Influence of 4 weeks of bovine colostrum supplementation on neutrophil and mucosal immune responses to prolonged cycling

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Running head: Bovine colostrum and innate immune responses

Abstract

Bovine colostrum (COL) has been advocated as a nutritional countermeasure to exercise-induced immune dysfunction. The aims of this study were to identify the effects of 4 weeks of COL supplementation on neutrophil responses and mucosal immunity following prolonged exercise. In a randomised double-blind, parallel group design, participants (age 28 ± 8 years; body mass [BM] 79 ± 7 kg; height 182 ± 6 cm; maximal oxygen uptake [$\dot{V}O_2$ max] 55 ± 9 mL·kg-$^{-1}$·min^{-1}) were assigned to 20 g per day of COL (n = 10) or an isoenergetic/isomacronutrient placebo (PLA) (n = 10) for 4 weeks. Venous blood and unstimulated saliva samples were obtained before and after 2.5 h of cycling at 15% Δ (~ 55-60% $\dot{V}O_2$ max). A significantly greater fMLP-stimulated oxidative burst was observed in the COL group compared with PLA group ($p < 0.05$) and a trend toward a time × group interaction ($p = 0.06$). However, there was no effect of COL on leukocyte trafficking, PMA-stimulated oxidative burst, bacterial-stimulated neutrophil degranulation, salivary secretory IgA, lactoferrin or lysozyme ($p > 0.05$). These findings provide further evidence of the beneficial effects of COL on receptor-mediated stimulation of neutrophil oxidative burst in a model of exercise-induced immune dysfunction.

Keywords: immune function, innate immune system, leukocyte, antimicrobial peptides, exercise

Introduction

It is now well established that exercise of a strenuous and/or prolonged nature can lead to significant transient perturbations of immune function (commonly referred to as immunodepression) which includes, but is not limited to, decreases in both cell-mediated and mucosal parameters (Nieman, 2007). This ‘‘open window’’ period of immune dysfunction has long been hypothesised to contribute to the frequency of symptoms of upper respiratory illness (URI) in individuals exposed to prolonged exercise on a regular basis (Gleeson, 2007, Walsh et al., 2011). Various nutritional interventions have been investigated as strategies to minimise immune perturbations and URI risk following prolonged exercise, but the majority lack evidence to support their use (Gunzer et al., 2012).

Bovine colostrum (COL) is the initial milk produced by a cow in the first few days following parturition. Our laboratory, and others, have reported reduced incidence of URI in exercising populations during periods of COL supplementation (20 - 60 $g \cdot day^{-1}$) (Brinkworth & Buckley, 2003; Jones et al., 2014). The mechanism(s) behind such effects remain unclear but it has been proposed that COL acts as a nutritional countermeasure to perturbations in immune cell functions and salivary antimicrobial peptides (AMPs) following acute exercise (Davison, 2013). However, there remains a lack of investigations on the effects of COL on specific parts of the human immune system in a post-exercise state.

Animal and *in vitro* culture studies provide evidence of direct effects of COL on phagocytosis and oxidative burst of polymorphonuclear cells (i.e. neutrophils) (Benson et al., 2012; Sugisawa et al., 2001, 2003). Neutrophils form a critical part of the human body’s first line of defence as the most abundant leukocyte in the

57 circulation. While blood neutrophil count is increased following an acute bout of
58 prolonged exercise (> 1.5 h), the killing mechanisms (production of cytotoxic reactive
59 oxygen species and release of hydrolytic enzymes) per neutrophil are reduced
60 (Gleeson, 2007). Previous work in our laboratory showed that 4 weeks of COL (20
61 $g \cdot day^{-1}$) supplementation can limit the immunodepressive effects of an acute
62 physical stressor (2 h of cycling) by enhancing neutrophil stimulated-degranulation
63 (elastase release) post-exercise (Davison and Diment, 2010). However, little is
64 known about the effects of COL supplementation on other aspects of neutrophil
65 functional capacity (e.g. neutrophil oxidative burst) under such physical exertion.

66 Enhancement or restoration of mucosal defences following exercise may be of
67 relevance to host defence against URI due to proximity to sites where most
68 pathogens enter the body (i.e. respiratory tract). COL supplementation has been
69 shown to prevent prolonged exercise-induced decrements of salivary lysozyme
70 (sLys) concentration and secretion (Davison and Diment, 2010). Given the
71 importance of mucosal immune parameters towards host defence (West et al.,
72 2010), further investigation of the effects of COL on other salivary AMPs (e.g.
73 salivary lactoferrin, sLac) is warranted. Salivary secretory SIgA is the immune
74 marker which has been most studied as a risk factor for URI during exercise training
75 (Walsh et al., 2011). COL supplementation for 2-12 weeks has been shown to
76 increase resting levels of SIgA (Crooks et al., 2006; Mero et al., 2002), an immune
77 modulation proposed to be an important underlying mechanism of reduced URI with
78 COL. This hypothesis has not been confirmed and a change in resting output of
79 salivary SIgA (concentration and/or secretion) is not consistently reported (Crooks et
80 al., 2010; Jones et al., 2014). Despite lower incidence of URI during 12 weeks of
81 COL supplementation, we did not observe any significant increases in SIgA

concentration or secretion rate in active males (Jones et al., 2014). This supports proposals that the effect of COL on host defence may occur through other (or a combination of) mechanisms (Shing et al., 2007). There are, however, a limited number of studies that have investigated the effect of COL on SIgA responses and other markers of immune function following prolonged exercise. Indeed, it is apparent that the magnitude of change in immunity immediately following each bout of strenuous exercise may have more clinical implications than training-induced alterations in resting immunity (Abbasi et al., 2013; Nieman et al., 1994; Pedersen and Bruunsgaard, 1995). Therefore, the primary aim of this study was to determine the effects of 4 weeks of COL supplementation on neutrophil oxidative burst following prolonged cycling with secondary aims to investigate responses of neutrophil degranulation and salivary AMPs.

