

Title: Repeated Supra-Maximal Sprint Cycling With and Without Sodium Bicarbonate Supplementation Induces Endothelial Microparticle Release

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Abstract

Under normal homeostatic conditions, the endothelium releases microparticles (MP), which are known to increase under stressful conditions and in disease states. CD105 (endoglin) and CD106 (vascular cell adhesion molecule-1) are expressed on the surface of endothelial cells and increased expression in response to stress may be observed. A randomised-controlled double-blinded study aimed to examine the use of endothelial microparticles as a marker for the state of one's endothelium, as well as whether maintaining acid-base homeostasis affects the release of these MP. This study tested seven healthy male volunteers, who completed a strenuous cycling protocol, with venous blood analysed for CD105+ and CD106+ MP by flow cytometry at regular intervals. Prior to each trial participants consumed either 0.3 g·kg⁻¹ body mass of sodium bicarbonate (NaHCO₃), or 0.045 g·kg⁻¹ body mass of sodium chloride (NaCl). A significant rise in endothelial CD105+MP and CD106+MP ($p < 0.05$) was observed at 90 minutes post exercise. A significant trend was shown for these MP to return to resting levels 180 minutes post exercise in both groups. No significance was found between experimental groups, suggesting that maintaining acid-base variables closer to basal levels has little effect upon the endothelial stress response for this particular exercise mode. In conclusion, strenuous exercise is accompanied by MP release and the endothelium is able to rapidly recover in healthy individuals, whilst maintaining acid-base homeostasis does not attenuate the MP release from the endothelium after exercise.

Key Words: ENDOTHELIAL FUNCTION, MICROPARTICLES, ANAEROBIC EXERCISE, SODIUM BICARBONATE, VCAM-1

Introduction

Microparticles (MP) are phospholipid micro-vesicles derived from cell membranes, ranging in size from 0.05 to 1.5 μm (Roos et al. 2010). They contain membrane proteins of their parental cells, and although they may be released from almost all cell types, most investigators have focused more specifically on MP from platelet, leukocyte, and endothelial cell origin (Roos et al. 2010). Endothelial MP (EMP) concentration in circulating blood are a marker of the state of the endothelium *in vivo* (Lekakis et al. 2011), with such MP analysed with specific antibodies by flow cytometry (Horstman, Jy, Jimenez, & Ahn, 2004). The mechanism of MP release is either through cellular activation (Horstman et al. 2004), injury, stress, or via cellular apoptosis or necrosis (Madden et al. 2008). It is important to note that circulating MP are found in the plasma of healthy participants, being constantly shed into the circulation (Berckmans et al. 2001; Freyssinet, 2003; Leroyer et al. 2007) as well as in various pathological conditions (Chironi et al. 2009).

It has been previously shown that EMP may express adhesion molecules harboured by mature endothelial cells that reflect the integrity of the endothelium (Jimenez et al. 2005; Vince, Christmas, Midgley, McNaughton & Madden, 2009a; Madden et al. 2010; Lekakis et al. 2011). CD105 and CD106 are expressed by endothelial cells upon apoptosis and cellular activation (Lekakis et al. 2011), as well as under normal physiological conditions (Freyssinet, 2003). CD106+MP has been shown *in vitro* to be released after tumour necrosis factor- α (TNF- α) stimulation of cultured vascular endothelial cells (Horstman et al. 2004). TNF- α has since been shown to significantly increase immediately after maximal exercise in both highly trained and

sedentary subjects (Denguezli-Bouzgarrou et al. 2006), and after moderate intensity exercise in healthy volunteers (Goebel et al. 2000).

In disease states, particularly those that are characterised by endothelial dysfunction, elevated concentration of EMP are found (Horstman et al. 2004). Dignat-George and Boulanger (2011) recently described EMP's as complex vascular structures that are shed from endothelial cells known to play key roles in inflammation, endothelial function, coagulation, and angiogenesis. Additionally, the maintenance of endothelial function has a preventative role in loss of homeostasis, and subsequent cardiovascular risk factors (Madden et al. 2008).

