Effect of Oral Glutamine Supplementation on Human Neutrophil Lipopolysaccharide-Stimulated Degranulation Following Prolonged Exercise

Neil P. Walsh, Andrew K. Blannin, Nicolette C. Bishop, Paula J. Robson, and Michael Gleeson

Recent studies have shown that neutrophils can utilize glutamine and that glutamine supplementation can improve neutrophil function in postoperative and burn patients. The present study investigated the influence of oral glutamine supplementation on stimulated neutrophil degranulation and oxidative burst activity following prolonged exercise. Subjects, 7 well-trained men, reported to the laboratory following an overnight fast and cycled for 2 hrs at 60% VO2max on two occasions a week apart. They were randomly assigned to either a glutamine or placebo treatment. For both trials, subjects consumed a sugar-free lemon drink at 15-min intervals until 90 minutes, then a lemon flavored glutamine drink (GLN) or sugar-free lemon drink (PLA) was consumed at 15-min intervals for the remaining exercise and the 2-hr recovery period. Venous blood samples were taken pre-, during, and postexercise. Glutamine supplementation had no effect on the magnitude of postexercise leukocytosis, the plasma elastase concentration following exercise (which increased in both trials), or the plasma elastase release in response to bacterial stimulation (which fell in both trials). Neutrophil function assessed by oxidative burst activity of isolated cells did not change following exercise in either trial. These findings therefore suggest that the fall in plasma glutamine concentration does not account for the decrease in neutrophil function (degranulation response) following prolonged exercise.

Key Words: immune function, postexercise recovery, cortisol, oxidative burst, degranulation

Several studies indicate that various aspects of immune function are temporarily suppressed following prolonged exercise (17, 23, 30). Prolonged exercise reportedly decreases the plasma concentration of glutamine (22), an important fuel and precursor for DNA and RNA synthesis in cells of the immune system, including

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lymphocytes and macrophages. Thus, such a decrease could impair immune function (1). The overtraining syndrome is associated with a reduction in plasma glutamine levels (15), and this may be at least partly responsible for the immunosuppression apparent in this condition. Although there is no direct evidence implicating low plasma glutamine (either after exercise or in the overtraining syndrome) with impaired immune function, epidemiological data showing low plasma glutamine and an increased occurrence of upper respiratory tract infection (URTI) has raised interest in glutamine supplementation (34).

Castell et al. (4) have provided the only prophylactic evidence that a glutamine solution (5 g in 330 ml water) consumed immediately after and 2 hrs after a marathon reduces the occurrence of symptoms of URTI in the 7 days following the race. A glutamine solution (0.1 g·kg⁻¹ body mass) given at 0, 30, 60, and 90 min following a marathon race has been shown to prevent the fall in plasma glutamine concentration but not the fall in mitogen-induced lymphocyte proliferation and lymphocyte activated killer cell activity (29). These findings suggest that a decreased plasma glutamine concentration is not responsible for the depression in lymphocyte function following prolonged exercise.

Neutrophils constitute 50–60% of the blood leukocyte pool and play an important role in nonspecific host defense. Various aspects of neutrophil function can be assessed including chemotaxis, phagocytosis, degranulation of cytoplasmic granules, and oxidative burst activity (32). Although some disagreement exists in the literature, there is a body of evidence showing that most changes in neutrophil function following acute exercise are intensity dependent (25). High intensity exercise suppresses most neutrophil functions with the exception of the degranulation of cytoplasmic enzymes, which increases after exercise independent of exercise intensity and indicates that exercise activates neutrophils (32).

Moderate intensity exercise has been shown to both increase and decrease the oxidative burst during recovery (26, 33). A recent study in our laboratory comparing cycle exercise to exhaustion at 55 and 80% VO₂max (mean time to exhaustion: 164 and 37 min, respectively) found that oxidative burst activity and the in vitro degranulation response to bacterial lipopolysaccharide fell after exercise at both intensities (28). However, falls in neutrophil function were greater after exercise to fatigue at 55% VO₂max, which we suggested could be attributable to the larger rise in plasma cortisol concentration after the prolonged exercise having a direct inhibitory effect on neutrophil function. The fall in neutrophil function after exercise at 55% VO₂max coincided with a fall in plasma glutamine concentration compared with the trial at 80% VO₂max, which did not alter the plasma concentration of glutamine.