Materials and methods

Participants

Twenty healthy, recreationally active males (age 28 ± 8 years; body mass [BM] 79 ± 7 kg; height 182 ± 6 cm; maximal oxygen uptake [$\dot{V}O_2$max] 55 ± 9 mL·kg^{-1}·min^{-1}) volunteered to participate in this investigation. Aberystwyth University Research Ethics Committee approved all experimental procedures prior to the recruitment of any participants. Participants provided both verbal and written consent following information on experimental procedures. All participants were non-smokers, non-allergic to dairy products and reported no symptoms of infection or taking any medication or dietary supplements 4 weeks prior to commencement of study. All laboratory visits involving exercise also required completion of a physical activity readiness questionnaire.

Supplementation

Participants were randomly assigned to a COL group (n = 10, age: 29 ± 8 years, BM: 79 ± 8 kg, height: 183 ± 6 cm, $\dot{V}O_2$ max 54 ± 9 mL·kg^{-1}·min^{-1}) or a placebo (PLA) group (n = 10, age: 27 ± 9 years, BM: 78 ± 8 kg, height: 180 ± 7 cm, $\dot{V}O_2$ max 56 ± 9 mL kg^{-1}·min^{-1}). In a double blind manner, participants were asked to consume 20 g per day (split into a morning and evening 10 g dose on an empty stomach) of COL (Neovite UK, London) or an isoenergetic/isomacronutrient PLA (as used in Davison and Diment, 2010) for 4 weeks (28 days).

Preliminary testing

On day 14 of the study period (where day 0 is the day on which baseline samples were obtained (see below) and supplementation of COL or PLA was commenced),

gas exchange threshold (GET) and $\dot{V}O_2$max were determined via a continuous incremental test (30 W·min^{-1} ramp rate following 3 min of unloaded baseline pedalling) to volitional exhaustion on an electrically braked cycle ergometer (Lode Excalibur, Groningen, The Netherlands). Throughout the duration of the incremental test, expired gas was analysed by the use of an online breath-by-breath gas analysis system (Jaeger Oxycon Pro, Hoechberg, Germany). The test was terminated when the participant's cadence fell 10 rpm below their preferred cadence for more than 10 s as used previously (Davison and Diment, 2010). For each participant $\dot{V}O_2$ max was determined by the highest 30 s average during the test. GET was estimated for each participant via the V-slope method (Lansley et al., 2011). The exercise intensity was set to the power output that would elicit 15% Δ (15% of the difference between power output at GET and $\dot{V}O_2$ max) which was equivalent to ~ 55-60% of the participant's $\dot{V}O_2$ max. The use of % Δ was used to provide a stricter control on the relative intensity and limit inter-subject variability in physiological responses (Lansley et al., 2011). On day 21, a familiarisation trial took place to accustom participants to the testing procedures and physical stress expected in the main experimental trial. Participants performed a 2.5 h exercise bout on the electronically braked cycle ergometer (specified above) at an intensity of 15% Δ. Expired gas was analysed during the 10th, 30th, 60th, 90th and 120th min of exercise to verify that the selected workrate did elicit the target intensity. Heart rate (HR) and RPE were monitored every 15 min during the protocol using a telemetric device (Polar S610, Polar Electro Oy, Kempele, Finland) and Borg scale respectively.

140 Experimental trial procedures

141 On day 28 of the study, participants completed the main experimental trial. During
142 the 48 h preceding the experimental trial, participants were asked to refrain from
143 heavy exercise and alcohol consumption. On the morning of the experimental trial,
144 participants reported to the laboratory at 09:00 after an overnight fast of at least 10 h.
145 The participants were asked to consume 500 mL of water 2 h before arrival to
146 encourage euhydration. Participants remained seated for 10 min prior to collection of
147 a resting blood sample from an antecubital vein and an unstimulated saliva sample
148 (see details on sampling below). Following collection of samples, participants
149 immediately commenced 2.5 h of cycling at 15% Δ. All participants were permitted
150 diluted cordial (four volumes of water to 1 volume of sugar-free cordial at 2 $mL \cdot min^{-1}$
151 kg of BM) every 15 min during the exercise but not at end of the exercise (to limit
152 contamination to saliva samples). Expired gas was analysed during the 30th, 60th,
153 90th and 120th min of exercise (Jaeger Oxycon Pro, Hoechberg, Germany). HR and
154 RPE were monitored every 15 min during the protocol. Participants remained fasted
155 for further blood and saliva samples immediately and 1 h post-exercise.

156 Blood sampling

157 Participants remained seated, performing minimal movement for 10 min prior to each
158 blood sample with the exception of immediately post-exercise samples which were
159 drawn within a few min of exercise cessation. Blood was collected prior to
160 commencing the above supplementation (Baseline, day 0), pre-exercise, post-
161 exercise and 1 h post-exercise. Blood samples were collected by venepuncture
162 (with a 21 gauge precision needle [Becton-Dickinson, Oxford, UK]) from an
163 antecubital vein into vacutainers (Becton-Dickinson, Oxford, UK) containing
164 tripotassium ethylene diamine tetraacetic acid (K_3EDTA) or lithium heparin.