It is known that exercise induces several types of physiological stress, such as increased heat production, reactive oxygen species and shear stress (Marsh & Coombes, 2005), and that oxidative stress activates the endothelium (Lehoux, Castier, & Tedgui, 2006; Ungvari, Wolin, & Csiszar, 2006). The study of EMP after exercise in healthy individuals is scarce, with a limited number of investigations being conducted in this area. An increase in levels of EMP, specifically CD106+MP was shown following a simulated (hyperbaric chamber) dive after breathing air at depth in healthy participants (Vince et al. 2009b); suggesting this may be a sign of endothelial activation. Bartzeliotou et al (2007) investigated circulating levels of adhesion molecules, including CD106, induced by a "Spartathlon" ultra distance foot race. CD106 was significantly greater at the end of this 246 km race, with levels returning to normal 48 hours after the end of the race. In a recent study Sossdorf, Otto, Claus, Gabriel, and Losche (2011) showed that a moderate bout of cycling exercise was enough to elicit an increase in the cell-derived MP concentration in healthy individuals.

In addition to the limited research in the effects of exercise upon MP numbers, there is also limited data on the impact of sodium bicarbonate (NaHCO₃) ingestion on the stress response following exercise. NaHCO₃ is an effective buffering agent for enhancing performance

(McNaughton, Siegler, & Midgley, 2008), and can improve acid–base recovery following exercise of an anaerobic nature (Siegler, Keatley, Midgley, Nevill & McNaughton, 2008). NaHCO₃ is proposed to increase the body’s natural bicarbonate reserve, acting as a buffer due to its ability to accept a proton to form carbonic acid ($H^+ + HCO_3^- \leftrightarrow H_2CO_3$). In turn, this mechanism increases the extra-cellular reserve enhancing H⁺ efflux from the cells (McNaughton et al. 2008). In addition, it is possible that the extra buffering of H⁺ may result in a reduced production of free radicals (Kellum, Song & Li, 2004), and thus reduce exercise induced oxidative stress, a possible stimulus for EMP release (Vince et al. 2009b). It has been shown previously in cultured cells and cardiac myocytes that acidosis is a major trigger of apoptosis (Thatte et al. 2004). A decrease in endothelial cell pH is part of the physiological response to exercise (Morikawa, Inubushi, Kito, & Tabata, 1994), and avoiding acidosis through the use of a buffering agent may attenuate MP release, and therefore endothelial damage/activation. Based on the evidence given here, we hypothesised that the plasma concentration of MP may be influenced by a strenuous cycling protocol. Thus the aim of this randomised-controlled, double-blinded trial was to assess damage/activation of the vascular endothelium as a result of a high intensity cycling exercise through quantification of EMP in healthy participants. An additional aim of the study was to investigate the effects that ingesting NaHCO₃ prior to strenuous exercise may have, if any, upon the numbers of circulating EMP and whether the acidosis response to high intensity exercise may be attenuated in healthy human participants.

Methods

Participants

Seven healthy, non-smoking male participants, free from cardiovascular, metabolic or any significant other disease were recruited for this study (mean \pm s, height, body mass, age, peak power output (PPO) and physical activity: 182 ± 0.06 cm, 81.3 ± 8.4 kg, 22.1 ± 3.2 years, 298.4 ± 23.5 W, 3.9 ± 1.0 h week⁻¹). None of the participants had been training excessively in the past two months, nor had they been supplementing their diet with any ergogenic aids prior to testing. Ethical approval was granted by the Departmental ethics committee at the University of Hull, and all the participants involved gave written, informed consent. Participants were treated in accordance with the Declaration of Helsinki.

Experimental Design

Participants were instructed to abstain from alcohol, caffeine, and exercise 24 hours prior to the testing, and were asked to fast overnight until after the final blood draw. Participants reported to the laboratory on three separate occasions, each separated by one week. Visit 1 was a PPO test, which preceded a familiarisation to the intermittent cycling protocol. The following two visits were organised in a randomised and double-blinded manner, which consisted of either NaHCO₃ or placebo trials. NaHCO₃ was administered at a dose of 0.3 g·kg⁻¹ body mass, contained in approximately 15-20 gelatine capsules that were taken with water. The placebo was administered at a dose of 0.045 g·kg⁻¹ body mass of sodium chloride (NaCl) made up with flour to provide the same amount of pills. Pills were consumed 60-min prior to exercise (Siegler, Midgley, Polman, & Lever, 2010). All testing was performed at the same time of day, with the pills being consumed at 0830 am, in order to control for any circadian variations that may affect results, which has been shown previously in the expression of CD106+MP (Madden et al. 2008) and exercise performance (Drust, Waterhouse, Atkinson, Edwards, & Reilly, 2005). Capillary blood samples were taken for the measurement of acid–base variables (pH, HCO₃⁻,

H⁺, base excess, Radiometer, ABL800, Copenhagen, Denmark) before commencement of exercise. Venous blood samples were drawn by a standard venipuncture technique from the antecubital vein in to a tri-sodium citrate Vacuette (Greiner, UK) tube for subsequent MP quantification. Blood draws were made immediately before exercise (Rest), immediately post exercise (Immediate), 90 minutes post exercise (90-min), and 180 minutes post exercise (180-min).