A recent study in rats has shown that neutrophils contain the enzyme glutaminase (5), suggesting that they are capable of utilizing glutamine. Isolated neutrophils from both burn and postoperative patients with low plasma glutamine showed enhanced bactericidal function following the addition of glutamine (8, 20). Hence, there is some indirect evidence in support of the notion that plasma glutamine levels may influence neutrophil function.

To determine whether neutrophil function following exercise is modified by oral glutamine supplementation, we compared the neutrophil degranulation response and oxidative burst activity during and following a prolonged bout of exercise undertaken both with and without oral glutamine supplementation.
Methods

Subjects were 7 recreationally active men, ages 28±6 yrs (mean±SEM), height 178±5 cm, weight 77±9 kg, and VO\(_2\)\(_\text{max}\) 55±8 ml·kg\(^{-1}\)·min\(^{-1}\). Approval for the study was obtained from the University of Birmingham Ethics Committee. The purpose of the study and the procedures involved were explained to subjects before their written consent was obtained. For the assessment of maximal oxygen uptake (VO\(_2\)\(_\text{max}\)), subjects undertook a continuous incremental test to volitional exhaustion on an electrically braked cycle ergometer (Lode Excalibur, Holland). Respiratory gas exchange was monitored throughout the test using Douglas bag collection of expired gas which was analyzed using oxygen and carbon dioxide analyzers (Servomex, Crowborough, UK) and a dry gas meter (Harvard Apparatus Edenbridge, UK). The British Association of Sport and Exercise Sciences criteria for attainment of VO\(_2\)\(_\text{max}\) were adopted (13).

For the main exercise trial, subjects arrived at the laboratory following an overnight fast and cycled for 2 hrs at a work rate equivalent to 60% VO\(_2\)\(_\text{max}\) (194±16W) on two occasions a week apart. They were randomly assigned to either a glutamine (GLN) or placebo (PLA) treatment. For the GLN trial, subjects consumed 250 ml of a lemon-flavored placebo drink at 15-min intervals during exercise until Minute 90 when 250 ml of a lemon-flavored 1.2% (w/v) glutamine drink was consumed at 15-min intervals for the remaining exercise and during 2 hrs of resting recovery. For the PLA trial, subjects consumed only the placebo drink (same volumes and times). Room temperature was 22±1 °C and relative humidity was 68±5%. Heart rate was measured continuously during both the VO\(_2\)\(_\text{max}\) test and exercise trial using a short range telemetry device (Sport Tester PE-3000 Polar Electro, Finland). Ratings of perceived exertion [RPE on a scale of 1 to 10] (3) were recorded at 10-min intervals. Blood samples were obtained from seated subjects by venepuncture from an antecubital vein before exercise, at 90 minutes of exercise, and at 0, 40, 80, and 120 min postexercise. Subjects were required to refrain from any strenuous physical activity for 24 hrs preceding and following the exercise task and were also required to abstain from drinking alcohol and taking any medication during this period.

Analytical Methods

Blood samples were collected into three vacutainer tubes (Beckton Dickinson, Oxford, UK), two containing lithium heparin and the other containing K\(_2\)EDTA (for haematological analysis). The sample (4 ml) in the K\(_2\)EDTA tube was stored at 4 °C and analyzed within 16 hrs. Haematological parameters, including haemoglobin concentration, packed cell volume, red blood cell count, platelet count, and differential leukocyte count were measured using a laser system (Technicon H-2 system). Changes in plasma volume were calculated according to Dill and Costill (6). One of the samples (7 ml) in the tubes containing lithium heparin was centrifuged (1500g for 10 min at 5 °C) within 15 min of sampling.

