Minimal muscle damage after a marathon and no influence of beetroot juice on inflammation and recovery

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Abstract

This study examined whether beetroot juice (BTJ) would attenuate inflammation and muscle damage following a marathon. Using a double blind, independent group's design, 34 runners (~16 previous marathons completed) consumed either BTJ or an isocaloric placebo (PLA) for 3 days following a marathon. Maximal isometric voluntary contractions (MIVC), countermovement jumps (CMJ), muscle soreness, serum cytokines, leucocytosis, creatine kinase (CK), high sensitivity C-reactive protein (hs-CRP) and aspartate aminotransferase (AST) were measured pre, post, and on the 2 days after the marathon. CMJ and MIVC were reduced after the marathon (P<0.05) but no group differences were observed (P>0.05). Muscle soreness was increased in the day after the marathon (BTJ; 45±48 vs. PLA; 46±39 mm) and had returned to baseline by day 2, irrespective of supplementation (P=0.694). Cytokines (Interleukin-6; IL-6, interleukin-8, tumour necrosis factor-α) were increased immediately post-marathon but apart from IL-6 had returned to baseline values by day 1 post. No interaction effects were evident for IL-6 (P=0.213). Leucocytes increased 1.7 fold after the race and remained elevated 2 days post, irrespective of supplement (P<0.0001). CK peaked at 1 day post marathon (BTJ: 965±967 & PLA: 1141±979 $IU \cdot L^{-1}$) and like AST and hs-CRP, was still elevated 2 days after the marathon (P<0.05); however, no group differences were present for these variables. Beetroot juice did not attenuate inflammation or reduce muscle damage following a marathon, possibly because most of these indices were not markedly different from baseline values in the days after the marathon.

Key words: EXERCISE RECOVERY; BETALAINS; RUNNING; MARATHON; FUNCTIONAL FOODS; NEUROMUSCULAR FUNCTION.

Introduction

It has long been established that running a marathon race can cause marked and prolonged damage to skeletal muscle fibres and the surrounding connective structures (Hikida et al. 1983; Warhol et al. 1985). From a functional perspective, the ultrastructural damage often manifests as a loss in the muscle force generating capacity and feelings of muscle pain and tenderness (Howatson et al. 2010; Areces et al. 2014; Hill et al. 2014). These symptoms, particularly muscle soreness, become more apparent in the days after the marathon (Howatson et al. 2010; Hill et al. 2014). Such functional impairments can hinder an individual's ability to perform exercise, that might also increase the propensity for injury (Smith 1992).

The precise aetiology of exercise-induced muscle damage (EIMD) and therefore the causes of the impaired functional capacity in the days after strenuous exercise are not yet fully understood. However, physical damage to sarcomeres (Morgan and Proske 2004) and the opening of stretch activated ion channels, which leads to a build-up of intracellular Ca^{2+} (Sonobe et al. 2008), are thought to play prominent roles in EIMD and the loss of muscle function after exercise. Researchers in the field have also proposed that EIMD is likely to develop in a biphasic manner (Toumi and Best 2003; Howatson and van Someren 2008); insofar as, the initial mechanical and metabolic stress induced during an exercise task, also known as the primary phase, provokes deformation of contractile and non-contractile apparatus and induces biochemical changes that cause further muscle damage, typically referred to as the secondary phase (Howatson and van Someren 2008). The hallmarks of the secondary phase are an acute inflammatory response and disturbance of redox balance; effects that can be observed in both the muscle and circulation in the days after the inciting bout (Lapointe et al. 2002; Toumi and Best 2003; Raastad et al. 2010).

Of most importance, the aforementioned cellular changes might result in a further or prolonged loss in muscle function in the hours and days following the initial exercise. Consequently, it has been suggested that an intervention that manages, or at least dampens these responses might be of use for accelerating recovery from exercise (Lapointe et al. 2002; Howatson and van Someren 2008; Howatson et al. 2010). A marathon race can evoke a profound systemic inflammatory response, evidence for which is provided by a number of studies that observed large systemic increases in cytokine and leucocyte activity (Suzuki et al. 2003; Howatson et al. 2010; Scherr et al. 2011; Laupheimer et al. 2014). Thus, it would be reasonable to assume that an intervention targeting the acute inflammatory response might help to minimize secondary muscle damage, which, in turn, might accelerate the recovery process after a marathon race.

In recent years, there has been a growing interest in the anti-inflammatory effects of so-called functional foods for the purpose of enhancing exercise recovery (Myburgh 2014; Sousa et al. 2014). These foods are proposed to provide an additional value beyond their caloric content that might be beneficial for health and well-being (Corbo et al. 2014). This interest stems from the observation that some functional foods contain a variety of naturally occurring compounds, such as polyphenols (Myburgh 2014), which appear to exhibit a broad range of physiological benefits that include, but are not limited to, antioxidant (AOX) and anti-inflammatory effects (Nikolaidis et al. 2012; Sousa et al. 2014). There are now several reports that supplements rich in these phytonutrients, such as Montmorency cherry juice (Howatson et al. 2010; Bowtell et al. 2011; Bell et al. 2014; Bell et al. 2015), blueberry juice (McLeay et al. 2012), and pomegranate juice (Trombold et al. 2010; Trombold et al. 2011) can attenuate indices of EIMD and accelerate recovery. However, only one of these studies examined the effects of a functional food supplement after a marathon run (Howatson et al. 2010). In this study, cherry juice accelerated the recovery of isometric strength and attenuated

markers of inflammation and oxidative stress in the 2 days following the race, providing evidence of their potential benefit for recovery following a marathon.