Peak Power Output Test and Experimental Trials

Each participant had completed a PPO test in visit 1 on a cycle ergometer (Lode Sport Excalibur, Netherlands). The PPO test consisted of a 5-min warm-up at 50 W followed by a ramp protocol, whereby workload increased at a rate of 30 W·min⁻¹ until participants felt they could no longer persist with the test. Participants then completed a cool-down period of 5-min at 50 W. The PPO attained during the test was used to determine the workload for the intermittent exercise protocol performed during the experimental trials. The intermittent protocol began with a 5-min warm-up at 50 W, which was followed by 10 x 15-second sprints at 120% PPO determined in visit 1, which were separated by 45 seconds of active recovery at 50 W. During the experimental trials, all participants were vocally encouraged to achieve maximal power output. Participants were also asked to rate themselves on their perceived readiness ratings (PRR) (Karu et al. 2000) prior to each sprint using a scale from 5 down to 1 (where 5 relates to completely ready to begin, and 1 is not at all ready to begin). Upon immediate cessation of exercise, participants were asked to leave the cycle ergometer and a venous sample was taken.

MP Quantification

Citrated blood was analysed by obtaining platelet rich plasma through centrifugation (180 x g, 10-min). Platelets were removed by further centrifugation (12,000 x g, 10-min). From this, samples of platelet free plasma (PFP, 25 μ L) were incubated with 4 μ L of either IgG1 negative control FITC conjugate (AbD Serotec, UK), IgG1 CD-105:FITC conjugate (AbD Serotec, UK), or IgG1 CD106:FITC conjugate (AbD Serotec, UK), in the dark at room temperature for 30-min. Quantification was achieved by adding filtered (0.1 μ m) PBS (150 μ L) and counting beads (25 μ L, Caltag Laboratories, UK) immediately prior to analysis by flow cytometry (BDFACSCalibur). A MP region was established using megamix beads (Biocytex, France) according to the current International Society on Thrombosis & Haemostasis Scientific and Standardization Subcommittee protocol (Lacroix et al. 2010), and 25,000 events were counted for MP analysis, with positive MP being defined as an increase in mean fluorescence intensity over the isotope matched negative control, and were quantified in relation to counting beads according to manufacturers' instructions.

Statistical Analysis

All statistical analyses were performed using IBM SPSS STATISTICS 19.0 (SPSS Inc, Chicago, IL). Central tendency and dispersion of the sample data are represented as the mean (s). Any changes in biochemical markers across condition and time were analysed using linear mixed models. Post hoc tests with Sidak-adjusted p values were used to locate significant differences where a significant F ratio was observed. The change in acid-base status from pre- to post-ingestion within the two experimental conditions, and comparisons across conditions was investigated using paired samples t tests. All of the data are presented as means and standard errors of the mean (s_x). Two-tailed statistical significance was accepted as $p < 0.05$.

Results

Acid-Base Homeostasis

The efficacy of the supplementation of NaHCO_3 is shown by significant rises in capillary blood pH, HCO_3^- and base excess, as well as a significant decrease in H^+ from pre to post-ingestion in the experimental group only ($p < 0.05$), with no significant changes in the placebo group for any of these variables (Figure 1).

