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Conducting maximal and submaximal endurance exercise testing to measure physiological and biological responses to acute exercise in humans

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June 21st, 2018

Dr. Alisha DSouza
Senior Review Editor

Dr. Nandita Singh, Ph.D.
Senior Science Editor, JoVE

Dear Editors,

Please see our revised manuscript titled **“Conducting maximal and submaximal endurance exercise testing to measure physiological and biological responses to acute exercise in humans”** (JoVE58417) for publication in the Journal of Visualized Experiments.

We have addressed the reviewer comments and also the editorial comments and are grateful for the constructive comments. Thank you again for your consideration of this manuscript. We appreciate the efforts to make this a better manuscript.

Sincerely,

Michael P. Gustafson, Ph.D.

TITLE:

Conducting Maximal and Submaximal Endurance Exercise Testing to Measure Physiological and Biological Responses to Acute Exercise in Humans

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Keywords:

exercise testing, maximal oxygen consumption, VO₂ testing, gas exchange, submaximal endurance exercise, physiological responses

Short Abstract:

To assess the influence of exercise intensity on physiologic and biologic responses, two different exercise testing protocols were utilized. Methods outlining exercise testing on a cycle ergometer as an incremental maximal oxygen consumption test and endurance, steady state submaximal endurance test are described.

Long Abstract:

Regular physical activity has a positive effect on human health, but the mechanisms controlling these effects remain unclear. The physiologic and biologic responses to acute exercise are predominantly influenced by the duration and intensity of the exercise regimen. As exercise is increasingly thought of as a therapeutic treatment and/or diagnostic tool, it is important that standardizable methodologies be utilized to understand the variability and to increase the reproducibility of exercise outputs and measurements of responses to such regimens. To that end, we describe two different cycling exercise regimens that yield different physiologic outputs. In a maximal exercise test, exercise intensity is continually increased with a greater workload resulting in an increasing cardiopulmonary and metabolic response (heart rate, stroke volume, ventilation, oxygen consumption and carbon dioxide production). In contrast, during endurance exercise tests, the demand is increased from that at rest, but is raised to a fixed submaximal exercise intensity resulting in a cardiopulmonary and metabolic response that typically plateaus. Along with the protocols, we provide suggestions on measuring physiologic outputs that include, but are not limited to, heart rate, slow and forced vital capacity, gas exchange metrics, and blood pressure to enable the comparison of exercise outputs between studies. Biospecimens can then

be sampled to assess cellular, protein, and/or gene expression responses. Overall, this approach can be easily adapted into both short- and long-term effects of two distinct exercise regimens.

Introduction:

Physical activity is defined as any bodily movement produced by skeletal muscles that require energy expenditure¹. Exercise is a physical activity that involves repetitive bodily movement done to improve or maintain one or more components of physical health². At one time, physical activity was not recommended for those who were seriously ill. For individuals with cancer, heart failure, or even for those who were pregnant, bed rest was preferred over physical activity. Clinical practice has since drastically changed, as the benefits of exercise on overall health are becoming clearer³. Regular exercise has been shown to help reduce cardiovascular disease risk, all-cause mortality, cancer risk and hypertension, improve blood sugar control, facilitate weight loss or maintenance, and prevent bone and muscle loss⁴⁻⁸.

The extensive benefits of exercise have now led many to utilize exercise as a type of “medicine” and an alternative or adjunct treatment option for a variety of conditions³. Shulman *et al.* demonstrated that a combination of treadmill and resistance exercise could result in improvements in gait speed, aerobic capacity and muscular strength which could improve motor control and overall quality of life in patients with Parkinson’s disease⁹. In heart failure patients, exercise intolerance and inadequate pharmaceutical interventions contribute to a poor quality of life¹⁰. Initial results from heart failure patients undergoing exercise training in the HF-ACTION trial demonstrated improvement in quality of life and reductions in hospitalizations and mortality¹¹. Additionally, the application of exercise to alter the cardiotoxic effects of anthracycline-containing chemotherapy (*e.g.*, doxorubicin) has demonstrated that regardless of when it is initiated with respect to the patients chemotherapy administration (before, during or after), exercise can provide beneficial effects such as reducing the decline in aerobic capacity, attenuating the left ventricular dysfunction and reducing oxidative damage¹².

The benefits of exercise in health and wellness are not just in its application as a medicine/treatment, but also as a diagnostic tool. Exercise testing is, for example, used to diagnose exercise intolerance, ischemia in the heart, or to understand the cause of shortness of breath¹³. Perhaps more importantly, exercise testing may be utilized to identify subclinical dysfunction. The human body is in most situations “overbuilt,” such that dysfunction or pathophysiology can often remain hidden and unapparent to an individual for months or years. This observation may explain why conditions such as pulmonary arterial hypertension or pancreatic cancer can silently increase in severity such that by the time symptoms are noticed; these conditions tend to be very advanced and extremely difficult to treat². In some of these situations, exercise testing can provide a stress stimulus to the body which increases demand above that of daily living and at times can identify dysfunction (cardiac, respiratory, metabolic) that was not seen at rest, helping to diagnose a disease and begin treatment earlier.

In order to fully maximize the therapeutic and diagnostic potential of exercise, standardized methods to quantify the responses to physical activity are needed to accurately assess the contributions of exercise to overall immune health. Variations in workload, inclination, duration,

type of exercise, and the timing of sample collection can all influence measurements of physiological responses. Here, we outline methods for maximal and submaximal endurance exercises for the purpose to gather physiological data while collecting samples for biological responses. This methodology was used understand how acute exercise affected the distribution and frequency of leukocyte populations in peripheral blood¹⁴ by measuring ^{14,15,16} immune cell populations at various time points before and after exercise by flow cytometry with 10-color flow protocols that permit the quantification of all major leukocyte subsets simultaneously¹⁷. The following protocol can be used as a standardized method for two distinct exercise regimens for measuring physiological and biological responses to exercise.

Protocol:

The protocol was approved by the Mayo Institutional Review Board and conformed to the Declaration of Helsinki. All participants provided written informed consent before participating in the testing described.

1. Calibration and setup of metabolic cart

1.1. Flow and volume (pneumotach) calibration

Note: Specific materials and equipment are listed in **Table 1**.

1.1.1. Open the pulmonary function and gas exchange software to calibrate for the test.

1.1.2. Click the “calibrate” button to open the calibration window. At the bottom of the calibration window, note the room temperature, barometric pressure, and the humidity — ensure that these values are accurate using a barometer.

1.1.3. Insert the umbilical and sample line into the pneumotach and then insert the pneumotach into the 3 L syringe.

1.1.4. Before starting calibration, click the “zero flow” button to ensure that there is no flow going through the syringe.