Haemoglobin, total and differential leukocyte counts were measured in each K_3EDTA vacutainer using an automated haematology analyser (Pentra 60 C+ Haematology analyser, HORIBA Medical, Montpellier, France). Haematocrit was determined from an aliquot of whole blood (heparin anti-coagulated) by a standard microcentrifugation method (using a Hawksley microcentrifuge). This was used along with the previously attained haemoglobin concentration, to estimate changes in blood and plasma volume from pre- to post-exercise as previously described (Dill and Costill, 1974). The remaining blood in heparin vacutainers were centrifuged at 1500 g for 10 min at 4°C with subsequent plasma being stored at -80°C for later analysis of plasma lactate and glucose concentrations using an automated analyser (YSI 2300 Stat Plus, Yellow Springs, Ohio, USA) and measurement of unstimulated elastase using an enzyme-linked immunosorbent assay (ELISA) kit (Merck Calibiochem, Darmstadt, Germany).

In vitro blood neutrophil function

Whole blood from the K_3EDTA treated tubes at pre-exercise, post-exercise and 1 h post-exercise was placed in a microcentrifuge tube and stored at room temperature (no longer than 2 h) prior to measurement of *in vitro* stimulated neutrophil oxidative burst response to PMA and/or fMLP using a commercially available chemiluminescence (CL) kit (ABEL, Knight Scientific Ltd, Plymouth, UK) in accordance with previous studies (Davison et al., 2012). The sample was diluted as described below before (~ 5 min) commencing the assay. The CL per well was measured by a microplate luminometer (FLUOstar OPTIMA, BMG Labtech, Aylesbury, UK). The contents of each microplate well that contained a stimulated sample were as follows: 10 µL of diluted whole blood (blood and Hank's balanced salt solution; HBSS, without calcium and magnesium, at ratio of 1:100), 90 µL assay

buffer (HBSS with calcium and magnesium), 50 µL Pholasin and 20 µL adjuvant K (substance that enhances the luminescence of Pholasin during assays involving diluted whole blood).

These mixtures were gently shaken and incubated at 37°C for 30 s in the luminometer, prior to the addition of 20 µL of PMA (5 µg mL^{-1}) or 20 µL fMLP (10 µm) to provide an end total volume of 200 µL per well, a 1:1010 final blood dilution and a PMA or fMLP concentration of 0.5 µg·mL^{-1} or 1µM respectively. The doses of fMLP and PMA for these assays are supramaximal in order to provide responses that are reproducible (attributable to neutrophils only) and prevent any rate limiting effect from utilisation of the stimulants. PMA penetrates the cell (independent of a receptor), triggering a long lasting, strong stimulation via protein kinase C and activation of NADPH oxidase throughout the cell. In contrast, fMLP, induces a rapid but short, receptor-dependent stimulation of neutrophils via activation of NADPH oxidase at the cell membrane. Although unstimulated samples in the PMA assay were also placed through similar mixing and incubation as the stimulated wells, 110 µL of HBSS was added to wells to ensure that the end volume of each well was standardised (replacing the combination of 90 µL HBSS and PMA). In all samples which were stimulated by PMA, CL was recorded in duplicate as relative light units (RLU) at 20 s intervals for 30 min and the area under the CL curve was calculated. The area under the unstimulated CL curve for each sample was subtracted from the mean area of the duplicate stimulated sample to determine the PMA-stimulated CL. When fMLP was the stimulant used, CL was recorded every second for 300 s. The unstimulated state in the fMLP was calculated as the mean CL of the well during the initial 2 s prior to addition of fMLP. The area above the stable unstimulated state was used to determine fMLP-stimulated CL.

To account for oxidative burst responses on a per cell basis, it was assumed that the CL responses were attributable largely to the neutrophils within the samples (Morozov et al., 2003). Thus, PMA-stimulated and fMLP-stimulated area under the CL curve were divided by the number of neutrophils present in each well to give CL in RLU (i.e. oxidative burst response) per neutrophil. To facilitate inter-subject comparisons, post-exercise and 1 h post-exercise stimulated oxidative burst responses were expressed as a percentage of the pre-exercise value, in accordance with Davison et al. (2012).

Whole blood (primarily via leukocytes) may contribute to exercise-induced increases in ROS and a state of oxidative stress. In accordance with Davison et al. (2012) the unstimulated 30 min CL responses from the PMA oxidative burst assay were expressed per L of whole blood to provide a measure of spontaneous CL (a snapshot of the level of ROS). This would allow for an investigation of the effect of COL on the antioxidant potential of whole blood following exercise.

The neutrophil degranulation response was assessed in accordance with Davison and Diment (2010). The measurement of neutrophil degranulation involved adding 1 mL of the heparinised blood sample to microcentrifuge tubes containing 50 μL of bacterial stimulant (840-15, Sigma, Poole, UK). The tubes were initially mixed by gentle inversion before being incubated at 37°C for 1 h. All tubes were gently mixed halfway through the incubation period. Following incubation, the tubes were centrifuged for 2 min at 16,000 *g*, with the supernatant being immediately removed and stored at -80°C until further analysis. Upon thawing at room temperature, neutrophil degranulation response was based on measuring the amount of stimulated elastase release per neutrophil using an ELISA kit (Merck Calibiochem,

239 Darmstadt, Germany). Bacterial-stimulated elastase release was based on
240 subtracting elastase concentration of unstimulated samples (heparinised plasma at
241 same timepoint) away from stimulated samples. The unstimulated samples were
242 processed immediately to provide background plasma elastase concentration at the
243 specific timepoint (i.e. not incubated alongside stimulated samples).