A functional food that has received enormous attention in the sporting domain in recent years is beetroot (*Beta Vulgaris* L.). To date, beetroot has mostly been studied for its ability to enhance athletic performance, effects that are thought to be mediated by nitrate after its conversion to nitric oxide (NO) (Jones 2014). To generate NO, dietary nitrate is first reduced to nitrite in the oral cavity, a process facilitated by bacterial species located at the posterior aspect of the tongue (Clifford et al. 2015; Lundberg et al. 2008). Salivary nitrite can then be metabolised to NO in the stomach by a variety of reductase enzymes before entering the circulation; hence, this process is known as the nitrate-nitrite-NO pathway (Clifford et al. 2015; Hobbs et al. 2012; Lundberg et al. 2008). Augmenting NO bioavailability has been shown to exhibit biological effects that might be of benefit for exercise performance, such as vasodilation and improved contractile efficiency (Jones 2014); however, in addition, a number of recent animal studies have also revealed that it might attenuate inflammation (Justice et al. 2015) and inhibit exercise-induced muscle proteolysis (i.e., calpain activation), preserving muscle function (Lomonosova et al. 2014). This raises the possibility that NO donors, such as beetroot, might also help to enhance recovery after muscle-damaging exercise in humans.

There are also other phytonutrients in beetroot such as the betalain pigments or polyphenol compounds that might be of benefit for exercise recovery (El Gamal et al. 2014; Vidal et al. 2014). Indeed, there is now a growing body of evidence in both human and animal studies suggesting that akin to other functional foods shown to enhance EIMD, these chemical compounds are endowed with potent anti-inflammatory and AOX effects (Reddy et al. 2005; Pietrzkowski Z 2010; Jadert et al. 2012; El Gamal et al. 2014). The betalains for instance, the pigments that give beetroot its violet colour, are endowed with potent radical scavenging

activity, which has been proposed to help attenuate EIMD (Howatson et al. 2010; McLeay et al. 2012). However, it is important to note, that somewhat paradoxically, because nitrate-rich beetroot juice (BTJ) has been shown to enhance blood flow (Ferguson et al. 2013), ostensibly via the nitrate-nitrite-NO pathway, beetroot also has the potential to exacerbate free radical mediated cell damage by increasing muscle perfusion (Suematsu et al. 1994; Suzuki et al. 1995). Such effects might actually nullify any potential for AOX effects from these other compounds. With that said, the potential for these deleterious effects are yet to be demonstrated with beetroot. Nevertheless, AOX effects might not be the main mechanism by which BTJ could benefit recovery; instead this could primarily be through anti-inflammatory effects or reduced proteolysis. In support of an anti-inflammatory mechanism, the betalains in beetroot have been shown to inhibit cyclooxygenase-2 (COX-2) activity, which, via synthesis of prostanoids, play a key role in the development of the acute inflammatory response (Vidal et al. 2014), and have been linked to the development of muscle soreness (Murase et al. 2013).

In light of the potential anti-inflammatory effects of beetroot, and the evidence in animal models that NO is associated with muscle recovery, we speculated that a BTJ drink could attenuate secondary muscle damage after exercise. We recently provided evidence to support such effects; consuming BTJ for 3 days after strenuous exercise helped to minimize symptoms associated with EIMD (Clifford et al. 2016a). There was also no evidence that the BTJ had any adverse effects for muscle function recovery (Clifford et al. 2016a). Nonetheless, the generalizability of these findings to real-world athletic events are limited, given that it was performed in a controlled laboratory environment, and in untrained males. Furthermore, evidence that BTJ can simultaneously attenuate exercise-induced inflammation and enhance recovery is not yet available.

Given the above discussion, the primary aim of the present study was to examine whether consuming BTJ for 3 days after a marathon race could attenuate indices of muscle damage and inflammation and thereby facilitate exercise recovery. As such, a comprehensive array of inflammatory makers proposed to play a role in the secondary muscle damage process were measured alongside the primary outcome measures (muscle function). As in the previous studies investigating functional foods and EIMD (Trombold et al. 2010; Trombold et al. 2011; Howatson et al. 2010; Bell et al. 2014; Bell et al. 2015), due to the invasive nature of collecting muscle tissue, inflammation was measured in the periphery, to give an indication of the acute inflammatory response to the marathon. We hypothesized that the consumption of BTJ, when compared to a placebo (PLA), would facilitate recovery, as evidenced by accelerating the return of muscle function, reducing muscle soreness, and dampening the exercise-induced inflammatory response.