1.1.5. Click “start” to begin the calibration. Withdraw first, then inject. Repeat 4 more times (5 total), except at different flow rates each time. Maintain constant flow rate for each respective withdrawal/injection, then vary flow rate for the next iteration.

1.2. Gas calibration

1.2.1. Attach sample line from respiratory mass spectrometer/gas analyzer/metabolic cart to the calibration/home port on the system.

1.2.2. In the pulmonary function software, select the O₂/CO₂ analyzers tab in the calibrate window. Open the reference (room air 21% O₂, 0.04% CO₂) and calibration (12% O₂ and 5% CO₂) gas tanks.

1.2.3. Select the calibrate button.

Note: The software will operate the solenoid valves in the metabolic cart system to switch between the reference and calibration gases being sampled at the calibration port. From this, the software will evaluate for any offset, sampling the delay due to sample line length and the 2-90% response time. A green “Calibration Successful” message will appear when calibration is complete.

1.3. Final Setup

1.3.1. Remove the sample line from the gas calibration port and insert it back into the pneumotach. Then attach a mouthpiece onto the pneumotach.

1.3.2. Wait for the subject to arrive for exercise test.

1.3.3. Upon arrival of the subject, inform them about the study and what their involvement will be, review the consent form with them and have him/her sign it, and as the visit proceeds continually discuss with them what (s)he will be doing next and provide explanation of the procedures before doing them.

Note: Inclusion and exclusion criteria will vary based on the purpose of the exercise testing, for this study those recruited were healthy, non-smokers, with no known cardiopulmonary or immune disease and not taking any steroids or immune modulating drugs.

2. Pulmonary function test (PFT)

Note: The pulmonary function test methods described are a brief summary of those published by the American Thoracic Society and European Respiratory Society, for additional details please refer to their publications^{18,19}.

2.1. Slow vital capacity (SVC) maneuver

2.1.1. Instruct the subject to be seated with straight back and feet flat on the floor with legs uncrossed.

2.1.2. Instruct the subject to put his/her mouth around the mouthpiece, and bite down; also fit the subject with a nose clip to seal off the nasal cavity.

2.1.3. Start the maneuver on the software. When beginning the maneuver, instruct the subject to continue to breathe normally.

2.1.4. Observe the subject's tidal breathing and have him/her continue to breathe while waiting for him/her to reach a stable breathing pattern.

2.1.5. Instruct the subject to inhale maximally and then exhale slowly. The subject will keep emptying his/her lungs until they can no longer exhale. This point will be apparent by a plateau in the flow tracing. At this point, instruct the subject to take a maximal breath in.

2.1.6. Stop the measurement and instruct the subject to release the mouthpiece and remove the nose clip to take a break if needed.

Note: A minimum of three SVCs are performed. To meet American Thoracic Society standards, they must agree within 5% or 150 mL of each other for both the largest VC and inspiratory capacity (IC) values. Up to 4 maneuvers can be performed in order to obtain three that agree^{18,19}.

2.2. Forced vital capacity (FVC) maneuver

2.2.1. Instruct the subject to grasp the mouthpiece and attach the nose clip while remaining in the same seated position.

2.2.2. Start the maneuver on the software and instruct the subject to continue to breathe normally. Ensure the subject has established a stable breathing pattern with a minimum of four tidal breaths.

2.2.3. Instruct the subject to inhale fully and rapidly, and then to immediately exhale (pause of < 1 s) as quickly and forcefully as possible.

2.2.4. Tell the subject/patient to continue to try and blast/push all the air out of his/her lungs reaching a full exhalation while remaining in an upright posture. This is seen as a plateau in the volume-time curve. Ask them to continue trying to exhale for 6 s or as long as they can.

2.2.5. Once this is achieved, instruct the subject to take a maximal breath in and stop the maneuver on the software.

Note: The test should be repeated at least two more times — ensuring that they agree within 5% or 150 mL of each other for both the two largest FVC and FEV₁ values. Up to 8 maneuvers can be performed in order to get two that agree.

2.3. Maximum voluntary ventilation (MVV) maneuver

Note: The goal of this maneuver is to have the subject move as much air as rapidly as they can. They will be coached to try to take as large breaths as possible while still breathing rapidly.

2.3.1. Instruct the subject to grasp the mouthpiece and attach the nose clip while maintaining the same seated position.

2.3.2. Start the maneuver on the software.

Note: A countdown bar at the top will indicate the number of breaths required (usually three) before data collection/measurements begin.

2.3.3. With one breath to go in the countdown, direct the patient to start breathing deeply and rapidly through the mouthpiece. They will continue this for 12 s.

2.3.4. Encourage the patient throughout the procedure to breathe deeply and fast. If the subject is unable to continue, stop the test.

2.3.5. At the end of 12 s, instruct the patient to resume normal breathing. They may feel light headed, so encourage them to sit back and take slow deep breaths.

Note: For repeatability the test should be performed a minimum of two times and the suggested variability should be less than 20%.

3. Exercise tests

3.1. Electrode placement

3.1.1. Prepare the skin for the electrodes by shaving hair away from electrode placement site if present. Rub the site with an alcohol pad and then with an abrasive pad to remove any dead skin cells.

Note: Electrode can be placed once this is completed, but be sure that the electrode has adequate gel and is not dry.

3.1.2. Equip the subject with electrodes for a 12-lead electrocardiogram using the following electrodes placement.

3.1.2.1. Position limb lead electrodes as follows: RA: right side subclavicular fossa; LA: left side subclavicular fossa; RL: right back just above posterior superior iliac spine; LL: left back just above posterior superior iliac spine

3.1.2.2. Position precordial leads as follows: V1: right of sternum in the 4th intercostal space; V2: left of sternum in the 4th intercostal space (in line with V1); V3: left side directly between V2 and V4; V4: left side in the 5th intercostal space at the midclavicular line (typically under breast/nipple); V5: place horizontally with V4 on anterior axillary line (down from the edge of the armpit (anterior axillary fold of the upper arm); V6: place horizontally with V4 and V5 on the middle auxiliary line.