244 Saliva sampling

245 Participants remained seated, performing minimal movement for 10 min prior to each
246 saliva sample with the exception of immediately post-exercise samples which were
247 obtained within a few minutes of exercise cessation. Saliva samples were collected
248 following blood samples at baseline, pre-exercise, post-exercise and 1 h post-
249 exercise. For all saliva samples the mouth was rinsed with plain water at least 10
250 min before the collection period. The participant was requested to swallow in order to
251 empty the mouth before each saliva sample. To obtain the sample, the participant
252 remained seated with the head tilted slightly forward and passively dribbling into a
253 pre weighed 7 mL sterile bijou tube while keeping orofacial movement to a minimum.
254 The final duration of collection was recorded and the tube was re-weighed to allow
255 for calculation of saliva flow rate when the density of saliva was assumed to be 1.0
256 $g \cdot ml^{-1}$ as used previously (Davison and Diment, 2010).

257 After collection of saliva, samples were centrifuged for 5 min at 16,000 *g* to pellet
258 debris leaving the remaining clear supernatant to be aliquoted and stored at – 80°C
259 for later analysis. All saliva samples were thawed at room temperature only once
260 prior to analysis. Following the thawing of saliva, samples were again centrifuged for
261 5 min at 16,000 *g* to precipitate mucins and other debris and allow for the resulting
262 clear supernatant to be analysed. With the use of a freezing point depression

263 osmometer (Osmomat 030, Gonotec, GmbBH, Berlin, Germany), saliva osmolality
264 was determined to allow for concentration of salivary immunological parameters to
265 be expressed relative to saliva osmolality.

266 Aliquots of saliva were screened for blood contamination by the determination of
267 salivary transferrin concentration using an ELISA kit (Salivary blood contamination
268 enzyme immunoassay kit, Salimetrics, State College, Pennsylvania, USA). If salivary
269 transferrin concentration was greater than 1 $mg \cdot dL^{-1}$, the sample was considered to
270 be contaminated with blood and all other salivary data for that sample was excluded
271 (except SIgA due to assay being specific to secretory IgA and hence not affected by
272 blood contamination) from the study.

273 Salivary secretory IgA and antimicrobial peptides

274 The concentration of salivary SIgA ($mg \cdot L^{-1}$) and antimicrobial peptides were
275 determined in accordance with the methods of Jones et al. (2014). Briefly, diluted
276 saliva supernatants (1:8000 with phosphate buffered saline, PBS) were analysed
277 using a sandwich enzyme-linked immunosorbent assay (ELISA) approach (specific
278 to the secretory component of human IgA). Following a 1000 and 8000 fold dilution
279 of saliva supernatants (with PBS) commercially available ELISA kits were used to
280 measure the concentration of sLac and sLys respectively (Assaypro LLC, St-Louis,
281 MO).

282 Statistical analysis

283 Data shown in the text, tables and figures are presented as mean ± standard
284 deviation unless stated otherwise. Statistical analysis of all data was performed via
285 the statistical computer software package SPSS (v20.00; SPSS Inc., Chicago, IL,
286 USA). Initially, a two factor mixed ANOVA (group × time) was carried out on

287 immunological measures to determine if the effect of time was different between
288 COL and PLA (group). Any significant main effects of time identified in the ANOVA
289 were further analysed by *post hoc* 2 tailed paired t-tests with Holm-Bonferoni
290 correction. Independent t-tests were used to determine any significant differences
291 between groups in HR, oxygen uptake ($\dot{V}O_2$) or RPE during the main experimental
292 trial. Statistical significance was accepted at $p < 0.05$.

293 **Results**

294 Physiological variables and RPE

295 There was no significant difference in $\dot{V}O_2$ expressed in either absolute terms ($p =$
296 0.479) or relative to $\dot{V}O_2$ max (; $p = 0.445$) between the COL (2406 ± 187 $mL \cdot min^{-1}$;
297 57 ± 4% $\dot{V}O_2$ max) and PLA trial (2497 ± 351 $mL \cdot min^{-1}$; 58 ± 4% $\dot{V}O_2$ max). There
298 was no difference ($p = 0.989$) in HR between COL (73 ± 2%) and PLA (74 ± 3%)
299 trials when expressed as a proportion of peak HR obtained during the incremental
300 test $\dot{V}O_2$ max determination. There was also no significant difference in RPE ($p =$
301 0.158) between the COL (12.4 ± 1.9) and PLA (13.5 ± 1.2) trial.

302 Similar patterns of plasma volume changes were observed from pre-exercise
303 between trials: COL; post-exercise (-6.4 ± 4.8%); 1 h post-exercise (-3.1 ± 6.0) and
304 PLA; post-exercise (-4.5 ± 2.7%), 1 h post-exercise (-1.7 ± 3.3%). As there was no
305 significant difference between trials (group ^{x} time interaction; $p = 0.697$), it was
306 deemed unnecessary to correct any haematological parameters for plasma volume
307 changes. Although there was a significant change across time ($p = 0.004$), there was
308 no significant group ($p = 0.937$) or interaction effect ($p = 0.771$) on plasma glucose.

Post hoc analysis revealed a significantly lower plasma glucose at 1 h post-exercise (COL: 4.29 ± 0.46 mmol·L^{-1}, PLA: 4.28 ± 0.33 mmol·L^{-1}) compared to both pre-exercise (COL: 4.87 ± 0.34 mmol·L^{-1}, PLA: 4.79 ± 0.31 mmol·L^{-1}, $p < 0.001$) and post-exercise (COL: 4.54 ± 0.86 mmol·L^{-1}, PLA: 4.59 ± 0.52 mmol·L^{-1}, $p = 0.031$). There was also a significant change across time for plasma lactate ($p = 0.002$) but no effect of group ($p = 0.796$) or interaction ($p = 0.751$). Plasma lactate significantly increased from pre-exercise (COL: 1.36 ± 0.28 mmol·L^{-1}, PLA: 1.49 ± 0.46 mmol·L^{-1}) to post-exercise (COL: 1.93 ± 0.67 mmol·L^{-1}, PLA: 2.01 ± 0.73 mmol·L^{-1}, p 0.014) before decreasing towards resting levels at 1 h post-exercise (COL: 1.54 ± 0.56 mmol·L^{-1}, PLA: 1.46 ± 0.28 mmol·L^{-1}, $p = 1.000$).