CD105+MP

CD105+MP data at rest and during the 180-min post-exercise recovery for the two experimental conditions is displayed in Figure 2(A). The mean percentage change values of CD105+MP increased from rest to a maximum level at 90-min post exercise in both groups. In the placebo group, an increase of 29.5% was observed compared to an increase of 44.1% in the experimental group. The interaction effect of condition and time produced no significance ($F = 0.583$, $p = 0.631$). A significant main effect for time was present ($F = 4.262$, $p = 0.001$) suggesting the EMP response for CD105 was significantly higher 90-min after exercise, and levels had returned back to basal levels after 180-min. Post-hoc tests indicated that there was a significant increase in CD105+MP in both conditions immediately after exercise to 90-min, placebo group ($p = 0.034$), and experimental group ($p = 0.013$). The experimental group also displayed a significantly higher value from rest to 90-min post exercise ($p = 0.017$), and also a decrease from 90-min to 180-min ($p = 0.044$). There was a tendency for both groups to decrease back to basal levels 180-min after exercise, regardless of condition (placebo or experimental). No main effect significance was found for condition across trials, indicating that the results gained were not due to the condition but the exercise protocol itself.

CD106+MP

CD106+MP data followed a similar trend to that observed for CD105+MP (Figure 2(B)). In the placebo group, an increase of 35.6% was observed, compared with 12.5% in the experimental arm. The interaction effect of condition and time produced no significance ($F=0.775$, $p = 0.517$). A significant main effect for time was present ($F = 7.861$, $p = 0.000$) indicating the response to exercise was significantly increased at 90-min, and levels returned towards basal values after 180-min. The results of the post-hoc test showed that both groups show significance from immediately post exercise to 90-min, placebo ($p = 0.001$), experimental ($p = 0.006$). The experimental group showed a significant increase in CD106+MP from immediately post exercise to 180-min ($p = 0.029$). The placebo group also displayed significance from resting values to 90-min post exercise ($p = 0.024$), and also a significant decrease between 90-min and 180-min of recovery ($p = 0.049$).

Overall MP change regardless of experimental condition

Due to no significant differences between conditions, data has been grouped combining values from all participants regardless of their experimental condition, in order to represent the trend over time that both markers of endothelial function displayed (Figure 3). A significant main effect for time was found in CD105+MP ($F = 6.903$, $p = 0.001$) and CD106+MP ($F = 6.903$, $p = 0.001$). Figure 3 shows that there was significance for both markers across several time-points, indicating a significant change across time in endothelial function.

Perceived readiness ratings

PRR showed significant pairwise comparisons across almost all time points in both conditions, with a significant overall main effect for time found ($F = 28.503$, $p = 0.000$). There was however no significant main effect found between experimental groups for condition ($F = 0.604$, $p = 0.443$). These results suggest that regardless of experimental condition, as the exercise progressed, participants perceived each subsequent sprint more difficult than the previous. Figure 4 displays the trend for PRR scores as sprints increase.

Discussion

The main findings from this study are that high-intensity repeated sprint exercise stimulates a rise in EMP, which then returns to basal levels within 180 minutes of ceasing exercise. Another finding from the study is that pre ingestion of NaHCO_3 to maintain acid-base balance closer to homeostatic levels did not attenuate the release of EMP from the endothelium following exercise.

To date, there has been very little research conducted to describe the effect of physical exercise on the overall cell-derived MP release from endothelial cells in healthy humans. Results here show that this particular bout of acute exercise was capable of increasing the concentration of EMP in comparison to resting levels. Additionally, the levels of MP had returned near to resting values after 180-min of recovery. In comparison to another study of this kind, Chaar et al. (2011) were unable to detect any changes in MP production from endothelial cells as characterised by no detection at all in levels of CD106+MP. This may be due to pre-analytical methodology (Lacroix et al. 2010) or the exclusivity of enumerating MP that were also positive for annexin V staining, as not all MP express phosphatidylserine. Chaar et al. (2011) utilised a cycling protocol involving three separate progressive, maximal ramp exercise tests in healthy

males, which would be expected to elicit sufficient damage to the endothelium due to its high intensity nature.

We observed a rise in EMP following exercise that we believe to be as a result of the nature of the exercise. The reason for such a rise in these markers may be due to the sensitive nature of the endothelium to oxidative stress and shear rate (Ungvari et al. 2006; Lehoux et al. 2006). There is a shedding of these EMP from cell surfaces that occurs due to an increase in shear stress (Marsh & Coombes, 2005) and although we are unable to provide precise measures of shear stress in this study, it is something that has been evident since in further work (Chen, Chen & Wang, 2010; Sossdorf et al. 2011), and given the supra maximal nature of the exercise, it is reasonable to assume there was a significant increase in shear rate. Oxidative stress may also be a determining factor in increased MP release from cells, and previously we have shown EMP release in response to hypoxia (Vince et al. 2009a) and a heat shock protein response to a single bout of high intensity anaerobic exercise (Peart et al. 2011) suggesting a more global circulatory stress involving regulatory triggers could contribute to increased MP release.