Materials and Methods

Participants

Participants were recruited from a pool of runners taking part in the 2016 Druridge Bay Marathon, Northumberland, UK. Based on a previous study that examined recovery after a marathon race (Howatson et al. 2010), it was estimated that at 0.80 power and 0.05 significance, the minimum number of participants required to detect a ≥10% group difference (SD: 8%) in one of our primary outcomes, MIVC, would be $n = 13$ per group. We surpassed this target, with 36 runners initially volunteering to participate in this investigation. Two runners dropped out during the race after sustaining injuries unrelated to the study, thus, thirty-four runners completed all study requirements. A summary of their characteristics is presented in Table 1. To assess eligibility, participants completed a health screening questionnaire; those with a known food allergy, cardiovascular complications, musculoskeletal injury or receiving prescribed anti-inflammatory therapies were excluded

from participating. One participant was deemed not eligible to participate because they were taking a prescribed medication and thus was not invited for a consent session. The female participants were also required to complete a menstrual cycle questionnaire in order to determine menstrual cycle phase during testing. Of those still menstruating, testing fell during the early/mid luteal phase. Participants were also instructed to not exercise during the trial, and avoid the use of recovery therapies such as cold water immersion, compression garments, foam rolling, non-steroidal anti-inflammatory drugs (NSAIDS) and AOX vitamins. Finally, the use of antibacterial mouthwash throughout data collection was prohibited due its potential to blunt nitrate-nitrite conversion after BTJ ingestion (Webb et al. 2008). The protocol received ethical approval from the institutional ethics committee. Participants provided written informed consent prior to data collection.

Experimental design

This study employed a double blind, placebo-controlled, independent group design with two experimental arms. Participants were allocated to receive either BTJ; $n = 17$ or a PLA; $n = 17$ for 3 days after a marathon race, with the first supplement taken immediately after a series of post-race marathon tests had been completed. All participants recorded their dietary intake throughout the trial (24 h prior to until 48 h after the marathon). The groups were matched according to predicted marathon finish times and contained a similar number females and males (Table 1). Participants attended the lab on 3 occasions in total: the first was in the week leading up to the marathon to collect baseline measures, and the other two on consecutive days after the marathon. Pre-marathon (baseline), immediately post-marathon, and on two days after the marathon (day 1 and day 2) participants had a blood sample taken, rated their muscle soreness, completed a CMJ and MIVC test in that order. Participants were fully familiarized with these procedures on their baseline visit.

Supplementation

The macronutrient composition of the two supplements is provided in Table 2. The BTJ supplement was provided by Love Beets Beetroot Juice (Gs Fresh Ltd, Cambridgeshire, UK). Previous analysis of the juice revealed that each bottle provides ~400 mg of phenolic compounds (expressed as Gallic acid equivalents) and ~194 mg of the pigment, betanin (Clifford et al. 2016b). This specific batch of BTJ contained approximately 210 mg of nitrate. We have previously shown that this BTJ can significantly increase plasma NO concentrations for up to 8 h post-ingestion (Clifford et al. 2016b). The PLA contained maltodextrin (Myprotein, Manchester, UK) and flavourless protein powder (Arla Foods, Amba, Denmark) to match the BTJ for macronutrient composition, and a small amount of fruit squash (Kia Ora, Coca Cola, UK). With regards to timings, participants consumed 3 bottles (250 ml) of their assigned supplement on day 1 (immediately post-marathon, 3 h later and at 8:00); another 3 bottles on day 1 (upon waking, with lunch and with an evening meal) and 1 bottle (upon waking) on day 2. The rationale for providing 3 daily servings after the marathon was based on our previous findings (Clifford et al. 2016a) that showed three, 250 ml servings taken after muscle-damaging exercise attenuated muscle pain and muscle function deficits in the ensuing days. Furthermore, because it has been shown that systemic inflammation is still significantly elevated in the day after a marathon, three servings were also provided on day 1 post-marathon in an attempt to counteract this stress (Scherr et al. 2011; Hill et al. 2014). Participants were given written instructions on when to consume their supplements and were also asked to complete a checklist, which was to be returned to the principal investigator at the conclusion of the study to ensure compliance. To comply with the double blind design, both supplements were provided in masked bottles that were identical in size and appearance. Although the drinks were distinguishable by taste, the independent groups design ensured that participants were unaware of this difference.

Furthermore, participants were not made aware of the specific aims of the study and were only given the information that AOX drinks were being tested for their recovery benefits. This helped ensure the participants were unaware as to whether they were receiving the treatment or PLA beverage. These controls were effectively used in our previous work for double blinding purposes (Clifford et al. 2016a).

Marathon race

The race consisted of 4 laps of the Druridge Bay Country Park situated on the Northumberland coast (Morpeth, UK). The course is mostly flat and across a mix of grassy or concrete terrain; however, approximately 1 mile of each lap was on soft sand. At 09:00 when the race started the air temperature was 3.8˚C, humidity 82%, barometric pressure 1013 hpa and wind speed, 9 $km \cdot h^{-1}$. Towards the end of the race (13:00-14:00) there was an increase in air temperature (8.5˚C) and wind speed (14 $km \cdot h^{-1}$) with the humidity dropping (62%). It remained dry and mostly overcast for the duration of the race with intermittent sunny spells.