Circulating total and differential cell counts

There was no time × group interaction effect or main effect of group on circulating total leukocytes, neutrophils, lymphocytes, monocytes, and neutrophil:lymphocyte ratio (Table 1). A main effect of time ($p < 0.001$) was observed in all leukocyte counts (Table 1). There was a significant increase in total leukocytes, neutrophils, monocytes, neutrophil:lymphocyte ratio from timepoints pre-exercise (Baseline and pre-exercise) to post-exercise ($p \leq 0.01$) and 1 h post exercise (except lymphocytes) ($p < 0.01$). Total lymphocyte count significantly increased from baseline to post-exercise ($p = 0.022$). There was a significant increase in neutrophils, neutrophil:lymphocyte ratio ($p < 0.001$) and decrease in total lymphocytes ($p < 0.001$) and monocytes ($p = 0.036$) from post-exercise to 1 h post-exercise.

331 Neutrophil responses

332 Two-way mixed ANOVA revealed a significant main effect of time ($p < 0.001$) for
333 fMLP-stimulated CL per neutrophil (neutrophil oxidative burst) (Fig. 1A). There was a
334 significant decrease in fMLP-stimulated CL per neutrophil from pre-exercise to post-
335 exercise ($p = 0.001$) and 1 h post-exercise ($p < 0.001$). There was a main effect of
336 group for fMLP-stimulated CL per neutrophil ($p = 0.049$) and an interaction effect
337 (group ^{x} time) which approached significance ($p = 0.060$, Fig. 1A).

338 A significant main effect of time ($p < 0.001$) was observed for PMA-stimulated CL per
339 neutrophil (neutrophil oxidative burst) (Fig. 1B). There was a significantly higher
340 PMA-stimulated CL per neutrophil at pre-exercise compared with post-exercise ($p <$
341 0.001) and 1 h post-exercise ($p = 0.001$). There was a significant increase in PMA-
342 stimulated CL per neutrophil from post to 1 h post-exercise ($p = 0.014$). There was
343 no main effect of group ($p = 0.395$) or group ^{x} time interaction ($p = 0.464$) for PMA-
344 stimulated CL per neutrophil (Fig. 1B).

345 A significant main effect of time ($p < 0.001$) was observed for $CL \cdot L^{-1}$ blood (Table 2).
346 There was a significant increase in spontaneous $CL \cdot L^{-1}$ blood compared to pre-
347 exercise at post-exercise ($p < 0.001$) and 1 h post-exercise ($p = 0.001$).
348 Spontaneous $CL \cdot L^{-1}$ blood significantly decreased from post-exercise to 1 h post-
349 exercise ($p = 0.014$). There was no main effect of group ($p = 0.071$) or group ^{x} time
350 interaction ($p = 0.539$) for spontaneous $CL \cdot L^{-1}$ blood (Table 2).

351 There was no significant main effect of time ($p = 0.629$), group ($p = 0.538$) or group ^{x}
352 time interaction ($p = 0.687$) for stimulated elastase release per neutrophil (neutrophil
353 degranulation) (Table 2).

354 Mucosal responses

355 There were no significant main effects of group or group × time interaction for
356 salivary SIgA or antimicrobial peptides when expressed as absolute concentration,
357 secretion rate or relative to saliva osmolality (Supporting Information Table S1-S2).

358 There was no significant main effect of time for salivary SIgA concentration (p
359 $=0.903$) or salivary SIgA secretion rate ($p = 0.430$) (Supporting Information Table
360 S1). There was, however, a main effect of time saliva SIgA:osmolality ($p < 0.001$)
361 with *post hoc* analysis showing a significantly decreased saliva SIgA:osmolality from
362 baseline and pre-exercise to post-exercise ($p < 0.001$) and an increase towards
363 resting levels from post-exercise to 1 h post-exercise ($p = 0.022$).

364 Blood contamination was detected in the saliva samples from six participants, three
365 in each group, leaving $n = 7$ in each group for analysis of sLac and sLys responses.

366 There was a significant main time effect for sLac concentration ($p < 0.001$,
367 Supporting Information Table S2) with post hoc analysis showing that sLac
368 concentration was increased at post-exercise compared to baseline ($p < 0.001$) and
369 pre-exercise ($p = 0.002$) followed by a decrease from post-exercise to 1 h post-
370 exercise ($p = 0.005$). A main effect of time was also revealed for sLac secretion rate
371 ($p < 0.001$) but not sLac:osmolality ($p = 0.405$), (Supporting Information Table S2).
372 *Post hoc* analysis identified greater sLac secretion rate at post-exercise ($p < 0.001$)
373 and 1 h post-exercise ($p = 0.030$) compared to baseline as well as an increase from
374 pre-exercise to post-exercise ($p =0.026$).

375 A significant main effect of time was reported for sLys concentration ($p = 0.003$)
376 (Supporting Information Table S2), *post hoc* analysis revealed increases in sLys

concentration from baseline to post-exercise ($p = 0.012$). There was a main effect of time ($p = 0.009$) for sLys secretion rate but no effect on sLys:osmolality ($p = 0.127$) (Supporting Information Table S2). *Post hoc* analysis revealed an increase in sLys secretion rate from baseline to 1 h post-exercise ($p = 0.023$).

