Tobacco smoking and acute exercise on immune-inflammatory responses among relative short and longer smoking histories

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Abstract:

This study examined the acute effects of combined tobacco smoking and exercise on immune-inflammatory responses in smoker populations with shorter or longer smoking history. The cohort comprised 14 young male adult (YSM) and 12 middle-aged (MSM) male active cigarette smokers matched for aerobic fitness and smoking behavior. Following an initial familiarization and baseline testing session, participant's consumed two cigarettes (12mg tar, 1mg nicotine) within 15min, followed by a 10min recovery period and then 40min of cycling at 50% peak aerobic workload. Venous blood was obtained pre- and post-protocol for analysis of interleukin (IL)-6, IL-1receptor antagonist (ra), IL-1beta (β) and monocyte chemoattractant protein-1 (MCP-1) and total leukocyte count. There were no significant baseline differences between age groups for IL-6 or MCP-1 ($p>0.05$), although higher basal IL-1ra was evident in YSM ($p<0.05$). Further, no significant differences existed between groups for post-exercise IL-1ra or IL-6 responses; though MSM demonstrated an elevated MCP-1 at 4h post ($p<0.05$). No between-group differences for total leukocyte count, platelets, neutrophils, lymphocytes or monocytes ($p>0.05$) were observed. However, higher concentrations of basophils immediately post and 4h post-exercise, and higher eosinophils at 4h post-exercise were evident in MSM ($p<0.05$). The current study highlights that prolonged elevations in MCP-1, alongside leukocytosis, accompany inhalation of tobacco smoke prior to exercise. Pre-exercise smoking may blunt the IL-1ra response to exercise in a smoker population, which in turn may be related to smoking history.

Introduction

Tobacco smoking is an addictive lifestyle behavior and has been associated with alterations in immune and inflammatory function. Despite a lack of clarity on the acute immune-inflammatory mechanisms, the long-term effects of tobacco smoking are well documented, with an increased risk for non-communicable diseases such as diabetes, cardiovascular disease and cancers amongst the most notable (Sopori, 2002; Yanbaeva et al., 2007). Contrary to the effects of tobacco smoke, exercise offers protection against all-cause mortality, and produces favorable immune and inflammatory actions (Pedersen & Hoffman-Goetz, 2008). Exercise has become increasingly more popular as a therapeutic intervention for mediating disease risk, further improving symptoms associated with chronic disease (Lee & Skerrett, 2001). However, it remains unknown as to whether the exercise-based responses provide an anti-inflammatory response to tobacco smoke. Further understanding of these potentially opposing effects of exercise and tobacco smoke on inflammatory responses may assist to determine whether exercise can be considered as a tool to reduce inflammatory states associated with long-term smoking.

The mechanisms behind the physiological consequences of tobacco smoking are not well understood, though accumulating evidence suggests the adverse health outcomes from smoking may be related to alterations in immune function (Sopori & Kozak, 1998; Arson, Shoenfel, Amital, 2010; Sopori, 2002; Kastelein, Duffield & Marino, 2015). Long-term smoking is associated with augmentation of pro-inflammatory cytokines including, tumor necrosis factor- alpha (TNF-α), interleukins (IL)-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF) and subsequently inhibition of anti-inflammatory cytokines such as IL-10 (Arnson, Shoenfeld & Amital, 2010). Some of the reported effects from long-term smoking include reduced T-cell activity, augmented B-cell activity, activation

of leukocytes, decreased immunoglobulin's and natural killer (NK) cells (Moszczynski et al., 2001). The acute effects of tobacco smoke on immune and inflammatory processes also remain equivocal. Recently, we reported a singular bout of tobacco smoking alters the time-course response of both interleukin-6 (IL-6) and monocyte chemoattractant protein- 1 (MCP-1) (Kastelein, Duffield & Marino, 2015), which was related to duration of smoking history. Accordingly, given that habitual tobacco smoking imposes immune-inflammation alterations, including the up-regulation of IL-1β, IL-6 and TNF-α (Sopori, 2002), it seems important to determine the physiological responses to smoking exposure with differing smoking histories.