Plasma was removed and immediately stored at –70 °C. Plasma glucose was analyzed enzymatically using hexokinase (EC 2.7.1.1; Sigma kit HK-50). Plasma glutamine was analyzed by enzymatic spectrophotometric determination of the plasma ammonia concentration before and after treatment of the plasma with glutaminase (34). Plasma cortisol concentration was measured using an ELISA kit
(Boehringer Mannheim, Germany). An ELISA kit (Merck, Lutterworth, UK) was used for determining the elastase concentration in plasma before and after treatment with bacterial lipopolysaccharide (LPS) stimulant (Sigma, Poole, UK). The elastase released from LPS-stimulated neutrophils during a 1-hr incubation at 37 °C was determined according to Blannin et al. (2). The other sample (7 ml) in the tube containing lithium heparin was used for separating neutrophil granulocytes by a technique adapted from Hack et al. (12). Platelet-rich plasma was removed from the blood by centrifugation at 300g for 10 min and the pellet was resuspended in an equal volume of phosphate buffered saline (PBS; Sigma).

Neutrophils were separated from lymphocytes by Histopaque density-gradient centrifugation (Sigma, Poole, UK) for 30 min at 450g and 20 °C. Erythrocytes were destroyed by hypotonic lysis in the presence of 20 ml of 0.2% NaCl for 20 sec, followed by the addition 20 ml of 1.6% NaCl to restore isotonicity. After centrifugation for 10 min at 300g, the supernatant containing lysed erythrocytes was removed. This step was repeated until the pellet was free of erythrocytes. When the supernatant was translucent, the neutrophils were washed twice and resuspended in 3 ml PBS. A differential cell count was then performed and the volume of PBS was adjusted to give a count of $1 \times 10^6 \cdot ml^{-1}$. The cell suspensions were found to comprise more than 95% neutrophils.

Oxidative burst activity was determined by a technique adapted from Gaudry et al. (10) using phorbol-12-myristate-13-acetate (PMA; Sigma, UK, P-8139). A 1-ml volume of cell suspension ($1 \times 10^6 \cdot ml^{-1}$) was incubated with ferricytochrome C (Sigma, UK, C-2506) at a final concentration of 80 μmol · L$^{-1}$ for 10 min at 37 °C before addition of PMA to give a final concentration of 1μg · ml$^{-1}$. Oxidative burst activity was calculated from the absorbance change per minute at 550 nm against a cell-free blank (PBS) multiplied by the molar extinction coefficient of 21 · 10$^3$ M$^{-1}$ · cm$^{-1}$. For a control, 10μl superoxide dismutase (SOD; Sigma, UK, S-7008) was added prior to the addition of PMA to remove superoxide production.

**Statistical Analysis**

After first establishing that the data were normally distributed, the data were examined using a two-way ANOVA with a repeated-measures design, with post hoc Tukey tests. Student paired t-tests were also used where appropriate. Values in the text, figures, and tables are shown as means and standard errors of the mean (SEM). The accepted level of significance was $p < 0.05$.

**Results**

Maximum heart rate was 190 ± 3 bpm at the end of the VO$_2$max protocol. All subjects completed the exercise trials. Mean heart rate was 146 bpm after 20 min cycling in both trials, and 155 bpm by the end of both trials. RPE increased during exercise on both trials but tended to be higher on the GLN trial. After 60 minutes of exercise, RPE was 5.2 ± 0.4 and 5.5 ± 0.5, and during the last minute of exercise it was 6.5 ± 1.2 and 8.4 ± 0.6 on the PLA and GLN trials, respectively. Plasma glucose concentration was significantly lower at 90 min exercise, immediately postexercise, and at 40 and 120 min postexercise compared to preexercise values (main effect of time: $p < 0.01$) (Table 1) but was not significantly affected by glutamine
Table 1  Plasma Glucose and Cortisol During PLA and GLN Trials (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mM)</th>
<th></th>
<th>Cortisol (nM)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLA</td>
<td>GLN</td>
<td>PLA</td>
<td>GLN</td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>4.9 (0.2)</td>
<td>5.0 (0.3)</td>
<td>413 (50)</td>
<td>459 (63)</td>
</tr>
<tr>
<td>90 min</td>
<td>4.6 (0.2)</td>
<td>4.3 (0.3)*</td>
<td>467 (61)</td>
<td>580 (36)</td>
</tr>
<tr>
<td>Postexercise</td>
<td>4.5 (0.3)</td>
<td>4.2 (0.3)*</td>
<td>609 (91)</td>
<td>696 (61)</td>
</tr>
<tr>
<td>40 min post</td>
<td>4.2 (0.3)*</td>
<td>4.3 (0.2)*</td>
<td>492 (64)</td>
<td>607 (63)</td>
</tr>
<tr>
<td>80 min post</td>
<td>4.3 (0.2)</td>
<td>4.6 (0.2)</td>
<td>403 (56)</td>
<td>407 (51)</td>
</tr>
<tr>
<td>120 min post</td>
<td>4.3 (0.1)*</td>
<td>4.6 (0.2)</td>
<td>324 (28)</td>
<td>340 (53)</td>
</tr>
</tbody>
</table>