Discussion

The present study demonstrated that 4 weeks of COL supplementation results in greater attenuation of the decline in fMLP-oxidative burst responses compared to PLA in a model of exercise-induced immune dysfunction. This is the first study to investigate the effects of COL on the responses of this innate immune parameter to prolonged exercise. We did not, however, observe beneficial effects of 4 weeks COL supplementation on other innate and mucosal parameters in this study.

Similar to other investigations of prolonged exercise, there were significant increases in circulating leukocytes, neutrophils, lymphocytes, neutrophil:lymphocyte, and monocytes (Davison et al., 2012; Li and Gleeson, 2005; McCarthy and Dale, 1988). In line with previous evidence (Carol et al., 2011; Davison and Diment, 2010), there were no differences between the COL and PLA groups for leukocyte trafficking to exercise. Taken together, these studies suggest that any effects of COL on exercise-induced changes in immune function are not related to the attenuation of the perturbations in leukocytes that occur as a result of increases in catecholamine or hypothalamic–pituitary–adrenal axis activation (e.g. leukocytosis) (McCarthy and Dale, 1988).

The findings of *in vitro* culture demonstrate that COL possesses the ability to prime neutrophil function (Benson et al., 2012; Sugisawa et al., 2001, 2003). Neutrophils can be primed by a range of cytokines (granulocyte macrophage colony-stimulating factor, granulocyte colony-stimulating factor, interleukin-8, tumour necrosis factor α) which amplify responses to subsequent stimulation (Elbim et al., 1994). Sugisawa et al. (2003) proposed that ‘priming’ induced by COL is due to low molecular weight substances (e.g. proteose peptones) other than cytokines. Although further

405 investigations into the role of cytokines or proposed bioactive peptides are required,
406 the findings here suggest that consumption of COL does increase the bioavailability
407 of components which enhance receptor mediated neutrophil oxidative activity (e.g.
408 fMLP). Jensen et al. (2012) observed an increase in phagocytic activity from
409 baseline in human polymorphonuclear cells obtained at 1 h and 2 h following
410 consumption of a low-molecular weight fraction of COL. It is currently unclear how
411 long any priming effect of COL may last but it has previously been reported that
412 enhanced oxidative burst responses to fMLP can last ≥ 24 h following exposure to
413 priming agents (e.g. G-CSF) (Ichinose et al., 1990). It can, however, be suggested
414 from the findings presented here that the underlying mechanism(s) of COL
415 supplementation are, at least, not due to a modulation of antioxidant capacity due to
416 a lack of effect on spontaneous oxidative burst activity.

417 This study showed no attenuation of exercise-induced decreases in PMA-stimulated
418 oxidative burst with COL supplementation. Therefore, COL may not exert effects on
419 neutrophil responses via protein kinase C (PKC) and/or does not affect the maximal
420 capacity of neutrophils following prolonged exercise. Although the activation of PKC
421 has been shown to occur following stimulation by some physiological agonists
422 (Peake, 2002), PMA is not encountered *in vivo* and hence considered as an artificial
423 stimulus (Sheppard et al., 2005). Due to the irreversible nature of PMA stimulation, it
424 differs substantially to physiological agonists (e.g. fMLP) (DeCoursey and Ligeti,
425 2005). The effectiveness of PMA to stimulate pathways for ROS production *in vitro*
426 has perhaps masked the irrelevancy of the agonist towards *in vivo* neutrophil
427 function (Sheppard et al., 2005). In contrast, production of formylated proteins (e.g.
428 fMLP) is attributed to bacteria and mitochondria, where a receptor (FPR1) on the cell
429 surface of neutrophils recognises microbial moieties and tissue damage (Jaillon et

al., 2013). Thus, the potential *in vivo* significance of enhanced fMLP-induced oxidative burst by COL supplementation may be enhanced responses toward infectious and/or inflammatory challenge (Zhang et al., 2010).

Despite the exercise-induced fall in neutrophil oxidative burst, there was no effect of prolonged exercise on neutrophil stimulated-elastase release. This is in contrast to some (Davison et al., 2012; Laing et al., 2008) but not other previous investigations (Li and Gleeson, 2005). The comparison of this study to others is, however, limited by the lack of measurement in potential mediators (e.g. stress hormones, cytokines) but it is reasonable to suggest that the exercise stress, in particular the exercise intensity, was not sufficient to induce decrements in neutrophil degranulation observed in other studies (Davison et al., 2012; Laing et al., 2008). Furthermore, the findings of this study may support the hypothesis that oxidative burst capacity may be more susceptible to the duration rather than exercise intensity *per se* (Peake, 2002).

The absence of a fall in neutrophil degranulation (bacterial-stimulated elastase) in this study (hence a reduced scope for nutritional intervention) may also explain why there was a discrepancy to effects observed with 4 weeks of supplementation in Davison and Diment (2010). The azurophilic granules of neutrophils are also abundant in AMPs e.g. lysozyme. In contrast, to Davison and Diment (2010), there was also no enhancement in sLys within the present study. It is worthy to note that the large variability in sLys responses to exercise in this study is in accordance with others (West et al., 2010) who have recognised the variance as a source of limitation for the detection of intervention induced-changes in mucosal parameters, particularly in a parallel groups design. As shown previously (Allgrove & Gleeson, 2014), there

454 was an increased availability of sLac following prolonged exercise in both COL and
455 PLA groups. The reason for this increase remains unclear but may be indicative of
456 an inflammatory response induced by damaged epithelial cells (Davison et al.,
457 2009). Given that longitudinal studies of sLac have observed lower levels in athletes
458 compared to resting controls (West et al., 2010), further investigations are required
459 to determine whether responses to prolonged exercise on a regular basis leads to a
460 depletion of sLac availability. This study and previous evidence from our laboratory
461 (Jones et al., 2014), suggest that COL does not influence sLac availability either in a
462 resting state or in response to acute stress.