3.2. Incremental maximal cycling test – Visit 1

Note: Performing a maximal exercise test, comes with risks. The American College of Sports Medicine outlines how to identify individuals who are at higher risk for an adverse event during the test¹³. Those who would be considered to have significant risk have: known cardiovascular, pulmonary and/or metabolic disease; major symptoms: chest pain, shortness of breath (SOB) at rest or with mild exertion, dizziness or syncope, orthopnea, ankle edema, palpitations or tachycardia, intermittent claudication, known heart murmur, unusual fatigue or SOB with usual activities; or at least two of the cardiovascular disease risks factors: family history of myocardial infarction or sudden death, age (males ≥ 45 , women ≥ 55), current smoker, sedentary lifestyle (< 30 min of moderate intensity physical activity 3 days a week for at least 3 months), obesity (BMI ≥ 30 kg/m²), hypertension (Systolic blood pressure (SBP) ≥ 140 mmHg or Diastolic blood pressure (DBP) ≥ 90 mmHg), dyslipidemia (total cholesterol ≥ 200 mg/dL; LDL ≥ 130 mg/dL; HDL < 40 mg/dL, or on lipid lowering medication), prediabetes (fasting blood glucose > 100 mg/dL). All maximal exercise testing should be performed under the supervision of a health care professional trained in clinical exercise testing, with at least two people present, one to monitor the ECG and the other to be taking blood pressures and monitoring the patient. For those who are higher risk, a physician should also be present during the test, whereas in those that are at lower risk the test can be performed without a physician present; it is preferred to have the physician nearby and available immediately if needed. The personnel performing the test should have basic life support with an automated external defibrillator (AED) in the room and at least one or more performing personnel should have advance cardiac life support training. Those performing the test should know the plan for responding to a medical emergency and have the appropriate contact numbers.

3.2.1. Fit the subject to the bike making sure the seat and handlebars are positioned comfortably.

Note: The general suggestion for seat height is such that the leg has a slight bend at the bottom of the pedal stroke and the seat should be adjusted horizontally so that when the crank arm is parallel to the ground the knee should fall over the metatarsals which should be over the pedal spindle. The handlebars should be at the same height as the seat or slightly higher and close enough so that the subject has a slight bend in their elbows. The handlebar position will depend on the experience of the rider, more experienced cyclists will want to be more bent over, where are those who do not bike often will prefer a more upright position.

3.2.2. Place a pulse oximeter on the subject's forehead. Wipe the placement location with an alcohol wipe to remove any makeup or dirt, etc. and secure it to the forehead with a headband.

3.2.3. Review the exercise test procedure with the subject. Inform the subject that they will need to stay breathing through the mouthpiece for the entire duration of the test, and breathe only through his/her mouth as the nose will be plugged with the nose clip.

Note: Using a mouthpiece and nose clip is not the only option; masks are available which cover the nose and mouth allowing the participant to breathe through either their mouth or nose²⁰. Gas exchange metrics are continuously measured and recorded through the pulmonary function software. Heart rate (HR) and rhythm will be continuously monitored by the 12-lead electrocardiogram (ECG). Peripheral oxygen saturation (SpO₂) will be continuously monitored with the pulse oximeter. These external signals (HR, SpO₂) can be linked to the metabolic cart so that all measurements are documented together. If this isn't possible, HR and SpO₂ should be recorded on a worksheet every minute.

3.2.4. Draw a 5 mL blood sample from the antecubital vein (baseline exercise blood draw).

3.2.5. After 2 min of rest, begin data collection. Then, start the exercise protocol and instruct the participant start to pedaling. Ask them to reach a pedal rate between 60 and 80 rpms.

Note: For this study, the maximal exercise test protocol used was an initial workload of 50 W with 30 W increments every 2 min. The protocol used can vary depending upon the population and the goals of the test. For older individuals or patient populations, the first stage can be performed unloaded to allow the individuals to get their legs moving before resistance is added. In younger healthy individuals, this is usually not necessary as 50 W is a low enough workload to warm up. Pedaling at 0 W while maintaining the desired pedal rate is actually more challenging than starting with resistance from the beginning.

3.2.6. Have an assisting technician measure blood pressure (BP) 1 min into each stage while a second technician assists with the test. Then ask the subject to rate his/her exertion level on the Borg Rating of Perceived Exertion (RPE) scale where 6 indicates that the exertion is perceived as easy (like (s)he is sitting/standing doing nothing) and 20 indicates that the perceived exertion is at the hardest work they can imagine doing²⁰. Take a 12-lead ECG printout within the last 30 seconds of each stage.

3.2.7. Continue the test until exhaustion of subject which is designated by having at least two of the following occur: when 60-80 rpm on the bike can no longer be maintained, the subject's VO₂ plateaus and does not increase with an increase in workload, his/her respiratory exchange ratio (RER) is equal to or greater than 1.1 – 1.2, and/or the subjects rating of perceived exertion (RPE) ≥ 18 .

3.2.8. Stop the test if any of the following occur¹³: onset of angina or chest pain symptoms; drop in SBP of ≥ 10 mmHg with an increase in work; excessive rise in BP: SBP > 250 mmHg and/or DBP > 115 mmHg; shortness of breath, wheezing, leg cramps, or claudication; signs of poor perfusion: light-headedness, confusion, nausea, cyanosis, cold or clammy skin; failure of HR to increase with increasing exercise intensity; change in heart rhythm with symptoms; subject requests to stop; subject vocalizes or severe fatigue is observed; testing equipment not functioning properly.

3.2.9. Upon reaching exhaustion, proceed to the recovery phase: drop the resistance to the initial workload and instruct the subject to continue to cycle for another 2 min.

3.2.10. Draw another 5 mL of blood from the patient (post exercise blood draw) *via* antecubital venipuncture.

3.2.11. Direct the subject to return in 3 h, and 24 h following the completion of the test for the additional 5 mL blood draws. Instruct the subject to not partake in further exercise until completion of the 3rd post exercise blood draw at 24 h.

Note: These were the time points chosen for this study to evaluate the time line of changes in immune cells. The questions and parameters of interest will dictate when sampling should occur.

3.3. Endurance steady-state submaximal cycling test – Visit 2

3.3.1. Complete steps 1 for calibration, 3.1 for ECG preparation, 3.2.1 for bike fitting and 3.2.2 for forehead pulse oximeter installation.

3.3.2. Review the exercise test procedure with the subject. Inform the subject that they will be biking for 45 min, but unlike the maximal exercise test, they will only be asked to breathe through the mouthpiece with the nose clip for sections of test and not continuously.

Note: As with the maximal exercise test, HR and rhythm will be continuously monitored by the 12-lead ECG. SpO₂ will be continuously monitored with the pulse oximeter.

3.3.3. Draw 5 mL of venous blood sample from the antecubital vein (baseline endurance submaximal exercise blood draw) before starting the test.

3.3.4. Instruct the subject to grasp the mouthpiece by mouth and attach the nose clip.

3.3.5. Begin data collection, and then start the exercise protocol. Instruct the participant start pedaling, and ask them to reach a pedal rate between 60 and 80 rpms.

Note: For this study the endurance steady-state protocol is 45 min with a 3-5 min warm up at 50 W. Following warmup, the wattage is increased to 60% of the subject's maximal workload determined from visit 1. This is different from visit 1 in that the workload is constant and the bout is set for a specific duration, rather than increasing the workload until peak VO₂ is reached.