Maximal voluntary isometric contraction

As described previously (Howatson et al. 2010; Clifford et al. 2016a), maximal isometric voluntary contractions (MIVC) were measured with a portable strain gauge (MIE Medical Research Ltd., Leeds, UK). All trials were performed with the participants seated in an upright position with a perspex gauze attached to their right ankle just above the malleoli. After adjusting the strap to ensure a 90° knee joint angle was maintained (verified with a goniometer), participants were instructed to push against the gauze with maximal force for a 3 second contraction. The peak value (N) from 2 maximal contractions (separated by 60 seconds) was used for analysis. Verbal encouragement was provided for all trials. This measure has previously been shown to have good reliability in our lab (coefficient of variation; CV <1.5%).

Countermovement jump

Counter movement jump height (CMJ) was measured using an optojump system (Optojump next, Bolzano, Italy), which calculates height (cm) via flight time. When performing the jumps participants were instructed to keep their hands on their hips throughout the full movement. As in previous studies (Clifford et al. 2016a), participants were required to descend into a squat (to a ~90º knee angle) and jump vertically with maximum effort. Two maximal efforts were performed, separated by 30 seconds of passive (standing) recovery; as recently recommended, the average value was used for data analysis (Claudino et al. 2016). The CV for measuring CMJ with this procedure in our lab has been calculated as <2.5%.

Muscle soreness

Muscle soreness was measured as per previously described methods (Howatson et al. 2010). After performing a squat (at ~90˚ knee flexion), participants rated their perceived level of muscle soreness (lower limbs only) by drawing a vertical line on a visual analogue scale (VAS), in which 0 represented 'no soreness' and 200 mm represented 'unbearably painful'. The line placement was measured with a ruler and recorded.

Blood sampling

All blood samples were obtained from a branch of the basilica vein at the antecubital fossa using the venepuncture technique. At all 4 time points (pre-marathon, post-marathon, day 1 and day 2), blood was drawn into a 10 ml vacutainer for serum and a 4 ml vacutainer coated with di-potassium ethylene diamine tetra-acetic acid (EDTA). Serum was allowed to stand and clot for 30 minutes before being centrifuged at 3000 *g* for 10 minutes to separate the supernatant. This was subsequently stored in aliquots at -80º and only thawed for analysis in the morning of the analysis. The 4 ml EDTA vacutainer was transported to a local hospital for haematological analysis.

Biochemical analysis

Haemoglobin, haematocrit, leucocytes and monocytes were measured in whole blood using an automated haematology system (Sysmex XE-2100, Illinois, US). Haemoglobin and haematocrit were used to calculate pre-post marathon changes in plasma volume according to the methods of Dill and Costill (1974). According to data provided by the laboratory, the CV's for this procedure are typically <10%. Serum concentrations of cytokines and growth factors were measured according to the manufacturer's instructions using a multiplex sandwich chemiluminescent immunoassay kit (Evidence Investigator, Randox Laboratories, Northern Ireland, UK). Inter and intra assay CV's were below 5%. Creatine kinase (CK), high sensitivity C-reactive protein (hs-CRP) and aspartate transaminase (AST) were measured in serum using an automated system based on an electrochemiluminescence method (Roche Modular, Roche Diagnostics, UK). According to data provided by the laboratory, the typical CV for these measures with this procedure is <5%.

Data Analysis

All data are expressed as mean ± SD and statistical significance was set at $P < 0.05$ prior to analyses. Differences in participant group characteristics, training history and dietary intake were analysed with student t-tests. Nutritics dietary analysis software (Nutritics LTD, Dublin, Ireland) was used to analyse participant's food diaries. Dependent variables (MIVC, CMJ, VAS and all blood indices) were analysed using a mixed model ANOVA with 2 independent group levels (BTJ vs. PLA) and 4 repeated measures time points (pre, post, day 1 and day 2). All blood variables were adjusted for plasma volume changes prior to analyses. If the ANOVA indicated a significant interaction effect (drink*time) Fisher LSD *post hoc* analysis was performed to locate the significant differences. Homogeneity of variance was checked with Mauchly's test of sphericity and in the event of a significant result, Greenhouse-Geisser

adjustments were used. All data were analysed using IBM SPSS Statistics 22 for Windows (Surrey, UK).

Results

There were no between group differences for training history and personal characteristics suggesting that the groups were well matched before the marathon (Table 1). Marathon finish times did not differ between groups either ($P = 0.162$). Pre to post-marathon changes in body mass and plasma volume were modest and to a similar extent in both groups (Table 1).