463 Reduced mobilisation of salivary SIgA has been advocated as a marker of
464 dysfunctional mucosal immunity following prolonged exercise (Walsh et al., 2011).
465 Despite no change in concentration or secretion, there was an exercise-induced
466 decrease in saliva SIgA:osmolality that was unaltered by COL supplementation. The
467 majority of previous studies have investigated the effects of COL on resting salivary
468 SIgA concentrations whereby some report increases (Crooks et al., 2006; Mero et
469 al., 2002) whereas other investigators have found little evidence of changes with
470 supplementation (Crooks et al., 2010; Davison and Diment, 2010; Jones et al.,
471 2014;). Based on Mero et al. (2002), Davison and Diment (2010) proposed that the
472 dosage of COL may need to be split across the day or taken over longer periods to
473 stimulate changes in resting salivary SIgA production. Although not separated into
474 four doses (5 g each) as done by Mero et al. (2002), the present study did split the
475 COL dosage into a morning and evening dose (10 g each). The variance in results,
476 to date, may be explained by the composition of the COL and the assay used in the
477 determination of IgA. As large variability in salivary SIgA may also be a confounding
478 factor here, further studies with exercise protocols that combine both high-intensity

and long duration exercise, thus associated with greater impact (i.e. depressive) on salivary SIgA output are required to clarify the effect of COL on exercise-induced changes in this mucosal parameter.

In conclusion, the primary finding of the study was that COL supplementation has beneficial effects on receptor dependent (fMLP-stimulated) oxidative burst responses to prolonged exercise. There was, however, no effect of COL on all other parameters of innate and mucosal immunity, although it is possible that larger decreases/immunodepression is required for a benefit in these measures to become apparent. Further research is required to determine this and also elucidate the mechanisms underlying the effects of COL supplementation in order to define the optimal timing and/or dosage of COL.

Perspectives

This study is the first to demonstrate that COL supplementation better preserves stimulated oxidative burst responses of human blood neutrophils following prolonged exercise. Neutrophils form a critical part of the human body's first line of defence as the most abundant circulating leukocyte. Once described as a short-lived effector cell, the role of the neutrophil is now known to extend beyond the elimination of microorganisms and is now considered a major cell type involved in orchestrating an immune response (Scapini & Cassatella, 2014). In addition to recurrent bacterial infections observed in those with genetic neutrophil disorders (Summers et al., 2010), the innate cell is receiving increased recognition as a key mediator of host defence against viral infections (e.g. influenza) in otherwise healthy populations (Drescher & Bai, 2013). The overall significance of the augmentation in neutrophil function with COL supplementation in terms of athletes' resistance to pathogens is

still to be determined. We have previously shown that COL supplementation limited increases in salivary bacterial load in physically active males (at rest) during the winter months which coincided with decreases in incidence of URI (Jones et al., 2014). Indeed, the beneficial effects of COL were most evident during periods of greater prevalence of URI (hence a greater scope for intervention). It was suggested that participants were exposed to fewer or shorter transient immune perturbations as a result of COL supplementation which may limit conditions for changes in resident microorganisms and/or susceptibility to invading pathogens. Given the complexity of the immune system, it is unlikely that one immune parameter alone would explain changes in URI risk with COL supplementation, thus the importance of other immune markers (at rest or following strenuous exertion) need to be determined. Further investigation of neutrophils to clarify the underlying mechanisms of COL supplementation on human host defence is warranted. This study goes some way to achieving this by providing important new evidence regarding the potential for COL in priming receptor-mediated neutrophil responses to bacterial stimulation.

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629 inflammatory responses to injury. Nature 2010: 464: 104-107.

630 Table 1. Blood immune cell counts in COL and PLA groups

Cell count, $10^9 \cdot L^{-1}$	Baseline	Pre-exercise	Post-exercise	1 h post-exercise	p values group time interaction
Total leukocytes					0.528
COL	4.91 ± 1.10	5.14 ± 1.07	11.46 ± 5.73	11.13 ± 4.47	< 0.001*
PLA	5.33 ± 1.60	5.62 ± 1.37	11.95 ± 4.39	11.51 ± 3.62	0.965
Neutrophils					0.611
COL	2.57 ± 0.81	2.50 ± 1.28	7.84 ± 4.14	8.32 ± 3.81	< 0.001*
PLA	2.48 ± 0.79	2.78 ± 0.83	8.45 ± 3.74	8.78 ± 3.36	0.679
Monocytes					0.899
COL	0.43 ± 0.15	0.52 ± 0.12	0.95 ± 0.58	0.84 ± 0.43	< 0.001*
PLA	0.50 ± 0.23	0.54 ± 0.12	0.79 ± 0.22	0.72 ± 0.21	0.336
Total lymphocytes					0.622
COL	1.65 ± 0.46	1.90 ± 0.71	2.41 ± 1.12	1.77 ± 0.46	0.001*
PLA	2.02 ± 0.68	2.02 ± 0.58	2.32 ± 0.57	1.69 ± 0.33	0.278
Neutrophil:lymphocyte					0.883
COL	1.61 ± 0.61	1.45 ± 0.72	3.36 ± 1.21	4.74 ± 1.90	< 0.001*
PLA	1.32 ± 0.48	1.45 ± 0.50	3.70 ± 1.46	5.35 ± 2.33	0.176