3.3.6. Measure BP (use the same technician as before) and ask the subject to report exertion level intermittently (every 3-5 min) throughout the exercise.

3.3.7. Instruct the subject to release the mouthpiece from the 10th min to 25th min, and re-grasp the mouthpiece from the 25th min to 30th min and during last 5 min of 45 min bout (40th min to 45th min).

Note: Gas exchange metrics are being intermittently monitored only when the subject is on the mouthpiece; intermittent monitoring is done as the mouthpiece can be dry and uncomfortable for the subject when used for longer periods of time. Since the goal of this type of exercise test is to have the subject reach specified exercise intensity and then hold that as a steady-state, the subject's gas exchange does not need to be continuously monitored unless it is a primary outcome measure. In the case of this study it was the stimulus, and not the outcome of interest.

3.3.8. Ensure that the test remains at a steady-state by monitoring the following metrics:

3.3.8.1. Check that the VO_2 hasn't increased significantly ($\pm 5 \text{ mL/min/kg}$) when the subject is back on the mouthpiece.

3.3.8.2. Check that the subject's HR doesn't increase by more than 5 bpm.

3.3.8.3. Observe for the subject appearing tired or that his/her RPE rating is rising.

3.3.9. Drop the workload by $\sim 5 - 10\%$ to ensure completion of 45 min of cycling, if any of the above occurs.

3.3.10. Following 45 min, instruct the participant to complete a 2 min recovery period of easy pedaling, and then draw 5 mL of venous blood (post exercise blood draw).

3.3.11. Give the same post exercise instructions as in visit 1 *i.e.*, do not engage in exercise until after the 24-hour time point and return to the lab for blood draws 3 h and 24 h post exercise.

4. Blood analysis

4.1. Process blood samples for analysis.

Note: Potential methods may include, but are not limited to, flow cytometry of circulating leukocytes, cytokine analysis of plasma samples, and/or leukocyte gene expression analysis. Additionally, optimal time points may need to be empirically determined.

Representative Results:

The application of maximal or submaximal endurance exercise testing provides a stimulus or stressor in which the body responds to meet the increased physiological demands. Various modes of exercise can be used to compare the physiological and biological responses to a particular exercise by itself or when a drug/intervention is used, or to evaluate the differences in responses between different exercise loads. Maximal and endurance exercise loads differ in the duration (short/long respectively) and intensity (high/low respectively), while the mode, (*i.e.*, cycling), is held constant. When designing a study with exercise testing, it is important to establish what the goals of the use of exercise are and what type of response is desired. **Table 2** highlights the differences and similarities between submaximal endurance and maximal exercise testing, but researchers also need to be cognizant of the effects different modalities of exercise will have

on the parameters being evaluated. In a maximal exercise test, where demand or exercise intensity is continually increasing with increases in workload (resistance/wattage on a bike or speed and/or grade on a treadmill) the cardiopulmonary and metabolic response (heart rate, stroke volume, ventilation, oxygen consumption and carbon dioxide production) also continuously increase (**Figure 1A**). In contrast, during a submaximal endurance exercise test the demand is increased from that at rest, but is raised to fixed exercise intensity. As such, the cardiopulmonary response has an initial increase, but then plateaus as the body adapts to meet the consistent demand (**Figure 1B**). The difference in intensity and demand between maximal and submaximal endurance exercise testing is also apparent when reviewing the change in rating of perceived exertion (RPE) and the respiratory exchange ratio (RER) over the respective exercise bout which estimates the fuel being used to supply the body with energy. In a maximal exercise test RPE and RER will steadily increase until the end of the test (**Figure 2A**), where as in a submaximal endurance exercise test these parameters will plateau (**Figure 2B**).

Although not required, it can be beneficial to perform a pulmonary function test before performing an exercise test. Exercise elicits a cardiac and pulmonary response and the performance during the exercise test can be limited by metabolic function and the ability of the heart, lungs or both to respond. When evaluating if there is a pulmonary limitation, it is helpful to know resting pulmonary function which can identify obstructive or restrictive limitations through the slow vital capacity (SVC) and forced vital capacity (FVC) maneuvers. Performing the maximal voluntary ventilation (MVV) maneuver to determine ventilatory capacity is useful as this can then be utilized to determine how much ventilatory reserve is present or if the individual is encroaching upon their ventilatory limits. However, this value can also be estimated from the FEV₁. Before performing pulmonary function testing, one should review the standardized methods for spirometry provided by the American Thoracic Society and European Respiratory Society^{18,19}.

Figure and Table Legends:

Figure 1: Gas Exchange and Heart Rate Data for Maximal and Submaximal Endurance Exercise Tests. Physiological changes in response to increasing workload (resistance in watts) in maximal test (**A**) and changes observed in an endurance test (over time) (**B**). Panels show the change in oxygen consumption (VO₂, open downward triangles), carbon dioxide production (VCO₂, black triangles) on the left y-axis and ventilation (VE, black circles) and heart rate (HR, grey circles) on the right y-axis. Peak oxygen consumption (VO₂ Peak) and test duration or endurance submaximal workload are listed on each panel figure.

Figure 2: Parameters of Exercise Intensity for Maximal and Endurance Exercise Tests

Two panels show the change in the rating of perceived exertion (RPE, asterisk) on the left y-axis and respiratory exchange ratio (RER, black downward triangle) on the right y-axis in response to increasing work (Watts) for maximal exercise test (**A**) and time (min) for the submaximal endurance test (**B**) on the x-axis.

Table 1: Comparison of Maximal and Submaximal Endurance Exercise Tests. The table summarizes the differences and similarities between the two exercise tests described.

Discussion:

There is great potential for exercise to be incorporated as an adjunct/alternative therapeutic tool. Indeed, an emerging body of evidence strongly suggests that physical activity promotes good health. The use of exercise as a medicine or diagnostic tool would require an understanding of the right amount or “dose” of exercise to achieve the desired effect. The optimal dose of exercise should be estimated, as too much exercise may be detrimental to improving health. As such, an exercise regimen may need to be tailored to each individual to achieve the optimal benefit from exercise. To that end, the variables that contribute to the nature of the diverse responses to exercise need to be understood and controlled. Therefore, standardized methodologies to exercise testing will be critical in moving the field forward.