Signif. diff. from pre-exercise value, *p < 0.5. Plasma cortisol concentrations immediately postexercise were significantly higher than preexercise values (main effect of time, p < 0.01).

supplementation. Plasma cortisol concentration increased significantly immediately postexercise (main effect of time: p < 0.01) but returned to preexercise levels by 80 min postexercise in both rides. Again there was no significant effect of treatment.

The circulating leukocyte count increased significantly in both trials during exercise and peaked immediately postexercise (main effect of time: p < 0.01) (Table 2). The increase in leukocyte count during exercise was due to an increase in the neutrophil and lymphocyte counts (main effect of time: p < 0.01). Plasma elastase concentration was significantly increased following exercise, indicating some degree of neutrophil activation during exercise (main effect of time: p < 0.05) (Table 3). Neutrophil function, as assessed by plasma elastase release in response to bacterial LPS-stimulation, was significantly decreased in both trials immediately postexercise (main effect of time: p < 0.01). However, neutrophil function assessed by oxidative burst activity did not change significantly following exercise in either trial.

Resting preexercise plasma glutamine concentration was significantly lower in the placebo trial (638 ± 22 vs. 770 ± 31 μM for PLA and GLN, p < 0.05); however, at 90 min when the first trial drink was consumed, similar values were measured (667 ± 20 vs. 696 ± 57 μM for PLA and GLN, respectively) (Figure 1). During the last 30 min of exercise and during 2 hrs of resting recovery when trial drinks were consumed, a significantly lower plasma glutamine concentration was measured in the PLA trial. Plasma glutamine concentration fell to a nadir of 522 ± 29 μM at 80 min postexercise in the PLA trial but was maintained at ~770 μM throughout the GLN trial.

Plasma volume fell by 6.7 ± 1.5 and 6.0 ± 1.7% at 90 min during exercise in the PLA and GLN trials, respectively (main effect of time: p < 0.01) (Table 2) but had returned to preexercise values by 40 min postexercise. Plasma volume did not differ significantly from preexercise at any other time point (Table 2).
Table 2  Circulating Leukocyte Numbers (X10⁹ cells · L⁻¹) and Percentage Change in Plasma Volume (Δ%PV) Compared With Pre-exercise (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Leukocytes</th>
<th></th>
<th>Neutrophils</th>
<th></th>
<th>Lymphocytes</th>
<th></th>
<th>Δ%PV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLA</td>
<td>GLN</td>
<td>PLA</td>
<td>GLN</td>
<td>PLA</td>
<td>GLN</td>
<td>PLA</td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>5.6 (0.4)</td>
<td>5.3 (0.3)</td>
<td>3.0 (0.3)</td>
<td>2.7 (0.2)</td>
<td>1.9 (0.1)</td>
<td>1.9 (0.1)</td>
<td>-</td>
</tr>
<tr>
<td>90 min</td>
<td>9.1 (0.7)</td>
<td>9.3 (0.9)</td>
<td>5.4 (0.7)</td>
<td>5.6 (0.7)</td>
<td>2.9 (0.2)</td>
<td>2.8 (0.2)</td>
<td>-6.7 (1.5)</td>
</tr>
<tr>
<td>Postexercise</td>
<td>14.8 (1.1)</td>
<td>16.0 (1.4)</td>
<td>10.6 (1.0)</td>
<td>11.3 (1.2)</td>
<td>3.4 (0.4)</td>
<td>3.2 (0.3)</td>
<td>-4.5 (1.4)</td>
</tr>
<tr>
<td>40 min post</td>
<td>13.6 (0.9)</td>
<td>14.4 (1.1)</td>
<td>10.9 (1.0)</td>
<td>11.6 (0.9)</td>
<td>1.7 (0.1)</td>
<td>1.6 (0.1)</td>
<td>+1.9 (1.0)</td>
</tr>
<tr>
<td>80 min post</td>
<td>12.8 (0.5)</td>
<td>14.2 (0.8)</td>
<td>10.4 (0.6)</td>
<td>11.5 (0.7)</td>
<td>1.5 (0.2)</td>
<td>1.5 (0.1)</td>
<td>+1.3 (1.5)</td>
</tr>
<tr>
<td>120 min post</td>
<td>12.7 (0.6)</td>
<td>13.4 (0.7)</td>
<td>10.3 (0.7)</td>
<td>10.8 (0.7)</td>
<td>1.6 (0.2)</td>
<td>1.5 (0.1)</td>
<td>+2.3 (1.7)</td>
</tr>
</tbody>
</table>

