samples were collected before and immediately after YYIRT1. Analysis for macrophage polarization by flow cytometry, reactive oxygen species (ROS) by flow cytometry, biochemical parameters by chemical reactions, and serum cytokines by ELISA were performed. The rating of perceived exertion (RPE) and cardiovascular parameters were recorded.

The time to exhaustion was 714.1 ± 114.4 seconds. The oxygen uptake (VO2max) was 48.7 ± 5.6 mL/min/kg, RPE scale was 19 ± 1, resting heart rate and maximal heart rate were 64.9 ± 8.8beat/min and 181.9 ± 9.3beat/min, respectively, indicating a high level of cardiopulmonary fitness. The expression of macrophage-specific CD14 and M1 marker HLA-ABC, but not M2 marker CD206, was down-regulated after YYIRT1. The intracellular ROS levels in macrophages had no significant change. In biochemical profile, the serum levels of lactic dehydrogenase (LDH), a marker of muscle damage, increased after YYIRT1 whereas no significant alteration was noted in creatine phosphokinase (CPK), blood urine nitrogen, creatinine, aspartate transaminase (AST), alanine transaminase (ALT), and C-reactive protein. The serum levels of interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)-α had no significant change.

The YYIRT1 may induce muscle damage accompanied by modulation of macrophage polarization toward suppression of M1 phenotype in young football players.

Abbreviations: ALT = alanine transaminase, AST = aspartate transaminase, BUN = blood urine nitrogen, CD = cluster of differentiation, CK = creatine kinase, CPK = creatine phosphokinase, DCFH-DA = 2',7'-dichlorodihydrofluorescein diacetate, HLA = human leukocyte antigen, IFN-γ = interferon-γ, IL = interleukin, LDH = lactic dehydrogenase, ROS = reactive oxygen species, RPE = rating of perceived exertion scale, Th = T helper, TNF-α = tumor necrosis factor-α, VO2max = maximal oxygen uptake, YYIRT1 = level-1 Yo-Yo intermittent recovery test.

Keywords: biochemical parameters, intermittent recovery test, macrophage polarization

1. Introduction
Football is a high-intensity intermittent exercise consisted of continuous movement incorporating frequent bursts of high-intensity activity.[1] It is characterized by the high aerobic load and the extensive anaerobic energy turnover.[2]

To evaluate the ability to perform high-intensity intermittent exercise, the level-1 Yo-Yo intermittent recovery test (YYIRT) was designed as a specific tool.[3,4] For exercise such as adolescent handball players, YYIRT score could provide a useful composite index of the response to training or rehabilitation.[5] YYIRT has been widely utilized in monitoring cardiopulmonary fitness of football players because it correlates with match performance.[6]

The metabolic responses to the YYIRT had been reported, including the changes in levels of lactate, glucose, triglycerides, and uric acid.[7] Its correlation to the elevation of biochemical markers of muscular damage such as creatine kinase (CK), and lactate dehydrogenase (LDH) had also been investigated.[8–10] However, the effect of YYIRT1 on immune status related to exercise and athlete health remains unclear. The importance of other immune lineage cells, including T cells, NK cells, and dendritic cells, involved in immunomodulation by football exercise had been demonstrated.[11–13] Given that macrophages consist of the most abundant and first-line innate immune cells with antigen presentation ability, we focused on the modulation of macrophage polarization by YYIRT1 in football players.

Macrophages, cells of innate immunity, are belonging to one of the subsets of human antigen-presenting cells.[14] Plasticity and heterogeneity is a hallmark of macrophages which is characterized...
by classification of macrophages into 2 different polarized states, one is the classically activated macrophages phenotype (M1) and another one is the alternatively activated macrophages phenotype (M2). The M1 induced by lipopolysaccharides (LPS) and Th1 cytokine interferon-γ (IFN-γ) possess high antigen-presenting ability and are more effective at antigen clearance and switching T-cell responses toward T helper-1 immune response. In contrast, the M2 responding to Th2 cytokine interleukin-4, interleukin-10 (IL-10), and interleukin-13 activation are anti-inflammatory with immunosuppressive capacities. The M1 subtype expresses major histocompatibility complex class II, CD80, CD86, and can secrete IL-12. M2 populations are alternatively activated with expression of associated markers including CD163, CD206, CD209, and can secrete IL-10. Exercise, especially high-intensity sports, may cause inflammatory reactions and alteration in activation state of immunity. However, the effect of exercise on macrophage polarization remains undetermined.