The best method for normalizing exercise intensity for submaximal exercise testing continues to be the subject of debate. We chose to use 60% of the maximal workload achieved, but percentage of VO_2max /peak, HRmax or HRRmax are commonly used for prescribing exercise training intensity zones²¹. More recently, other methods have been suggested as being more effective at normalizing exercise intensity for research. One being the percentage delta concept, where the intensity is set to a specified percentage of the difference between the gas exchange threshold and VO_2max and has been shown to provide more consistent between-subject responses to endurance submaximal exercise testing than using a percentage of VO_2max ²². A second method for cycling exercise testing is critical power (CP) which describes power output that corresponds to the fatigue threshold. At this point cardiopulmonary and metabolic responses are most synchronized or unified. When exercise is performed below this threshold, peripheral fatigue does not limit the duration the exercise can be performed for, and exercise intensity can be stabilized. On the other hand, above CP, the amount of work that can be done or W' can be identified and the duration until W' is exhausted can be predicted²³. The best choice for how to determine the submaximal exercise intensity remains yet to be determined, but many in the field of exercise physiology field are moving away from the older methods and moving towards one of the newer methods described. The protocol chosen depends upon the study and the primary outcomes being evaluated. Additionally, in this study intermittent monitoring of gas exchange was chosen to make the test more comfortable for the participants as breathing on a mouthpiece for long periods is uncomfortable due to mouth dryness. Saliva can accumulate and holding the mouthpiece in the mouth can be tiring for the jaws. Since the primary outcome was a change in peripheral blood leukocytes and not a change in cardiopulmonary response to submaximal endurance exercise, intermittent monitoring of gas exchange to ensure that the exercise test remained at steady-state was sufficient.

We have outlined standardized exercise protocols, but additional steps can be taken preceding the exercise testing to further improve the consistency and reproducibility of exercise testing results. For example, have the same technician perform all of the blood pressure measurements for a particular study, or at minimum, have the same technician measure a subject over multiple repeat tests. Second, a proper calibration of all test equipment, especially the metabolic analyzer,

should be performed prior to each experiment. Finally, the variability of the subject population and how this will alter the individual response and comparisons between individuals should be considered and minimized. This can be mitigated in several ways through restricting the use of stimulants (*i.e.*, caffeine), and controlling food intake and exercise prior to testing and also ensuring that the subjects is well rested. The testing conditions (equipment, room temperature, time of day, *etc.*) should be kept consistent if tests are going to be repeated. In some scenarios, having female participants complete testing on a particular phase of their menstrual cycle (*e.g.*, early follicular phase) is also an important control. Additionally, the researcher will need to decide if they will allow supplements and medications to be taken, as these can alter the response to exercise. While there may be additional variables to control for in a particular study, we strongly recommend that these steps be incorporated into any study design involving exercise testing.

The exercise regimens described here can be utilized to study physiological responses to acute exercise. We have previously used this methodology to understand immunological changes in healthy individuals in the two different exercise regimens¹⁴. We collected blood samples prior to exercise testing with three blood samples collected at different time points after exercise. While both maximal and endurance exercise regimens led to a rapid accumulation of several leukocyte populations, the maximal regimen lead to a greater increase of most leukocyte subpopulations immediately after the testing was performed. We also found that CD56⁺CD16⁺ natural killer cells increased the most immediately after exercise, but CD15⁺ granulocytes had a delayed response by peaking at three hours post exercise. As it is well known that peripheral blood leukocytes rapidly accumulate into circulation following exercise (reviewed by Freidenreich and Volek¹⁵), our study demonstrated that the kinetics of mobilization are quite different and cell type specific. Natural killer (NK) cells and CD8⁺ cytotoxic T cells appear to be the most influenced upon exercise¹⁶, but other populations including myeloid cells and B cells also increase to some degree. While many studies have focused on acute effects of single exercise events, longitudinal training-based exercise regimens are likely needed to provide additional insight into how exercise affects long-term immunological performance.

The protocols described here provide a standardized methodology to incorporate exercise regimens for biologic and physiologic responses. These protocols can be easily modified for both single exercise tests as well as long-term longitudinal multiple testing. Physiologic measurements may include, but are not limited to, heart rate, blood pressure, oxygen consumption, body mass index (BMI). Biologic responses can be measured from a variety of specimens including peripheral blood, saliva, urine, and sweat. From these samples, multiple concurrent analyses can be performed *via* flow cytometry of cellular composition, proteomics analyses, gene expression arrays, or other types of biochemical and molecular approaches. In addition to understanding changes in the composition of peripheral blood leukocytes, others have looked at plasma markers of inflammation²⁴, cytokines²⁵, and how training regimens may be used to alter exercised-induced changes²⁶. Taken together, standardized protocols allow the measurement of physical activity of different durations and intensities with associated physiological parameters in a defined manner.

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Disclosures:

The authors declare that they have no competing financial interests.