Note: Total leukocyte and neutrophil counts were significantly (main effect of time: p < .01) higher than pre-exercise values at all time points after exercise; lymphocyte counts increased significantly at 90 min and immediately postexercise in both trials (main effect of time: p < .01); plasma volume fell significantly at 90 min in both trials.
Table 3  Plasma Elastase Concentration and Neutrophil Function Assessed by Elastase Release in LPS-Stimulated Whole Blood and Unstimulated Plasma and PMA-Stimulated Oxidative Burst Activity (mean ± SEM)

<table>
<thead>
<tr>
<th></th>
<th>Plasma elastase (μg·L⁻¹)</th>
<th>Elastase release (fg·cell⁻¹) stimulated</th>
<th>Oxidative burst activity (nmol·10⁻⁶ cells·min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PLA</td>
<td>GLN</td>
<td>PLA</td>
</tr>
<tr>
<td>Pre-exercise</td>
<td>78 (26)</td>
<td>72 (11)</td>
<td>170 (12)</td>
</tr>
<tr>
<td>Postexercise</td>
<td>136 (23)</td>
<td>157 (37)</td>
<td>113 (9)</td>
</tr>
<tr>
<td>120 min post</td>
<td>138 (37)</td>
<td>225 (84)</td>
<td>137 (7)</td>
</tr>
</tbody>
</table>

Note: Plasma elastase concentration was significantly (main effect of time: $p < .05$) higher at 120 min postexercise compared with pre-exercise values; neutrophil elastase release from LPS-stimulated whole blood was significantly (main effect of time: $p < .01$) lower immediately postexercise compared with resting values in both trials.
Figure 1 — Plasma glutamine concentration before, during, and after exercise in PLA (Δ) and GLN (■) trials (mean ± SEM). Significantly different from preexercise value: (a) $p < .01$. Significantly different from 90-min exercise value: (b) $p < .01$. Significant difference between PLA and GLN value: (*) $p < .05$, (**) $p < .01$.

Discussion

Changes in leukocyte cell numbers and function could explain the increased susceptibility to infection in athletes engaged in prolonged high intensity exercise (17). These changes may be mediated through the actions of stress hormones, particularly glucocorticoids and catecholamines (23). Also, changes in the plasma concentration of glutamine during exercise and recovery have been implicated as a mechanism through which leukocyte function may be altered (15, 21, 22, 34). Glutamine is utilized at high rates by rapidly dividing cells, including lymphocytes and macrophages, to provide energy and optimal conditions for nucleotide biosynthesis (1). As such, it is considered essential for proper immune function.