The increased CD106+MP levels observed in our study may appear to follow trend with those participating in the “Spartathlon” study (Bartzeliotou et al. 2007), but in fact there was a marked difference. The latter found a significant rise in CD106 immediately upon ceasing exercise after a speed walk, which lasted on average almost 32 hours in duration. These results also followed a similar tendency with Nielsen and Lyberg (2004), who found comparable undertakings when measuring plasma levels of CD106 before and after the Oslo marathon and half marathons respectively. No data was available following a recovery period that may indicate if the endothelium was able to recover as in accordance with the current study. However, in our results, the rise in EMP was not observed until 90-min post exercise, and values immediately post exercise were remarkably similar to resting levels. This response is reasonable as it is expected that the process is active in nature and so there is a time delay in

the release of MP (Piccin et al. 2007). Future protocols could include additional time points to further characterise the time course of MP release after an acute stress.

It could be assumed that from this work, and others previously mentioned (Jensen et al. 2004; Bartzeliotou et al. 2007; Sossdorf et al. 2011; Wahl et al. 2011) that high intensities are required to activate endothelial cells, and there is an intensity-dependent increase in MP, suggesting that in order to significantly stress the endothelium of healthy individuals, strenuous activity is required to see such a rise. This intensity-dependent increase is thought to be as a result of shedding of the cell adhesion molecules via adrenergic mechanisms (Rehman et al. 1997), but it may also be expected following a bout of exercise of this intensity, and so could be as a result of an increase in shear stress. As the time interval from cessation of the exercise protocol to the observed increase in EMP was relatively short, it may be postulated that increased levels of EMP are as a result of increases in shear stress due to increased heart rate. Endothelial cells are constantly shedding MP at a nominal rate (Freyssinet, 2003) and a sudden increase in shear stress may result in a one-off cleaving of MP that are in a process of being released from the endothelium, resulting in temporary increase in circulating EMP. Sossdorf et al. (2011) provided evidence that shear stress plays a major role in EMP release in a recent study. This group found that cardiopulmonary exertion was significantly higher in the trained group throughout the 90 minute exercise at a fixed percentage of participants' individual aerobic threshold. Heart rate and blood pressure was monitored at regular intervals throughout the exercise and immediately after, suggesting a greater shear stress was the reason for a transient increase in numbers of EMP at 45 minutes in the trained group versus the untrained group. This finding may also suggest biological differences in the effectiveness of the stress-induced mediators between trained and untrained participants. There were similarities between the current study and the aforementioned in terms of a time delay from cessation of exercise in order to see an increase in EMP, even in such a moderately intense protocol. Further to this,

our participants were fit and healthy individuals partaking in regular physical activity per week, and who were accustomed to high intensity exercise. Such characteristics could link to the trained group in the study by Sossdorf et al (2011) and if this is indeed the case, then it may be reasonable to assume that a similar time course was present in both studies. Although the exact timings of the sample collection were slightly different, it appears that trained individuals hit a peak of endothelial stress and subsequent recovery within a similar timeframe.

We attempted to determine if the stress response placed upon the endothelium as a result of exercise, by means of changes in pH, may be attenuated, and therefore MP release reduced by the inclusion of a NaHCO₃ buffer. This has previously been shown to be successful in other markers of exercise related stress (Peart et al. 2011). Recently, Wahl et al (2011) investigated the effects of exercise-induced acidosis on Vascular Endothelial Growth Factor (VEGF) levels following all-out cycle sprints. VEGF stimulates angiogenesis, thus resulting in increased oxygen delivery to tissues; however over-expression can be detrimental (Wahl et al. 2011). This group found that following an ingestion of NaHCO₃, there was no attenuation to the VEGF response following highly strenuous exercise. The endothelium is exposed to a more acidic environment during physical exercise (Morikawa et al. 1994) and the rate of VEGF production has been shown to be increased at acidic pH in an endothelial cell culture model system (Burbridge et al. 1999) suggesting a possible oxidative stress response. However, *in vitro* studies in bovine aortic endothelial cells have actually found that acidosis inhibits endothelial cell apoptosis and inhibits angiogenesis despite increased VEGF mRNA expression (D'Arcangelo et al. 2000) whilst Fukumura et al (2001) also demonstrated a more acidic environment increased VEGF expression. However, there was no attenuation of EMP release present in this study, which may suggest that MP release is not influenced by changes in blood pH in healthy human subjects.