In contrast to tobacco smoking, exercise promotes anti-inflammatory responses (Pedersen & Hoffman-Goetz, 2000). Specifically, a marked immune and inflammatory cellular influx accompanies an acute bout of exercise, with the magnitude of this cascade dependent upon physical stress induced from exercise intensity, duration and modality (Gleeson et al., 2011; Petersen & Pedersen, 2004). Anti-inflammatory cytokines including IL-6, IL-1ra and IL-10 and circulating leukocytes are amongst the myriad of cells induced by exercise stimulus and with regular exercise have been reported to produce favorable health outcomes and indeed, may offer protection against all-cause mortality (Gleeson et al., 2011). However, brief exposure to secondhand smoke compromises both the cardiorespiratory and immune responses to moderate-intensity exercise (Flouris et al., 2012), suggesting some interference in the exercise-induced inflammatory responses from smoke exposure. Despite the contrasting immune-inflammatory responses induced by exercise and smoking respectively, very few studies have examined whether one interferes with the other.

Given the significant alterations suggested to result from chronic tobacco smoking and the reported beneficial effects of exercise on immune function, it seems important to

90 determine the acute effects of tobacco smoking and exercise in a smoker population. Thus,
91 this study aimed to aim to compare the immune-inflammatory responses to exercise and
92 smoking in populations with longer or shorter smoking histories.
93
94 **Materials and Methods**
95 **Subjects**
96
97 Young adult (*n*=14; 18-25y; YSM) and middle-aged (*n*=12; 30-50y; MSM) active
98 cigarette smokers matched for fitness and smoking behavior (active smokers) were recruited.
99 Participants reported as apparently healthy and were free of any metabolic or
100 cardiopulmonary abnormalities or renal or hepatic disorders, immunological irregularities,
101 abnormal leukocyte sub-populations, periodontal disease, and any other diseases or
102 conditions associated with systemic inflammatory responses. The self-reported smoking
103 history for the YSM and MSM populations was 5.2 ± 1.7y of smoking and 12.3 ± 6.8
104 cigarettes per day and 14.6 ± 6.5y of smoking and 15.8 ± 7.3 cigarettes per day, respectively
105 (Table 1). Prior to the commencement of the study, all participants were required to provide
106 written and verbal consent following an outline of all procedures and measures. This study
107 conformed to the Declaration of Helsinki and was approved by the Research in Human Ethics
108 Committee at Charles Sturt University.
109
110 **Overview**
111 Prior to commencement of the study participants were required to complete pre-
112 exercise, health history and smoking questionnaires (healthy history questionnaire,
113 Fagerstrom Test for Nicotine Dependence (Heatherton et al., 1991) and adult pre-exercise
114 screening system (APSS). Following pre-screening, and if satisfying the inclusion criteria,

participants were enrolled into the study following provision of informed consent. Participants then completed an initial familiarization session prior to a baseline testing session, which included measures of anthropometry, a graded exercise test (GXT) and a dual-energy x-ray absorptiometry scan. Following approximately 7d rest, participants returned to the laboratory and were required to partake in a testing session. The testing session involved the consumption of two cigarettes and following a 10min recovery the completion of a standardized cycle ergometry exercise protocol of 40min at 50% of peak oxygen consumption (VO_{2peak}).

Baseline Testing

Participants reported to the laboratory between 0530h and 800h in a rested and fasted state for the baseline testing session, with all consent and health questionnaires completed prior to this session. Stature (Stadiometer: Customised, Australia), body mass (HW 150 K, A & D, Bradford, MA, USA), and waist and hip circumferences (steel tape, EC P3 metric graduation, Australia) were obtained as measures of anthropometry based on standardized techniques. In addition, a supine dual-energy x-ray absorptiometry (DXA) scan was conducted for the determination of body composition (XR800, Norland, Cooper Surgical Company, Trumbull, CT, USA). Scanning resolution and speed were set at 6.5 x 13.0mm and 130 $mm \cdot s^{-1}$, respectively. Whole body scans were analyzed (Illuminatus DXA, ver. 4.2.0, USA) for total body lean mass and total body fat mass and are reported in absolute (kg) and relative (%) terms. Participants were further fitted with a heart rate (HR) monitor (Rs800cx, Vantage NV, Polar, Finland) for the recording of HR. A baseline blood sample was collected to determine, resting concentrations of inflammatory markers and blood lipid profile (subsequently described).