In the present study, we evaluated the changes in macrophage subsets responded to YYIRT1.

2. Materials and methods

2.1. Subject

Fourteen elite young football players from Chinese Culture University (Taipei, Taiwan), were recruited. All the enrolled subjects were male. Each cyclist had been engaged in football training for 2–4 years before the study. In the subjects, the amount of alcohol intake and smoking had been restricted by the in-charge coach. No subjects participated in competitions in 3 months prior to the study. No clinical illnesses were noted, and no medical or surgical treatment was given during the 6 months prior to blood sampling. The pre-test blood was sampled after subjects had rested quietly for 30 minutes before the test. This study was approved by institutional review board (MMH-17MMHIS170) with informed consents.

2.2. The level-1 Yo-Yo intermittent recovery test (YYIRT1)

The YYIRT1 was performed according to the procedures suggested by Krstrup et al[3] with modification. The reliability of YYIRT1 was assessed by the intraclass correlation coefficient (ICC). The ICC of YYIRT1 was 0.98 (95% CI: 0.97–0.99). The reliability of YYIRT1 was assessed by the ICC.

Figure 1. Flow cytometric analysis for expression of surface antigens in macrophages. A, representative dot-plot figures and histograms before YYIRT1; (B) representative dot-plot figures and histograms after YYIRT1; and (C) bar charts for comparison of data. Data were expressed as mean ± standard deviation. ∗ P < .05 for comparison of data between before and after YYIRT1.
of YYIRT1 was established in a previous study. The test consisted of 20-meter shuttle runs with increasing speeds and 10 seconds of active recovery in 5-m distance between runs till exhaustion. The end of the test was defined as twice of failure to reach the front line in time. The total time (seconds) covered during the YYIRT1 was recorded. All participants had a warm-up consisting of the first four running bouts before the test. The total duration of the test was set in 6 to 20 minutes.

2.3. Rating of Perceived Exertion Scale (RPE)
The RPE scale assessment was performed to record the subjective exertion rating for the physical task of the participants.

2.4. Measurement of cardiopulmonary fitness
Maximal oxygen uptake (\(\text{VO}_{2\text{max}}\)) was measured using a graded maximal treadmill protocol applied in our previous study. In brief, oxygen uptake and ventilation were measured using a cardiopulmonary exercise testing system (Q-plus IW/Corival 400, Seattle, WA) equipped on a treadmill (Quinton-645). The participants warmed up by walking at 3.0 mph on a 10% grade initially and ran at increasing speeds with an increment of 0.5 mph per minute. The maximal heart rate was recorded by using a polar pacer heart rate monitor.

2.5. Assessment of macrophage polarization
After lysis of red blood cells, the mononuclear cells (MNCs) from blood samples were separated by centrifugation on a density gradient (Ficoll-Hypaque, 1.077 gm/mL, Pharmacia Fine Chemicals). Immunolabeling was performed using fluorescei-conjugated antibodies including isotype control, CD14 (for monocyte/macrophage) (Clone M5E2, BD Biosciences, NJ), HLA-ABC (for M1) (Clone G46–2.6, BD Biosciences), and CD206 (for M2) (Clone 19.2, BD Biosciences). Before surface marker analysis, the cell debris was excluded by forward and side scattering on flow cytometer.

2.6. Fluorocytometric analysis of intracellular reactive oxygen species (ROS)
Intracellular ROS level was assessed by staining with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) (Molecular Probes). Briefly, phosphate buffered saline-washed cells were incubated with 1 \(\mu\)M DCFH-DA for 15 minutes at 37°C before the fluorocytometric analysis. The DCF level was measured with the FL-1 channel (green fluorescence).