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Figure 1

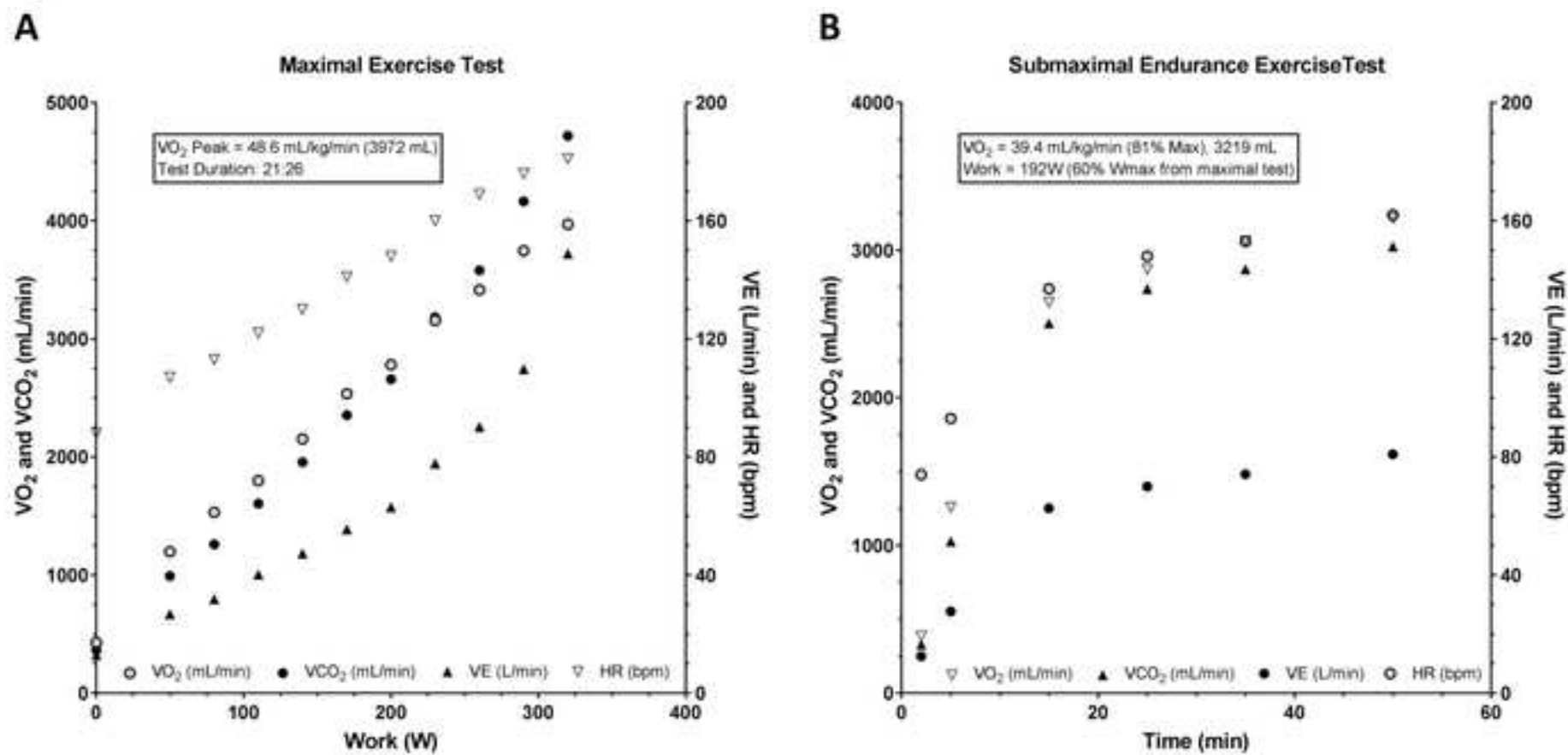
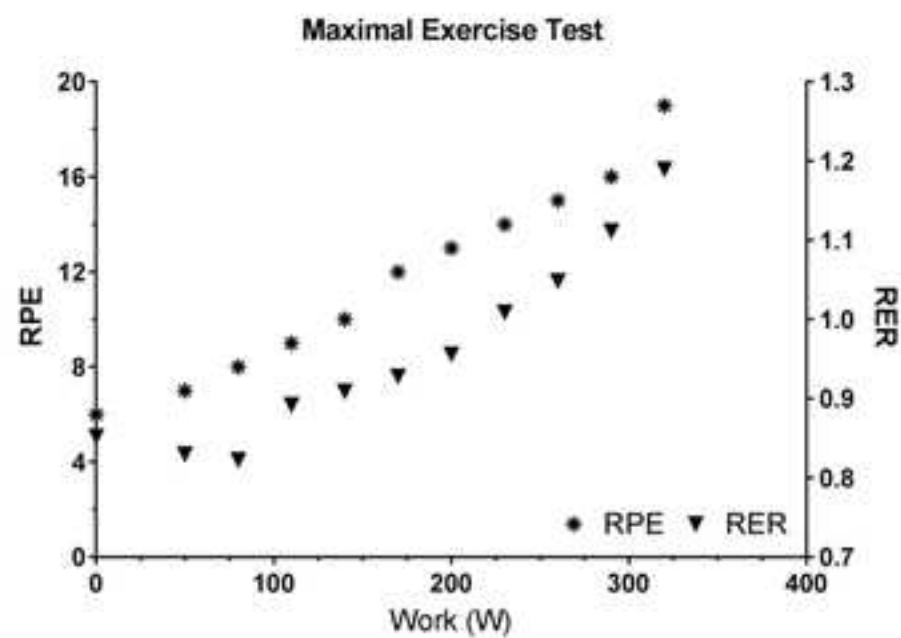
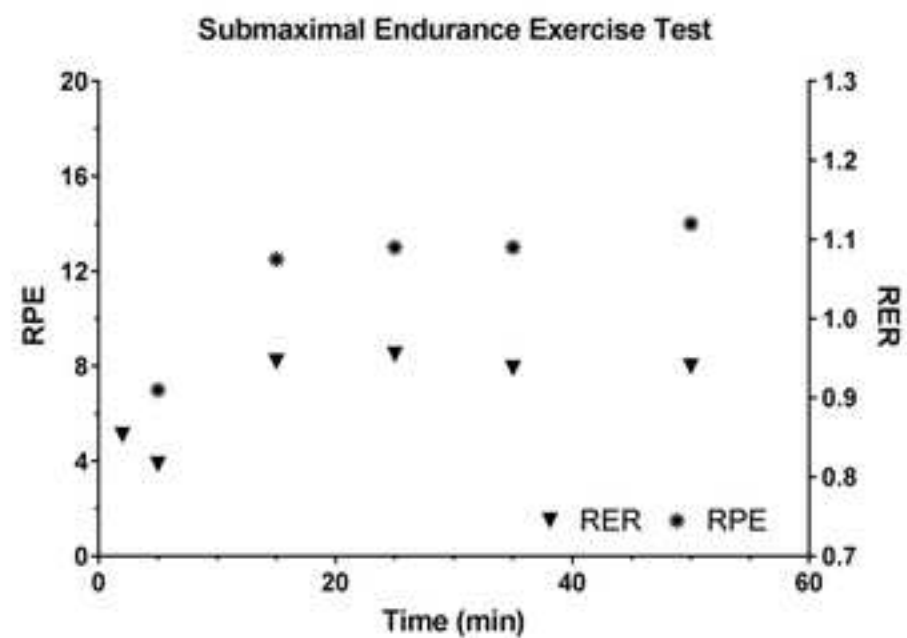


Figure 2

A



B



Maximal Exercise Test	Similarities	Endurance Submaximal Exercise Test
<ul style="list-style-type: none"> – Goal duration 10-20 minutes – Increasing intensity: ramping or stages – Gas exchange continuously monitored – HR, VO_2, VCO_2 and VE steadily increasing with increasing workload – RER is ≥ 1.1 – At end measurements will be the maximum that the individual's body can produce (HR, VO_2, VCO_2, VE, workload, etc.) 	<ul style="list-style-type: none"> – HR continuously monitored – BP measured at regular intervals – RPE measured – Pulse oximetry continuously monitored 	<ul style="list-style-type: none"> – Duration 30+ minutes – Steady state, desired intensity chosen and held – Intermittent gas exchange monitoring – HR, VO_2, VCO_2 and VE plateau and workload is decreased if start to rise as goal is to keep these steady – RER stays below < 1.0 – At the end measurements will be the percentage of the maximum that the individual's body can produce (HR, VO_2, VCO_2, VE, workload, etc.). Percentage is dictated by the intensity of submaximal exercise and/or the duration – To know what percentage of maximum it is a maximal exercise test would be needed to be performed usually at an earlier visit

VO_2 : oxygen consumption; VCO_2 : carbon dioxide production; VE: ventilation; HR: heart rate; RER: respiratory exchange ratio