Finally, a GXT was performed on an electronically-braked cycle ergometer (LODE Excalibur Sport, LODE BV, Groningen, The Netherlands) for the determination of $VO_{2\ peak}$. Pulmonary gas exchange was measured by determining O_2 and CO_2 concentrations and ventilation to calculate VO_2 using a metabolic gas analysis system (Parvo-Medics, True2400, East Sandy, UT, USA). The younger group (YSM) commenced the test at 100W and increase by 25W whereas the middle-aged group (MSM) commenced at 25W and increase 25W every minute until every minute until volitional exhaustion. HR was collected every minute and a rating of perceived exertion (RPE: Modified Borg CR10 scale) collected at completion of the GXT.

Pre-Exercise Cigarette Consumption

Prior to the acute exercise bout, participants were instructed to smoke two cigarettes (Winfield Blue, 12mg tar, 1mg nicotine) within 15 minutes in a private but open area near the laboratory. Participants were seated throughout the protocol with no or minimal movements to ensure standardized measurements. The smoking protocol was based upon previous research by Van der Vaart et al., (2005) who administered two cigarettes of the same brand (two cigarettes of 12mg tar, 1mg nicotine) within 30min. Given that smoking is highly individualized and variable we standardized procedures in terms of dose and duration of the acute protocol. Participants were instructed to smoke as per normal smoking behaviour, adequacy of smoking ensured by visual observation by the research team. Following a 10min seated rest after the consumption of both cigarettes participants commenced the exercise bout.

Exercise Protocol

Following cigarette consumption, participants were required to complete the acute exercise protocol which consisted of 40min of stationary cycle ergometry (Monark 828E, Monark Exercise AB, Varburg, Sweden) at 50% of VO_{2peak}. The workload was calculated as 50% of the pedaling resistance (W) achieved during the GXT and was converted into kilopond units and set as a fixed intensity for the exercise protocol. Selection of this exercise protocol was based upon previous research (Mendham, Donges, Liberts & Duffield, 2011) that demonstrated an inflammatory response to a bout of exercise of the same intensity and duration as the current study, yet within the tolerable limits for the population. Further, telemetry-based HR (Rs800cx, Vantage NV, Polar, Finland) and RPE (Borg CR10 scale) were recorded every 5min and blood pressure was collected every 10min throughout and post-protocol.

Blood Collection

A 21-guage catheter inserted into the medial antecubital vein for the collection of venous blood will be collected pre and post (0min, 1h, 4h) protocol. Approximately, 50ml of blood was collected and aliquoted into serum separator tubes (SST) for analysis of blood lipid profile and CRP, ethylene diamine tetraacetic acid (EDTA) tubes for analysis IL-6, IL-1ra, MCP-1, glycosylated haemoglobin (HbA1c), glucose and total and sub-population leukocyte count. EDTA tubes were centrifuged immediately at 3500rpm for 15min at 4^{o}C and SST tubes were left to clot at room temperature for 20min prior to centrifugation. Supernatants were immediately stored at -80 ^{o}C or -20 ^{o}C for EDTA and SST, respectively.

Blood Analysis

Samples were analysed for blood lipid profile, CRP, IL-6, IL-1ra, MCP-1 and total and sub-population leukocyte count. Total cholesterol was analysed using an enzymatic

method and polychromatic endpoint technique measurement (Dimension Xpand Plus, Siemens Healthcare Diagnostics, Sydney, Australia). HDL cholesterol was measured using accelerator selective detergent methodology. Triglycerides were assessed using an enzymatic method and biochromatic endpoint technique measurement. Further, HbA1c was measured using automated high-performance liquid chromatography methodology (Bio-Rad Variant, Bio-Rad Laboratories, Sydney, Australia). Leukocyte count was determined by a cell counter (Sysmex XT-1800i, Mundelein, IL, USA). Moreover, concentrations of CRP were determined using a solid-phase, chemiluminescent immunometric assay and concentrations of IL-6, IL-1ra, MCP-1 and were analyzed with a sandwich enzyme immunoassay technique, according to manufacturer's instructions (ELISAkit, Melbourne, Australia and Merk Millipore, Billerica, MA, USA).

Statistical Analysis

All data are reported as mean ± standard deviation (SD). Normal distribution was determined by Shapiro-Wilk's test and non-normally distributed data (inflammatory data) was logarithmically transformed prior to analysis. Two-way repeated measures analysis of variance (ANOVA) was used to determine within- and between-group differences. Where a group interaction was noted, one-way ANOVA tests were applied to determine the source of significance. Significance was set at $p<0.05$. All statistical procedures were performed using Predictive Analytic Software (PASW) (Statistical Package for the Social Sciences for Windows version 18.0, Chicago, IL, USA).