2.7. Measurement of serum biochemical profiles
The serum levels of lactate dehydrogenase (LDH), creatine phosphokinese (CPK), blood urine nitrogen (BUN), creatinine, aspartate transaminase (AST), alanine transaminase (ALT) and C-reactive protein were measured by using chemical reactions as previously described.

2.8. Measurement of serum cytokines
Serum levels of cytokines were quantified by a sandwich enzyme-linked immunosorbent assay (ELISA). IL-6, IL-10 and TNF-\(\alpha\) were measured using Quantikine ELISA kits (R&D Systems) according to the manufacturer’s instructions.

2.9. Statistical analysis
Data were expressed as mean ± standard error of the mean (SEM). The changes in the parameters before and after YYIRT1 were evaluated by paired t-test using SPSS 12.0 software (SPSS Inc, Chicago, IL). Statistical significance was defined as a \(P\) value of less than .05.

3. Results

3.1. Total duration of YYIRT1 and cardiopulmonary fitness parameters
The time to exhaustion for YYIRT1 was 714.1 ± 114.4 seconds. RPE scale was 19.1 ± 1.1. The resting and maximal heart rates of the cyclists were 64.9 ± 8.8 and 181.9 ± 9.3 beats/min, respectively. The mean \(\text{VO}_{2\text{max}}\) was 48.7 ± 5.6 mL/kg/min. These parameters indicated the participants did possess a high level of cardiopulmonary fitness.

3.2. The effect of YYIRT1 on macrophage polarization
The expression of macrophage-specific CD14 and M1 marker HLA-ABC, but not M2 marker CD206, was down-regulated after YYIRT1 (Fig. 1).

3.3. The effect of YYIRT1 on biochemical profiles
In biochemical profile, the serum levels of LDH, a marker of muscle damage, increased after YYIRT1, indicating a possible muscle damage by YYIRT1 (Fig. 2A). No significant alteration...
was noted in CPK, blood urine nitrogen, creatinine, aspartate transaminase, alanine transaminase, and C-reactive protein (Fig. 2A,B).

3.4. The effect of YYIRT1 on intracellular superoxide levels

The intracellular ROS levels in macrophages had no significant change (Fig. 3).

3.5. The effect of YYIRT1 on serum cytokine levels

The serum levels of interleukin (IL)-6, IL-10 and tumor necrosis factor (TNF)-α had no significant change (Fig. 4).

4. Discussion

Our findings indicate that YYIRT1 caused elevation of serum LDH levels and down-regulated the M1 polarization of macrophages in young football players.

The main reason for the small test sample number is that only elite and young football players were recruited. We enrolled these elite football players from the national representative team to ensure high and relatively homogenous cardiopulmonary fitness levels of subjects. However, the small sample size remains a limitation of this study.

At initial or acute phase of tissue injury, the recruited macrophages that infiltrate the ischemic muscle are polarized to a pro-inflammatory M1-like phenotype. These M1-polarized macrophages may increased significantly suppress the arteriogenic response to decrease the perfusion recovery. By contrast, the M2 polarized macrophages play an important role in the resolution of tissue and muscle damage through removal of necrotic cells to promote repair. Strategy of therapies that are aimed at decreasing pro-inflammatory and increasing the anti-inflammatory macrophage populations in ischemic muscle are regarded beneficial to repair of tissue and muscle damage. Data from young football players in the present study show that
YYIRT1 caused elevation of serum LDH levels, indicative of resultant muscle damage. Intriguingly, YYIRT1 suppressed the M1 polarization of macrophages in young football players. Taken together, it implicates that YYIRT1 may cause muscle damage with a stress reaction to suppress inflammation related to M1 polarization of macrophages.

The reversible nature of macrophage polarization between M1 and M2 phenotypes is characteristic to their plasticity.[15] Pharmacological targets, such as CREB-C/EBP beta cascade, sustaining M2 macrophage polarization to promote vascular remodeling and perfusion recovery in ischemic muscle have been developed.[15] YYIRT1 had no significant effect on polarization of macrophages towards M2 phenotype. The mechanism for differential effect of YYIRT1 on M1, but not M2, polarization remains unclear and warrants further investigation.

Author contributions

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