Average energy intake in the day before the marathon until the cessation of the trial did not differ between groups (BTJ, 2488 ± 607 vs. PLA, 2308 ± 480 kcal; $P = 0.438$), nor did the proportion (%) consumed from carbohydrates (BTJ 49.88 ± 0.07, PLA 46.76 ± 0.05; $P = 0.203$) and protein (BTJ 16.38 ± 0.02, PLA 14.71 ± 0.04; $P = 0.209$). The proportion of calories from fat was slightly higher in the PLA vs. BTJ group however (36.12 ± 0.04 vs. 32.06 ± 0.06; $P = 0.032$).

Muscle function and muscle soreness

Muscle function (CMJ and MIVC) and muscle soreness showed a main effect of time ($P < 0.0001$), indicating the presence of muscle damage after the marathon (Figures 1 & 2). Relative to baseline values, CMJ decreased to a similar extent in the BTJ and PLA groups immediately post-marathon (71.4 ± 18.5 vs. 69.2 ± 20.5 %, respectively) and remained similarly depressed at day 2 (94.5 ± 8.86 vs. 95.3 ± 5.9 %, respectively). No group or interaction effects were present at any time point ($P > 0.05$).

MIVC was less affected by the marathon than CMJ and had recovered to pre-marathon values by day 2 (BTJ: 100.6 ± 13.5 vs. PLA: 99.1 ± 10.6 % of baseline; Figure 1). MIVC recovery was independent of treatment with no group or interaction effect observed ($P > 0.05$). Muscle soreness increased (time effect; $P < 0.0001$), and was greatest immediately after the marathon

and was largely absent at day 2 (Figure 2). No group or interaction effects were present at any time point ($P > 0.05$).

Leucocytosis, inflammation and liver function

Total blood leucocyte counts demonstrated main effects for time ($P < 0.0001$) increasing 1.7 fold (average across groups) immediately after the race. Leucocytes were still raised above pre-marathon levels on day 1 ($P = 0.0007$) and day 2 post-marathon ($P = 0.01$); however, no group or interaction effects were present ($P > 0.05$; Table 3). Neutrophils and monocytes followed the same pattern, peaking immediately after the marathon race and not returning to baseline by day 2 post-marathon in both groups ($P < 0.05$). No group or interaction effects were present at any time point for these measures ($P > 0.05$).

Serum CK (Figure 2) and AST (Table 3) demonstrated main effects for time ($P < 0.0001$) but no group or interaction effects were present ($P > 0.05$). In both groups CK and AST increased immediately after the marathon, peaked day 1 post, and although were attenuated on day 2 still remained higher than pre-marathon values. Serum hs-CRP also demonstrated main effects for time ($P < 0.0001$) and was elevated on day 1 and day 2 post-marathon (Table 3). There were no group or interaction effects for hs-CRP ($P > 0.05$).

Cytokines, growth factors and chemokines

Changes in cytokine, growth factor and chemokine activity pre-post marathon are displayed in Table 4. Immediately post-race, the serum cytokines interleukin-6 (IL-6), interleukin-8 (IL-8), interleukin-10 (IL-10) and tumor necrosis factor-α (TNF-α) all increased (time effect; $P < 0.0001$). IL-6 was the only cytokine still significantly elevated on day 1 (~0.9 fold change in BTJ and ~0.5 fold in PLA) but no significant group or interaction effects were present ($P > 0.05$). Other cytokines measured (interleukin-1 receptor antagonist (IL-1ra), interleukin-2 (IL-2), interleukin-4 (IL-4) and interferon gamma (IFN-γ)) did not rise appreciably at any time point after the marathon in either group ($P > 0.05$). VEGF was

unchanged after the marathon but EGF showed main effects for time ($P < 0.0001$), initially decreasing but then increasing on day 1 and 2; however, no group or interaction effects were observed ($P > 0.05$). The chemokine, MCP-1, demonstrated a main effect for time ($P < 0.0001$) increasing in BTJ (2 fold-change) and PLA (1.9 fold-change) immediately after the marathon. MCP-1 was still elevated on day-2 post marathon but to a similar extent in both groups with no group or interaction effects present ($P > 0.05$).

Discussion

It was hypothesized that BTJ would enhance the recovery of muscle function and attenuate muscle soreness after a marathon race, possibly by reducing the acute inflammatory response shown to accompany long-distance running. However, contrary to our hypothesis, BTJ did not favourably affect the recovery of muscle function or attenuate muscle soreness in the 2 days after the marathon race. In addition, biochemical markers of inflammation and muscle damage measured before and up to 2 days after the race did not differ between the BTJ and PLA groups, suggesting that BTJ was ineffective for attenuating the acute inflammatory response after marathon running.

The findings in the present study are in contrast to a previous study that examined the effects of a phytonutrient rich drink (Montmorency cherry juice) on EIMD after a marathon race (Howatson et al. 2010). In this study, the cherry juice supplementation was shown to attenuate biomarkers of inflammation (IL-6) and oxidative stress (thiobarbituric acid reactive substances; TBARS) in the 48 h following the marathon; effects that were associated with an accelerated recovery of MIVC. There are, however, some differences in study design that could account for these discrepancies. Apart from the obvious difference in that cherry juice was used as opposed to BTJ and, therefore, the biological activities of the phytonutrients likely differ, the aforementioned study also provided supplements in the 5 days leading up to the marathon, as well as the 3 days after. By contrast, in the present study, supplementation

only began after the marathon race; thus, the discrepancy in findings between these two studies could be due, at least in part, to the different supplements and dosage strategies used. Speculatively, a more long-term dosage strategy (incorporating a loading phase) might have been required to mediate similar recovery benefits with BTJ after the marathon.