Results

Baseline data for blood lipid profile, anthropometric and smoking variables are reported in Table 1. No differences were observed between groups for VO_{2peak} ($p>0.05$). The

MSM group demonstrated greater absolute and relative fat mass than YSM ($p<0.05$). In regards to smoking history, as expected the MSM group had significantly greater smoking history in terms of years of smoking and pack-years than YSM ($p<0.05$); however, this did not differ in regards to the number of cigarettes smoked or level of dependence ($p>0.05$) according to the Fagerstrom Test for Nicotine Dependence. Exercise-induced HR responses (% of HR_{max}) were not significantly different between groups (76.2 ± 8.3%, 77.8 ± 6.6% for YSM and MSM, respectively; $p>0.05$). Additionally, there were no significant differences in RPE between groups (5.0 ± 0.3 and 4.4± 0.3 for YSM, MSM, respectively; $p>0.05$).

There were no baseline differences between groups for IL-6 or MCP-1 ($p>0.05$; Figure 1); however, YSM showed higher IL-1ra at baseline compared to MSM ($p<0.05$). No between group differences for IL-1ra or IL-6 were observed following exercise ($p>0.05$), although MSM demonstrated elevated MCP-1 at 4h post ($p<0.05$). Despite baseline differences for IL-1ra, no within-group change was observed for either group ($p>0.05$; Figure 1). Post-exercise elevations in IL-6 were evident for YSM ($p<0.05$), though not for MSM ($p>0.05$). Post-exercise elevations in MCP-1 were observed for MSM, yet not in YSM, who showed a decline from 1h to 4h post-exercise ($p<0.05$).

Total and fractional leukocyte responses are presented in Figure 2. There were no between-group differences for total leukocyte count, platelets, neutrophils, lymphocytes or monocytes at baseline or following the protocol ($p>0.05$). MSM showed higher concentrations of basophils at immediate post and 4h post-exercise, whilst eosinophils were higher elevated is MSM at 4h post-exercise ($p<0.05$). Both groups observed within-group post-exercise increases in WBC, which remained elevated at 4h post-protocol ($p<0.05$). Increased neutrophils counts were evident immediately post-protocol for YSM followed by a

decline to 1h ($p<0.05$). Similarly, MSM observed a decline to 1h followed by an increase to 4h ($p<0.05$) in neurtophils, while platelet values for both groups remained above pre at 4h post ($p<0.05$). Both YSM and MSM showed a decrease from post to 1h in lymphocytes followed by an increase to 4h ($p<0.05$), and only values for YSM remained above pre ($p<0.05$). Monocytes increased post-exercise in MSM ($p<0.05$); however values for YSM were significantly higher at 4h than pre-values ($p<0.05$), which was not observed in MSM ($p>0.05$). The smoking and exercise protocol induced declines in eosinophils immediately post to 1h in both groups, this decline continued to 4h for MSM and remained below pre-values ($p<0.05$), not observed in YSM. Finally, basophils in both groups increased post exercise and declined thereafter (to 1h).

Discussion

This study examined the combined effects of acute tobacco smoking prior to exercise on inflammatory responses in smoker populations with longer or shorter smoking histories. Accordingly, the novel finding from this study is that IL-1ra remains relatively unchanged following both smoking and exercise stimulus, suggesting smoking to have a suppressive effect of the IL-1ra response to an acute bout of exercise. Furthermore, the chemokine MCP-1 was significantly elevated at 4h post in MSM, which may be indicative of inflammatory cell recruitment resultant from the insult of tobacco smoke prior to exercise