A possible limitation to this study is that the EMP markers used here may not be the most specific marker attesting for the endothelial origin of MP, as suggested by the position statement from the European Society of Cardiology working group on peripheral circulation which was recently released (Lekakis et al., 2011). This group suggest that CD144, CD146, and CD62E may be more specific for EMP detection, however both CD105 and CD106, as well as other endothelial antigens, have been widely used previously. A further limitation to the study would be the lack of acid-base variables both during and post exercise, which would have provided an indication of the recovery between groups. However, the acid-base variables pre and post ingestion provide an indication of the efficacy of NaHCO₃ supplementation.

We hypothesised that changes in the circulating levels of EMP following strenuous activity would reflect the changes in the state of the endothelium. Taking this into account, it is reasonable to assume that the levels at which EMP were highest would represent the time at which the endothelial cell stress occurred, which was 90-min post exercise. Detecting the peak stress is something to consider in future work as it may even be that the EMP count was actually falling at 90-min and the true optimum was in fact prior to this time point. The effects of exercise on the vasculature are not particularly well characterised, and here we are able to draw attention to MP as potentially novel markers that are able to offer an insight into the state of the endothelium of healthy human participants during strenuous physical activity.

Conclusion

We are able to present evidence that a strenuous bout of exercise is accompanied by a rise in EMP within the circulation. The endothelium of healthy individuals is highly stressed after such exercise, but is able to rapidly recover within 180-min of ceasing exercise. Additionally,

EMP release was shown to be unaffected by changes in blood pH by means of NaHCO₃ ingestion prior to exercise, suggesting that shear stress may be the factor resulting in the increased circulating EMP due to its exhaustive nature, and warrants greater understanding. Finally, this work strengthens the notion that CD105 and CD106 appear novel biomarkers as to the state of the endothelium, specifically as shown here in healthy human participants.

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Conflict of interest

None declared. This is an original research article and we can confirm that this has not been submitted previously nor is under consideration with any other journal. We received no external financial support, nor did we require any sources of outside support for this research.

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Legends

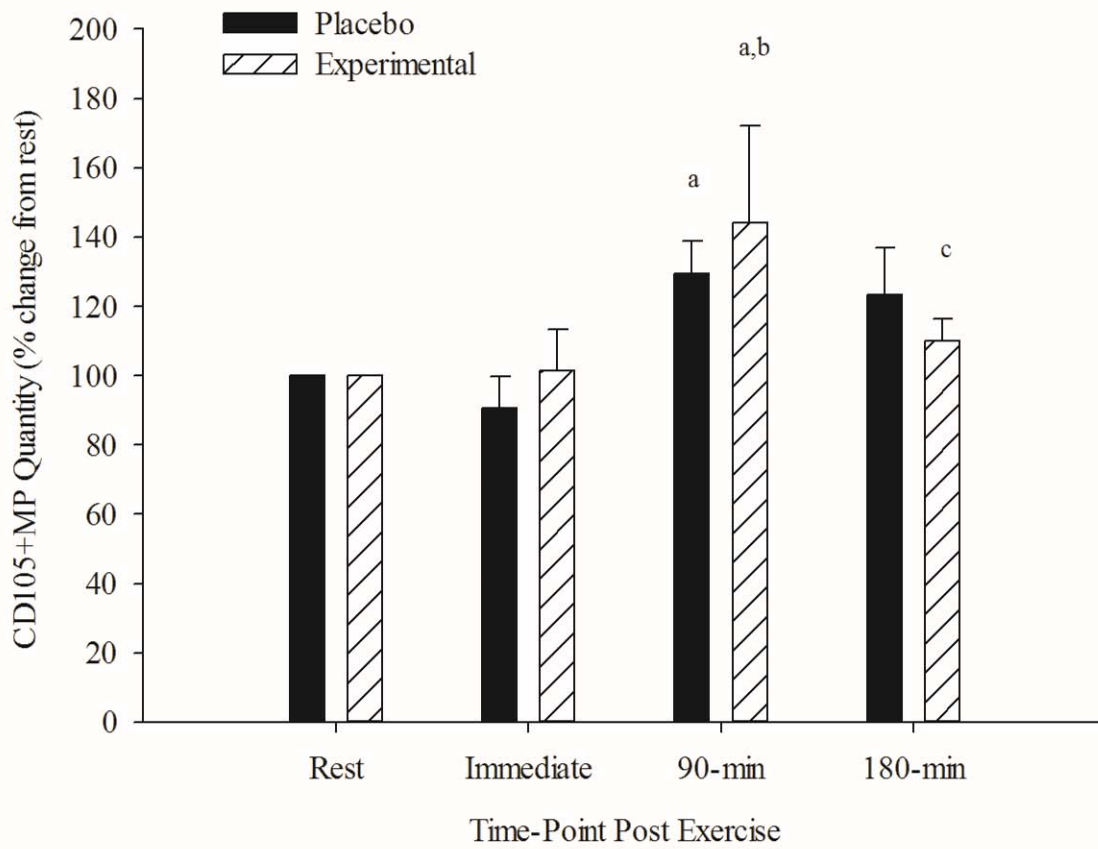
Figure 1 Acid-base variables pre- and post-ingestion of NaHCO₃ (black bars) and placebo (grey bars). Data are represented as mean \pm s. * significant difference to pre-ingestion ($p < 0.05$).