631 Significant main effect of time *p < 0.001).

632

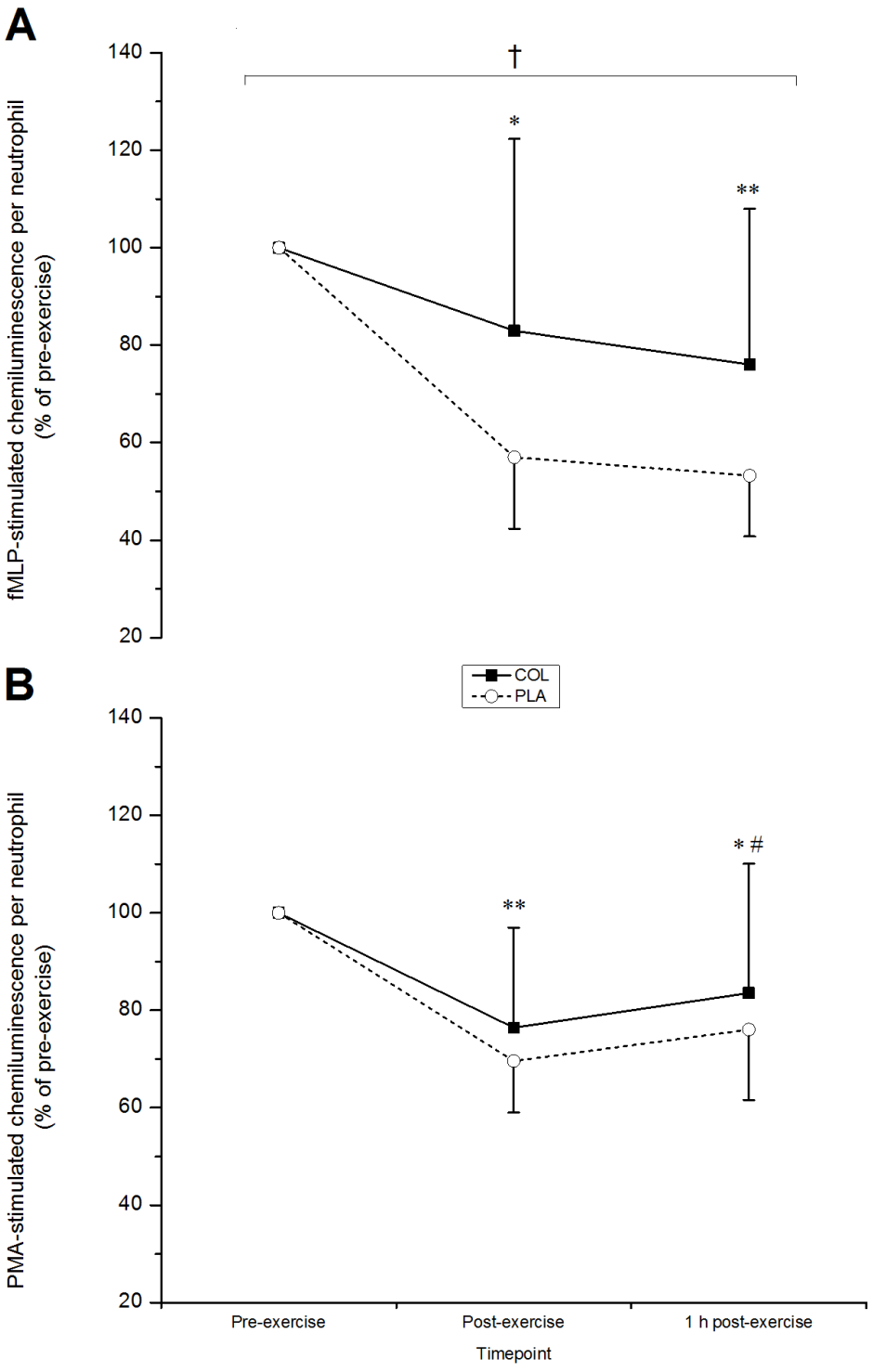
633 Table 2. Spontaneous ROS production and stimulated degranulation responses and
634 in COL or PLA groups.

Measure	Pre-exercise	Post-exercise	1 h post-exercise	p values group time interaction
Spontaneous CL·L^{-1} blood				0.071
COL	813 ± 193	1110 ± 323	1042 ± 326	< 0.001*
PLA	1114 ± 335	1502 ± 537	1309 ± 467	0.539
Stimulated elastase release per neutrophil (fg·cell^{-1})				0.538
COL	369 ± 85	428 ± 310	374 ± 159	0.629
PLA	444 ± 144	396 ± 166	447 ± 233	0.687

635 Significant main effect of time *p < 0.001).

636 Fig. 1 fMLP (A) and PMA (B) stimulated chemiluminescence per neutrophil following
637 4 weeks of COL or PLA. Significant change from pre-exercise: *p = 0.001, **p <
638 0.001. #Significant change from post-exercise (p < 0.05). †Significant main effect of
639 group for fMLP-stimulated chemiluminescence per neutrophil (p < 0.05).

640


A
fMLP-stimulated chemiluminescence per neutrophil (% of pre-exercise)
140
120
100
80
60
40
20
†
*
**
B
COL
PLA
PMA-stimulated chemiluminescence per neutrophil (% of pre-exercise)
**
* #
Pre-exercise
Post-exercise
1 h post-exercise
Timepoint

641

642 Fig. 1