Chronic smoking results in significant alterations to the immune system (Sopori, 2002; Mehta, Nazzal & Sadikot, 2008). As evidenced by the current study, even an acute bout of tobacco consumption has immune system implications. The current study revealed that pre-exercise smoking may inhibit the anti-inflammatory IL-1ra response to exercise, given IL-1ra remained unchanged, when previous research shows increased IL-1ra following

an acute exercise bout (Moldoveanu, Shephard & Shek, 2001; Petersen & Pedersen, 2005). IL-1ra is known as an anti-inflammatory cytokine as it acts to inhibit the actions of IL-1 and ultimately IL-1β (Arend, 2002), and thus presents anti-inflammatory actions (Arend, 2002; Moldoveanu, Shephard & Shek, 2001). Accordingly, the present results suggest pre-exercise smoking may inhibit the anti-inflammatory IL-1ra response to exercise. Further, the present data contrasts with previous research indicating an IL-1ra response to acute exercise in young smokers delivered following an exercise bout (Kastelein et al. 2017). Given the differences in tobacco smoking timing, pre-exercise smoking may have an inhibitory effect on the IL-1ra response to exercise and thus counter the acute anti-inflammatory benefits derived from exercise.

Accompanying the systemic inflammatory response is the presence of MCP-1, a potent chemoattractant. In the present study, a prolonged MCP-1 response in MSM was evident following the combination of smoking and exercise. Chemokines such as MCP-1 control the expression and migration of cells such as neutrophils and lymphocytes, and while Becker, Seul & Lindemann (2005) observed increased MCP-1 following a maximal exercise, it is not uncommon for MCP-1 to be elevated in chronic tobacco smoking, with Traves et al. (2002) reported MCP-1 to be elevated in the pulmonary microenvironment in smokers at rest. Previous findings from our group revealed middle-aged smokers demonstrated elevated MCP-1 above that of age-matched non-smoking counterparts; and further, moderate-intensity exercise resulted in elevations in MCP-1 in both young and middle-aged smokers and their age-matched non-smoking counterparts (Kastelein, Duffield & Marino, 2015). Further in response to an acute tobacco smoking bout, MCP-1 remained elevated for 4h post-consumption in middle-aged smokers, whereas values declined for younger smokers (Kastelein, Duffield & Marino, 2015). Similarly, the present study revealed MCP-1 to be

elevated 4h post-protocol in MSM and not YSM. This finding may be as a consequence of the longer smoking history of the MSM group, and whilst speculative, alterations in the expression of MCP-1 resultant from chronic tobacco smoking may contribute to the development of low grade inflammatory states and consequently pathogenic events associated with long-term inflammation (Yadav, Saini & Arora, 2010).

An acute bout of exercise generally causes a transient increase in IL-6 (Moldoveanu, Shephard & Shek, 2001), and smoking is suggested to cause perturbations in immune-inflammatory function particularly in response to exercise as previously reported (Kastelein et al., 2017; Kastelein, Duffield & Marino, 2015). The current study showed no between-group differences IL-6 responses. However, significant within-group elevations were observed for YSM and although following a similar pattern, values for MSM were not significant. As explanation, Koethe et al. (2000) reported cigarette smoke condensate to prime neutrophils thus making them more responsive to activating agent, additionally, primed neutrophils may function to amplify and prolong inflammation (Koethe et al., 2000); however in MSM, inhibition of immune response may be a consequence of long term smoking (Stampfli & Anderson, 2009; Sopori, 2002). In the present study, elevations in IL-6 for YSM are accompanied by neutrophilia, and Suzuki et al. (1999) previously proposes exercise induced neurtophilia may be in part responsible for the inflammatory response. These mechanism may be in part responsible for the IL-6 responses in YSM, and while were not measured here, the elevations in IL-6 may result from neutrophil priming amongst YSM in response to an acute bout of smoking and exercise.

Leukocytosis following an acute bout of exercise is well known (Gabriel & Kindermann, 1997; Suzuki et al., 2000). Similarly, it is commonly reported for chronic

smokers to exhibit leukocytosis as a result of long term cigarette smoke exposure (Frohlich et al., 2003; Kawada, 2004). However, minimal literature exists describing whether smoking exacerbates the leukocyte response to exercise following acute smoke exposure.

This study showed that smoking followed by an acute exercise bout induced elevations in basophils and eosinophils in MSM at 1h and WBC remained elevated at 4h, this is in contrast to our previous work, which suggest post-exercise tobacco smoking results in decreases in eosinophils and basophils (Kastelein et al., 2017). Interestingly, the elevated in WBC count are paralleled in prolonged elevations in MCP-1, particularly amongst MSM. Given MCP-1 is a chemoattractant, it is plausible to suggest that the physiological stress of smoking and exercise results in inflammatory cell recruitment; which, may provide insight to the mechanisms responsible for low grade inflammation observed in tobacco smokers (Yadav, Saini & Arora, 2010).