Figure 2 (A+B) Time dependent profile for the percentage change from resting values in CD105+MP (A) and CD106+MP (B) expressed at rest and immediately, 90-min, and 180-min post exercise during the placebo (dark bars) and experimental (white hatched bars) trials ($n=7$; mean \pm s_x). ^a significant difference to immediate. ^b significant difference to rest, ^c significant difference to 90-min, and ^d significant difference to immediate ($p < 0.05$).

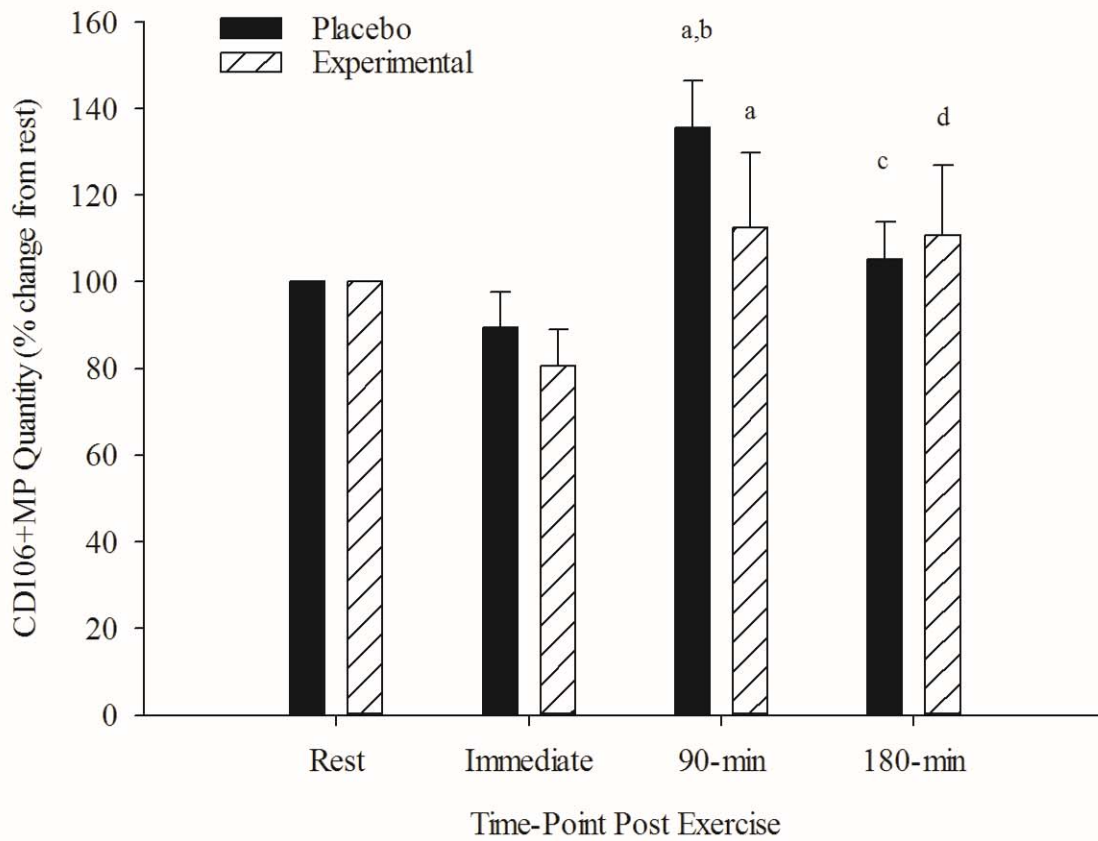
Figure 3 Time dependent profile for overall group means regardless of experimental condition, displaying percentage change from resting values in CD105+MP (●), and CD106+MP (○), expressed at rest, immediately, 90-min, and 180-min post exercise ($n=14$; mean \pm s_x). ^a significant difference to immediate, ^b significant difference to rest, ^c significant to 90-min, ^d significant to immediate. ($p < 0.05$).