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Metabolic cart/portable system	MCG Diagnostics	Mobile Ultima CPX System	The flow calibration syringe, and calibration gases should come with system. There are numerous possible options/alternatives.
Pulmonary function software (Breeze Suite)	MCG Diagnostics		Software used will depend on the metabolic system
Upright cycle ergometer	Lode ergoline	960900	Numerous possible options/alternatives
12-Lead ECG	GE Healthcare	CASE Exercise Testing System	Used for 12 lead ECG capture, control bike. Having a full 12-lead is ideal for maximal exercise test so can monitor for arrhythmias, but alternative for just HR would be a wireless chest strap heart rate monitor
Pulse oximeter	Masimo	MAS-9500	Usually multiple probe options: finger, forehead, ear lobe. Usually avoid finger as tight handlebar grip can cause measurement inaccuracies
Pneumotach (preVent Flow Sensor)	MCG Diagnostics	758100-003	Alternative systems can use a turbine
Nose piece (disposable)	MCG Diagnostics	536007-001	Numerous possible options/alternatives
Mouthpeice with saliva trap	MCG Diagnostics	758301-001	Suggest filling the saliva trap with paper towel/gauze and tape cap to limit dripping
Headband	Cardinal Health	292866	Used to secure the forehead pulse oximeter and the lines for the cart
Stethoscope	3M Littman	3157SM	Numerous possible options/alternatives
Blood pressure cuff	HCS	HCS9005-7	Cuff size will depend on the population planning to test
ECG Electrodes	Cardinal Health	M2570	only needed with lead based ECG/HR monitoring
K ₂ EDTA tube 5mL	Becton Dickinson	368661	

*The table provides a list of the supplies and equipment utilized in this protocol and comments related to the equipment. Brand name/company is provided, but the use of other brands will not affect the results, key is to keep it consistent throughout testing in a particular study.



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We have addressed these changes in the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a relevant article describing 2 different exercise testing protocols where traditional physiological measurements can be combined with either blood samples (or as suggested in the end of the article, saliva, urine and sweat) to further understand the exercise induced biological mechanisms improving health.

With minor revision this article can be published.

Major Concerns:

First of all, in the protocol section, I really miss a description of the safety arrangement around, especially, the maximal exercise test procedure as well as a description of indications for termination a maximal exercise test, including absolute, relative indications and subjective symptoms. Also a part of the expertise knowledge for those supervising the tests should be included. (For detailed information and reference see ACSM's Guidelines for exercise testing and prescription). Please add this to ensure that the protocol presented also account for the patients safety!

We apologize for this oversight. We have added a section regarding safety and included the reference to ACSM Guidelines.

Also, the attempt to explore the dynamic response of immunological or growth factors or other biomarkers is not new. We have published a series of article where maximal exercise testing was combined with measurements of markers from blood sample taken at submaximal workload and at maximal workload, see references at the end. Please add information in your Introduction section and also in your Discussion section, compare your protocol with information from our studies.

Yes we understand that that what our attempt to measure biological samples before and after exercise is not new and we did not state that in our manuscript. We were

aware of the work that was suggested to us as by the reviewer. However, we are not sure that comparing these protocols is appropriate in this manuscript because it does not fit the scope of this manuscript. Additionally, there are many studies that have looked at measuring responses after exercise and are too numerous to adequately cite them all. In the references provided, the main method for exercise and measured biological outputs were different, so we are not sure a direct comparison would be fruitful at this point. That being said, we did include a couple suggested references into the discussion.

Title

add

Conduction maximal and submaximal endurance exercise testing....

Comment; Using the term submaximal exercise test usually refer to a 6-10 min test with constant workload, where the steady-state heart rate combined with the workload are combined to estimate VO₂max. This is different from the submaximal exercise test that you present in your article. Thus, in your article please ensure that you include endurance whenever you refer to your submaximal exercise test

Minor Concerns:

Abstract

83 add and rearrange ... increasing cardiopulmonary and metabolic response (heart rate, stroke volume, ventilation, oxygen consumption and carbon dioxide production).

86 add...., but are not limited, heart rate, slow....

Introduction

97 delete The World Heath Organization defines

change to Physical activity is defined as any bodily..... (Reference: Caspersen et al. Physical activity, exercise and physical fitness: definitions and distinctions for health-related research. Public Health Rep. 1985, 100 (2) 126-31).

120 add Exercise testing is, for example, (Reference: ACSM's Guidelines for Exercise Testing and Prescription)

129 change peripheral to metabolic

131 add ... long-term moderate to high....

Comment; The current guidelines for physical activity is 150 min weekly aerobic exercise at moderate intensity or 75 min weekly at high or vigorous intensity

133 add... not fully clear

142 add.... exercise tests output

143 add and delete; or work load but are not limited to, mode, the test protocol (workload, inclination, duration of the exercise test) standardized methods to measure the output in response....

In the protocol section the test procedure is well described

311-312 add...(RPE) scale where indicating 6 is the exertion perceived at easy....and indicating 20 is the exertion perceived at the hardest....

360 see 311-312 above

380 Reviewers question; Why have you chosen 60% of the subject's maximal workload? Why

not a % of VO₂max or of HRmax or HRRmax, which is a more common way to guide intensity? See Norton K, Norton L, Sadgrove D (2010) Position statement on physical activity and exercise intensity terminology. J Sci Med Sport 13: 496-502. Please add some comment on this in your discussion section!

Please see the second paragraph of the discussion as we addressed these questions.

393-95 Reviewers question; Why have you chosen not to measure using ergospirometry during the whole 45 min test? Please add some comment on this in your discussion section!

Intermittent gas exchange was chosen to make the test more comfortable for the participants as breathing on a mouthpiece for long periods is uncomfortable as the mouth becomes dry, saliva can accumulate and holding the mouthpiece in the mouth can be tiring for the jaw. Since the primary outcome for this particular study was changes in peripheral blood leukocytes not changes in cardiopulmonary response to submaximal endurance exercise, intermittently monitoring gas exchange to ensure the exercise test was remaining a steady-state was sufficient. We have made this note in the methods and commented on this in the discussion.

424, 428, 431,, 437 add submaximal endurance exercise test

428 add ...duration (short/long respectively) and intensity (high/low respectively)

429 add... a study with exercise testing,

435 add and rearrange ...cardiopulmonary and metabolic response (heart rate, stroke volume, ventilation, oxygen consumption and carbon dioxide production).

449 add.... to respond, as well as by the metabolic function.

469 Delete From our experience, the exercise protocols outlined here provide relatively reproducible results. However, we have found that

Comment; You have not shown that in this article, no test-retest are presented, thus re-write.

Suggestion re-write; We have outlined exercise protocols and have found that additional steps....

474 add... proper calibration of all test equipment, especially the metabolic...