The lack of benefit with BTJ in the current study is also in contrast to the findings from our previous work (Clifford et al. 2016a), in which BTJ was shown to enhance the recovery of CMJ performance and attenuate muscle soreness in the days following high intensity plyometric exercise (100 drop jumps). The differences in exercise protocol (marathon race vs. drop jumps), participant cohort (marathon runners vs. untrained) and techniques used to assess EIMD (VAS vs. pressure pain-threshold; PPT) between the present and previous studies are all factors that could provide a potential explanation for these disparate results. Another possible explanation is related to the magnitude of the muscle damage response, which, in the present study, was markedly lower, and largely absent by 2 days (see Figure 1). There was also no secondary loss in muscle function in the post-exercise period as there was in the previously mentioned study. It is therefore possible that the small magnitude of muscle damage in this study might have limited our ability to detect any subtle differences between groups. Indeed, it would be reasonable to assume that any recovery benefits associated with BTJ or any recovery intervention would only be evident when muscle damage is still present. Given these data, it could be speculated that the experienced marathon runners in this study might have already been sufficiently well protected from marked and prolonged symptoms of muscle damage and, thus, BTJ conferred no benefits for recovery. Lending some support to this idea is the fact that better trained individuals experience less muscle damage, inflammation, and oxidative stress than non-trained individuals after the same bout of exercise (Evans et al. 1986; Said et al., 2006; Newton et al., 2008; Bloomer et al., 2008). Nonetheless, this possibility needs to be clarified with future research that directly compares

muscle damage responses in untrained and trained runners. Notwithstanding, these data draw into question the usefulness of BTJ (and indeed any intervention) to enhance recovery in well-trained individuals, especially those who regularly participate in long-distance running events.

After strenuous exercise, leucocytes, mostly neutrophils and monocytes, migrate from the circulation into damaged muscle in order to degrade structural components, and thus are thought to play a major role in secondary muscle damage after exercise (Pizza et al. 2005; Butterfield et al. 2006). As alluded to in the introduction, several of the phenolic compounds and pigments in BTJ have been shown to exhibit ant-inflammatory activity (El Gamal et al. 2014; Vidal et al. 2014). There is even evidence that NO, generated from the nitrate-nitrite-NO pathway (Jadert et al. 2012; Wylie et al. 2013), is a critical regulator of inflammation (Waltz et al. 2015). As such, it was hypothesized that BTJ might inhibit leucocytosis, which, in turn, would preserve muscle cell integrity and functional strength. However, contrary to this hypothesis, leucocyte accumulation in the circulation was unaffected by BTJ supplementation. As shown in Table 3, while the marathon evoked a large inflammatory response, with total leucocyte counts, neutrophils and monocytes still elevated above pre-marathon values 2 days' post, the magnitude of change was not different in the PLA and BTJ groups. These results suggest that BTJ, at least at this dose and in marathon runners, did not modulate any systemic leucocyte response after exercise. Based on these data, it is unsurprising this study did not find any beneficial effect of BTJ on muscle pain and muscle function.

BTJ also had no effect on AST, hs-CRP or a host of cytokines in the days after the marathon race (Tables 3 & 4). Several cytokines were increased immediately after the marathon race (IL-6, IL-8 and TNF-α), which is in agreement with previous studies that examined the early cytokine response after long distance running events (Howatson et al. 2010; Scherr et al.

2011; Shanely et al. 2014). However, some of the cytokines measured did not change at all after the marathon (IL-1β, IL-1ra, IL-2, IL-4, IFN-y) and only IL-6 was elevated above pre-exercise values in the days after the marathon. Il-1β did appear to be elevated on day 1 post, but a wide heterogeneity was observed, which might have limited our ability to detect a statistical difference. Some of the changes in these cytokines are in contrast to a previous study that found experienced runners still exhibited elevated levels of TNF-α and IL-10 >1 day after a marathon race (Scherr et al. 2011). The study of Scherr et al. (2011) did contain a larger cohort of participants than the present study however (n = 105), so perhaps this study was underpowered to detect such small changes in these markers. Nonetheless, the present findings suggest that cytokines, at least at the systemic level, might not play a significant role in the secondary muscle damage process in the days after the marathon. Instead, their activity might be primarily limited to the muscle and surrounding tissues, as previously suggested after other types of exercise (Peake et al. 2015). It is possible that if blood samples been taken at additional time points however, specifically between the end of the marathon and day 1, several of these cytokines would have still been elevated. This might have been a better time in the post-exercise period for detecting potential differences between the groups. Likewise, if supplements had been consumed in the days before the marathon, as in the study by Howatson and colleagues (2010), group differences might have been apparent at the post-marathon time point, or have at least been easier to detect, given the majority of biomarkers were significantly elevated at this point (Table 3 and Table 4).