Numerous in vitro studies suggest that falls in plasma glutamine concentration may have a detrimental effect on the function of immune cells but that the addition of glutamine can restore immune cell functions such as lymphocyte proliferation and neutrophil bactericidal function (1, 8, 20, 21). The present study has shown a fall in plasma glutamine concentration during and after exercise in the placebo trial, which is in agreement with several studies showing that prolonged exercise is associated with a fall in the plasma concentration of glutamine (22, 27, 34). The fall in the plasma glutamine concentration following prolonged exercise more than likely results from an increased demand and uptake for glutamine by
tissues and cells that require it, such as the liver where glutamine acts as a major
 gluconeogenic precursor. Indeed, during prolonged exercise the plasma levels of
cortisol (Table 1) and glucagon rise (9), which both increase the uptake of glutamine
by the liver for gluconeogenesis and acute phase protein synthesis. Alternatively,
the fall in plasma glutamine following prolonged exercise could be due to an in-
creased uptake of glutamine by activated lymphocytes for energy provision and
bionucleotide synthesis (18).

Following more intense exercise resulting in metabolic acidosis, it is conceiv-
able that renal uptake of glutamine increases to provide for ammoniagenesis (11).
However, increased renal uptake of glutamine is unlikely to explain the fall in
plasma glutamine in this study, as the exercise was not intense enough to cause
significant metabolic acidosis. Decreased production and/or altered transport kinet-
ics of glutamine which results in diminished glutamine release from muscle could
also contribute to the fall in plasma glutamine following prolonged exercise (34).

Glutamine supplementation has been shown to have favorable outcomes for
immune cell function (14, 19), although the mechanism by which glutamine has its
prophylactic benefits remains to be clarified. In rats infected with Escherichia coli,
a glutamine supplemented diet for 7 days has been shown to raise plasma and
intramuscular glutamine levels, increase the total blood leukocyte counts, and
increase the proliferation of lymphocytes in response to concanavalin A (35). In
humans, only Castell et al. (4) found a prophylactic effect of glutamine supplemen-
tation, albeit through indirect evidence, that glutamine supplements following a
marathon can reduce the incidence of respiratory tract infections in the week follow-
ning the race. The present study demonstrates that glutamine supplementation during
exercise and recovery prevents the fall in plasma glutamine concentration.

In agreement with studies looking at other leukocyte functions (29, 31), we
also showed that maintaining plasma glutamine concentration at ~770 μM during
exercise and recovery does not affect the magnitude of the postexercise leukocyto-
sis or the fall in LPS-stimulated neutrophil degranulation postexercise. In somewhat
similar studies (29, 31), both natural killer cytolytic activity at rest and lymphokine
activated killer cell activity following exercise were not altered by glutamine supple-
mentation.

In accordance with other studies (7, 28), plasma elastase was significantly
increased immediately postexercise, indicating there was some degree of neutrophil
activation during exercise. However, neutrophil function assessed by the in vitro
degranulation response to bacterial stimulation fell after exercise. This fall in neu-
 trophil function may be a direct result of the elevated plasma cortisol concentration
(Table 1) having an inhibitory effect on neutrophil function. Alternatively, this may
be due to a higher proportion of less mature cells entering the circulation from the
bone marrow under the influence of cortisol. Another explanation could be that
following an initial activation during exercise, neutrophils enter a refractory state
and are less responsive to subsequent stimulation (24, 28). However, we found no
significant change in the neutrophil oxidative burst activity after exercise.

This finding contrasts with others (16, 28) who have found an immediate fall
in neutrophil oxidative burst activity following prolonged exercise of a similar
nature. Since we found no change in neutrophil oxidative burst activity performed in
PBS cell suspension, but a fall in the neutrophil degranulation response in whole
blood following exercise, this might indicate that the fall in degranulation response
is caused by an inhibitory bloodborne factor (e.g., catecholamines, cortisol,
cytokines). It is possible that by removing the cells from the normal blood environment following exercise and performing the oxidative burst assay in isolated cell suspensions in PBS, we removed one or more of the bloodborne factors that inhibit neutrophil function following exercise.

In conclusion, the present study indicates that glutamine supplementation during exercise and recovery can maintain the plasma glutamine concentration and thus prevent the postexercise fall in plasma glutamine concentration. However, the provision of glutamine did not alter the magnitude of the postexercise leukocytosis, or whole-blood neutrophil degranulation response to bacterial LPS. These findings therefore suggest that the fall in plasma glutamine concentration is not a causal factor in the decrease of LPS-stimulated neutrophil degranulation following prolonged exercise.

References


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