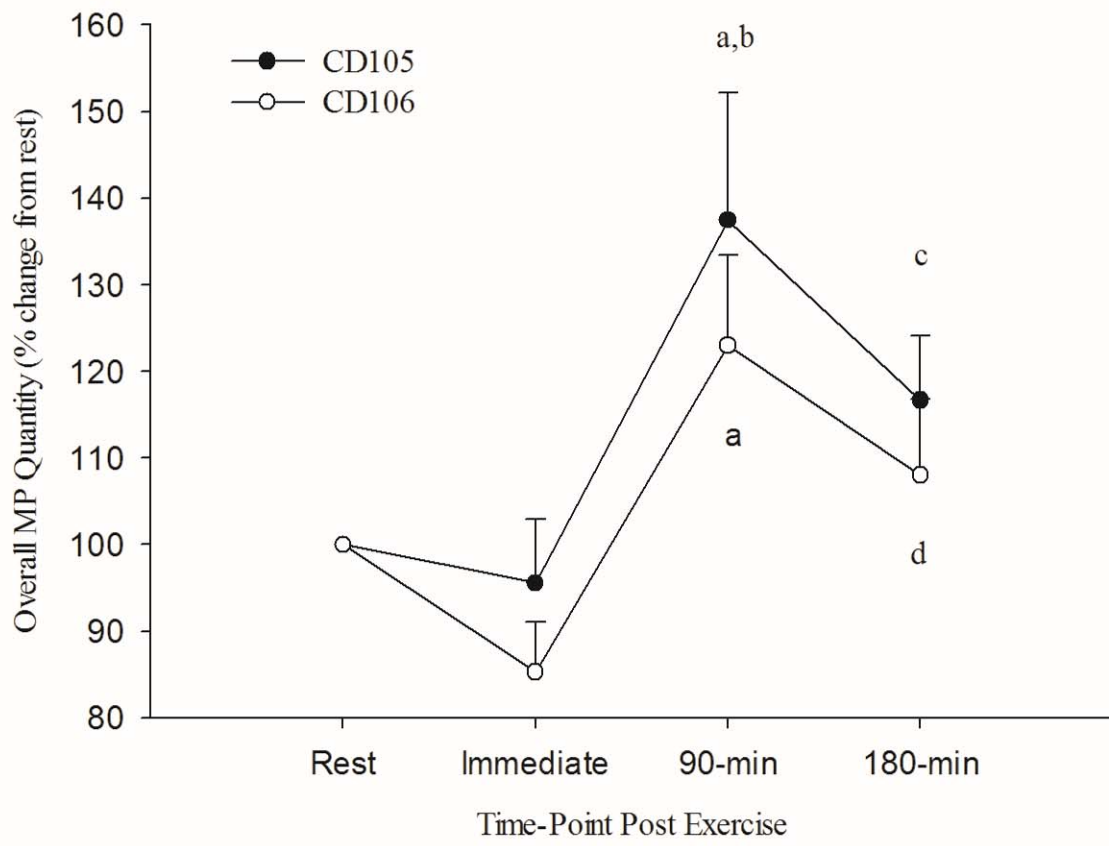
Figure 4 Time dependent profile for the PRR scores in each experimental group expressed from sprint number 1 to 10 during the placebo (●) and experimental (○) trials ($n=7$). Error bars have been excluded for clarity.

A



B





4