In contrast to many of the cytokines, the chemokine, MCP-1, and the growth factor, EGF, were still significantly elevated at 2 days' post-marathon, suggesting that they have more prominent roles in muscle damage after exercise. Indeed, MCP-1, also known as CCL2, is thought to play a particularly important role in resolving muscle damage after exercise, via its effects on facilitating macrophage infiltration and activating resident satellite cells (Warren et

al. 2004; Hubal et al. 2008; Urso 2013). Nonetheless, as with the other inflammatory markers measured in this study, MCP-1 was unaffected by BTJ supplementation.

As seen in previous marathon studies, serum CK activity was markedly increased, peaking 1 day after the race (Howatson et al. 2010; Hill et al. 2014). The magnitude of CK release did not differ between the groups however, which is consistent with our previous work with BTJ (Clifford et al. 2016a) and the work of others who report no benefit of functional foods akin to BTJ on CK efflux after muscle damaging exercise (Howatson et al. 2010; Trombold et al. 2010; Bell et al. 2014; Bell et al. 2015).

It is important to acknowledge that a key limitation of this study is that we did not measure circulating NO levels to establish that BTJ actually increased NO bioavailability. Instead, we assumed that because a BTJ with similar nitrate content had previously increased plasma NO levels (Clifford et al. 2016b), a similar effect would occur this study. However, because NO generation via the nitrate-nitrite-NO pathway can vary widely between individuals, and can be attenuated in well-trained athletes (Jones 2014), we cannot rule out the possibility that the NO concentrations were simply not sufficiently raised to actually mediate any beneficial effects for recovery. Additionally, we did not measure the bioavailability of any of the phenolic and betalainic compounds in the BTJ to test whether they reached the circulation for biological effects. Thus, as with nitrate, we cannot rule out the possibility that BTJ was ineffective because the bioactive compounds it contains were not present in sufficient quantities to exert effects that might beneficially influence recovery. This limitation needs to be addressed in future studies.

In conclusion, this study reports that consuming BTJ for 3 days after a marathon race does not attenuate muscle soreness, enhance the recovery of muscle function or attenuate biochemical markers of inflammation and muscle damage in marathon runners. Our data could have been limited by the fact than several of the markers used to assess recovery did

not differ from the baseline values in the 2 days after the marathon and, thus, there was not much muscle damage for BTJ to attenuate. Nevertheless, the present findings do not support the use of BTJ as a recovery intervention following a marathon race, at least in already experienced runners. Notwithstanding, future studies might benefit from including a supplement loading phase in the days leading up to the marathon, as this has previously been shown to attenuate inflammation and enhance functional recovery after a marathon (Howatson et al. 2010).

Conflict of interest

This study was funded as part of a doctoral degree that receives financial support from Gs Fresh Ltd. The funders supplied the supplements used in this study but had no role in the conception of the study, its design, preparation, analysis and writing of the manuscript. The authors declare no conflict of interest.

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Table 1 - Participant characteristics and training history for the BTJ and PLA groups.

	BTJ (*n*=17)	**PLA (*n*=17)**
Age (yrs)	42 ± 10	39 ± 12
Sex (M/F)	10/7	11/6
Height (m)	1.71 ± 0.09	1.72 ± 0.08
Mass (kg)	69.7 ± 10.4	71.0 ± 11.3
Yrs running	13 ± 11	7 ± 6
Average weekly mileage	34 ± 11	32 ± 10
Longest training run (miles)	20 ± 2	21 ± 2
Previous marathons	12 ± 14	19 ± 38
Predicted finish time	04:04:06	04:09:24
Actual finish time	04:05:39	04:28:29
Pre-post change in body mass (kg)	1.3 ± 0.9	1.3 ± 0.8
Pre-post change in plasma volume (%)	0.5 ± 5.3	-1.9 ± 5.7

Values are mean ± SD. There were no differences between groups for any variable ($P > 0.05$).

Table 2. Macronutrient composition of the BTJ and PLA drinks.

Supplement	BTJ	PLA
Energy (Kcal)	81.0	76.8
Volume (ml)	250	250
Carbohydrate (g)	16.4	16.4
Protein (g)	2.8	2.8
Fat (g)	0.4	Trace

Table 3 - Serum inflammation and liver function response before and after the marathon in the BTJ and PLA groups.