475 change subject population to individual response

476 add food intake and also a line of standardization of the room temperature, test conditions, time (if test are going to be repeated)

Figure legends

511, 519 add... Data for Maximal and Submaximal Endurance...

514 change Subscribe 2, that is VO₂ and VCO₂

516 Comment and change; If using peak oxygen uptake, then remove max and use VO₂peak change last sentence to; Peak oxygen consumption (VO₂peak) and test duration are shown in panel A. Submaximal oxygen uptake (VO₂) and workload are shown I panel B.

522 add...and time (minutes) for the submaximal endurance test (B)....

Figure 1

change Subscribe 2, that is VO₂ and VCO₂ (A and B in several places) and change VO₂max to

VO₂peak

add in heading B, Submaximal Endurance Test

add in B, Work = 192 W (60% W_{max} from maximal test)

Comments;

1. It is not relevant to have 37 yo male in the heading, please remove in A and B

2. If you are using the unit ml at the y-axis, also give the corresponding value of VO₂peak in ml (and also in ml/kg/min as you have) in A and B

Table 2

add RER in both columns

We have addressed all of the minor concerns as sufficiently as we could.

References that might be useful to include in introduction and or in discussion section

Using maximal exercise test with a submaximal continuous workload included

Blood samples at rest, submax, max and recovery

1. Gustafsson A, Ventorp F, Wisén A, Ohlsson L, Ljunggren L, Westrin Å.

Effects of acute exercise on circulating suPAR in patients with major depressive disorder.

Biomarkers Insights. 12:1-6 (2017)

<http://insights.sagepub.com/effects-of-acute-exercise-on-circulating-soluble-form-of-the-urokinase-article-a6290>

2. Wisen AGM, Ekberg, K, Wohlfart B, Ekman R, Westrin A. Plasma ANP and BNP during exercise in patients with major depressive disorder and in healthy controls. J Affect Disord 129, 371-375 (2011) http://ac.els-cdn.com/S0165032710005707/1-s2.0-S0165032710005707-main.pdf?_tid=24105f86-d1ae-11e6-b3d8-00000aab0f6c&acdnat=1483445834_9fdeba48c8b6a3f351e9ac1e686a9395

3. Hallberg L, Janelidze S, Engstrom G, Wisén AGM, Westrin A, Brundin L. Exercise-induced release of cytokines in patients with major depressive disorder. J Affect Disord 126, 262-267 (2010) http://ac.els-cdn.com/S0165032710002715/1-s2.0-S0165032710002715-main.pdf?_tid=0d8e2612-d1ae-11e6-90b0-00000aacb360&acdnat=1483445796_01da72121e7da3e9dd19fce2e5c17831

4. Gustafsson G, Lira CM, Johansson J, Wisén AGM, Wohlfart B, Ekman R, Westrin Å. The acute response of plasma brain-derived neurotrophic factor as a result of exercise in major depressive disorder. Pshychiatry Res. 244-8 Oct 30 (2009) http://ac.els-cdn.com/S0165178108001984/1-s2.0-S0165178108001984-main.pdf?_tid=3db2cf22-d1af-11e6-9d7e-00000aacb360&acdnat=1483446306_ace4c70d87add49bffa3c79085be28a

Using maximal exercise test, blood samples at rest, max

5. Marie E, Bengtsson-Lindberg M, Wilke L, Vestberg S, Jacobsson H, Wisén A. Exercise-induced release of cytokines/myokines in a single exercise test before and after a training intervention in patients with mild cognitive impairment. International Journal of Physical Therapy & Rehabilitation.

<http://www.graphyonline.com/archives/archivedownload.php?pid=IJPTR-138>

Reviewer #2:**Manuscript Summary:**

This manuscript presents the proposal of two different protocols of exercise (maximal and submaximal exercise testing) of potential interest to measure different physiological and biological responses to acute exercise in humans. The paper is overall well written and technically correct, and illustrate step by step the procedure of two cardiopulmonary exercise tests.

Major Concerns:

- The procedure of the maximal cardiopulmonary exercise test (and the spirometry that should be performed before it) is largely available within the published guidelines of different International societies.

Yes we agree with the statement. Upon invitation from the journal, our desire was to submit a manuscript to fit the scope of the journal for video demonstrations of protocols which facilitate scientific reproducibility and productivity using an exemplar study that was previously published by our group (*J Immunother Cancer*, 2017 5:30).

- The step-by-step procedure is exclusive only for a specific brand (device and software), and follows the manufacturers' procedures. [*Editor's Note: This is okay, but it would be useful to add a note stating that the protocol would need to be adapted if other instruments are used*]

We have put in a note in the table description that other instruments can be used.

- It is unclear to me why you use a specific timing of blood draws. Actually, I understand why, but I believe that this timing is not generalizable and appropriate for any biological response to be recorded.

We are not sure how to respond to this comment. Our timing of pre, post, 3 hours, and 24 hours as outlined in our *J Immunother Cancer* paper was chosen as a balance of what has been done previously, our estimations of capturing data that would reflect changes, and what was practical for healthy volunteer subjects availability for blood draws. We did in fact see many leukocyte changes/ biological responses at each time point. In our system, the peripheral immune system returned to normal homeostasis after 24 hours, so that time point was also helpful to gain context as well. We certainly acknowledge and encourage that depending on your system and methodology, one has to empirically determine the optimal timing of measuring responses. Thus we added a comment in protocol step 4.1.

- Considering your example, the athlete's VAT 1 (First Ventilatory Anaerobic Threshold) is above the proposed intensity of the prolonged, constant load, exercise (at 60% of maximal power). However, it may happen that untrained and sedentary people have a VAT 1 below this exercise intensity, thus developing a premature fatigue. By the way, for these reasons, the use of

a fixed percentage respect to the maximal power could also elicit different true physiological strains.

The reviewer is correct, this is why we monitored the “steady-state” as described in 3.3.9 to ensure fatigue wasn’t occurring or the intensity increasing with time, if we observed signs of fatigue we dropped the workload by 5-10% to ensure that the entire 45min exercise bout could be completed.

Minor Concerns:

- Gas calibration 1.2.2: while within the room air O₂ should be 20.93%, CO₂ calibration is not mentioned (room air CO₂: 0.03%)
- Please correct a few spelling mistakes (lines 98, 452, Table).
- Exercise Test 3.1.2: V5 position is unclear
- Exercise test 3.2.1 You could specify which should generally be a correct positioning of the seat
- Exercise test 3.2.7: why here you don't initially use an unloaded pedalling?
- Exercise test 3.2.9: no mention about Borg's Scale?

We have addressed all of the minor concerns as sufficiently as we could.

We would like to thank both reviewers for their comments that helped improve the quality of the manuscript. We appreciate their efforts greatly.