The addictive nature of tobacco smoke contributes to multiple tobacco-induced pathological states, of which immune-inflammatory mechanisms may play a role. While numerous studies report the immune and inflammatory response to smoking and exercise respectively (Moldoveanu, Shephard & Shek, 2000; Yanbaeva et al., 2008; Arnson, Shoenfeld & Amital, 2010), concurrent smoking and exercise responses remain relatively unknown. Pre-exercise smoking responses here show reduction in the post-exercise IL-1ra response, suggesting that smoking prior to exercise inhibits the anti-inflammatory response to an acute exercise bout. Further, the current study suggests that prolonged elevations in MCP-1, alongside leukocytosis, which may be indicative of pathological changes amongst MSM. These findings contribute to the understanding on the mechanisms responsible for the physiological consequences of smoking, further, examines the role of exercise as a tool for improving the health of smokers. While the current findings suggests that concurrent

smoking and exercise may inhibit the anti-inflammatory response to exercise future research should be directed toward smoking cessation and exercise programs, given the purported benefits of exercise for health risk reduction.

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Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

TK, RD and FM were involved in the methodological design. TK collected and analysed the data and drafted the manuscript. RD and FM provided critical feedback on the manuscript.

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Table 1. Mean ± SD Baseline descriptive, anthropometric, dual-energy x-ray absorptiometry (DXA), biochemistry, aerobic fitness and smoking variables within the young smoker (n=14) and middle-aged smoker (n=14) populations.

Anthropometric & Descriptive Data	*YSM*	*MSM*	*Desirable value*
Age (years)	*22.0 ± 1.57*	*33.27 ± 7.75**	
Height (m)	*1.82 ± 0.07*	*1.77 ± 0.07*	
Weight (kg)	*81.78 ± 12.07*	*81.22 ±12.87*	
VO_2 peak ($mL \cdot kg^{-1} \cdot min^{-1}$)	*36.67 ± 3.06*	*33.93 ± 8.74*	
Final stage Watts (GXT)	*275 ± 36.69*	*230 ± 46.48*	
Waist Circumference (cm)	*84.46 ± 8.44*	*87.67 ± 8.92*	<102
Waist to hip ratio	*0.86 ± 0 .05*	*0.86 ± 0.06*	<0.90
% Fat mass	*15.62 ± 5.78*	*24.75 ± 6.76**	
Lean Mass (kg)	*63.03 ± 9.05*	*59.02 ±6.61*	
Fat Mass (kg)	*12.37 ± 5.32*	*20.68 ± 6.83**	
Biochemistry			
CRP	*2.0 ± 1.90*	*1.98 ± 1.80*	*<2.9*
HDL ($mmol\ L^{-1}$)	*1.06 ± 0.34*	*1.22 ± 0.36*	>1.0
Triglycerides ($mmol\ L^{-1}$)	*1.28 ± .064*	*1.50 ± 0.84*	<2.0
Fasting glucose ($mmol\ L^{-1}$)	*5.07 ± 0.73*	*4.79 ± 0.46*	<5.5
HbA1c (%A1c)	*5.20 ± 0.31*	*5.48 ± 0.28*	<6.5
Smoking Variables			
Years of smoking	*5.21 ± 1.72*	*14.62 ± 6.55**	
Cigarettes per day	*12.31 ± 6.81*	*15.79 ± 7.34*	
Pack years	*2.86 ± 1.91*	*12.15 ± 9.61**	
Fagerstrom score	*2.31 ± 1.38*	*2.48 ± 1.28*	

*Denotes significantly different to YSM ($p<0.05$)

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Fig. 1. Mean ± SD for IL-1ra, IL-6 and MCP-1 pre, post, 1h and 4h post protocol for younger smokers and older smokers. * represents significantly different between YSM and MSM (P<0.05), # represents significantly different within condition for YSM (P<0.05), ¥ represents significantly different within condition for MSM (P<0.05).

Fig. 2. Mean ± SD for total and fractional leukocytes pre, post, 1h and 4h post protocol for younger smokers and older smokers. * represents significantly different between YSM and MSM (P<0.05), # represents significantly different within condition for YSM (P<0.05), ¥ represents significantly different within condition for MSM (P<0.05).