Biomarker	Pre-race	Post-race	Day 1 post	Day 2 post
Leucocytes (10^{*9} cells·L^{-1})				
BTJ	6.26 ± 1.11	15.24 ± 2.90*	7.46 ± 2.02*	7.28 ± 1.94*
PLA	5.25 ± 1.5	15.22 ± 3.60*	7.10 ± 1.44*	6.47 ± 1.47*
Neutrophils (10^{*9} cells·L^{-1})				
BTJ	3.55 ± 1.01	12.55 ± 2.55*	4.21 ± 1.23*	4.04 ± 1.13*
PLA	2.67 ± 1.07	12.52 ± 3.49*	3.88 ± 1.13*	3.38 ± 0.89*
Monocytes (10^{*9} cells·L^{-1})				
BTJ	0.51 ± 0.11	1.00 ± 0.38*	0.65 ± 0.21*	0.68 ± 0.16*
PLA	0.50 ± 0.17	1.06 ± 0.27*	0.61 ± 0.13*	0.62 ± 0.20*
Hs-CRP (mg·L^{-1})				
BTJ	0.88 ± 1.16	0.62 ± 0.62*	12.22 ± 9.03*	7.18 ± 4.49*
PLA	0.70 ± 0.82	0.57 ± 0.71*	11.37 ± 10.10*	6.10 ± 4.90*
AST (IU·L^{-1})				
BTJ	21.66 ± 7.43	30.68 ± 9.25*	49.97 ± 29.44*	41.16 ± 23.5*
PLA	23.25 ± 12.47	31.24 ± 7.36*	60.28 ± 48.80*	48.94 ± 31.79*

Values are mean ± SD, n = 16 per group. *Different from baseline ($P < 0.05$). hs-CRP, high sensitivity-C-reactive protein; AST, aspartate aminotransferase.

Table 4 - Serum cytokine, growth factor, and chemokine response before and after the marathon in the BTJ and PLA groups.

Biomarker (pg/ml)	Pre-race	Post-race	Day 1 post	Day 2 post
IL-1_{ra}				
BTJ	0.38 ± 0.27	0.31 ± 0.12	0.29 ± 0.09	0.30 ± 0.09
PLA	0.39 ± 0.27	0.37 ± 0.24	0.38 ± 0.22	0.37 ± 0.31
IL-1β				
BTJ	1.44 ± 0.72	3.09 ± 1.41*	1.76 ± 0.60	1.63 ± 0.77
PLA	1.79 ± 1.03	2.29 ± 1.21*	2.26 ± 1.70	2.45 ± 2.31
IL-2				
BTJ	2.89 ± 3.32	4.96 ± 8.72	2.85 ± 2.57	2.87 ± 2.52
PLA	4.19 ± 3.00	3.84 ± 2.97	4.34 ± 3.16	6.75 ± 10.03
IL-4				
BTJ	2.15 ± 0.61	2.13 ± 0.41	2.25 ± 0.45	2.56 ± 0.81
PLA	2.52 ± 0.78	2.32 ± 0.73	2.36 ± 0.68	2.38 ± 0.78
IL-6				
BTJ	1.15 ± 0.48	31.12 ± 15.93*	2.26 ± 1.89*	1.64 ± 0.73
PLA	1.02 ± 0.51	31.42 ± 25.12*	1.53 ± 0.58*	1.05 ± 0.47
IL-8				
BTJ	7.60 ± 3.57	19.34 ± 8.95*	7.00 ± 3.06	7.78 ± 4.03
PLA	8.38 ± 5.83	19.22 ± 10.91*	6.94 ± 4.93	6.83 ± 6.40
IL-10				
BTJ	0.94 ± 0.35	17.55 ± 16.42*	0.90 ± 0.18	1.34 ± 1.74
PLA	1.62 ± 1.01	16.58 ± 19.01*	1.36 ± 0.72	1.50 ± 1.02
TNF-α				
BTJ	2.98 ± 1.13	3.61 ± 1.34*	2.94 ± 0.84	3.17 ± 1.15
PLA	2.81 ± 0.70	3.17 ± 0.77*	2.70 ± 0.58	2.95 ± 1.01
VEGF				
BTJ	127.41 ± 86.69	131.57 ± 112.48	144.87 ± 101.78	144.74 ± 94.74
PLA	100.66 ± 77.58	97.24 ± 102.34	121.54 ± 99.59	120.5 ± 100.09
INF-γ				
BTJ	0.52 ± 0.51	0.51 ± 0.65	0.43 ± 0.61	0.61 ± 0.77
PLA	0.42 ± 0.32	0.34 ± 0.24	0.43 ± 0.31	0.66 ± 0.86
EGF				
BTJ	22.55 ± 20.36	17.68 ± 13.25*	59.24 ± 47.00*	72.21 ± 45.06*
PLA	27.68 ± 34.97	10.37 ± 10.15*	50.94 ± 37.99*	58.35 ± 43.95*
MCP-1				
BTJ	178.49 ± 85.24	537.22 ± 107.60*	235.82 ± 60.80*	225.92 ± 76.46*
PLA	172.75 ± 52.12	486.49 ± 190.83*	222.19 ± 78.08*	198.28 ± 69.59*

Values are mean ± SD, n = 16 per group. *Elevated above baseline values ($P < 0.05$). IL-1-ra, interleukin 1-receptor agonist; IL-1β, interleukin-1beta; Il-2, interleukin-2; Il-4, interleukin-4; Il-6, interleukin-6; Il-8, interleukin-8; Il-10, interleukin-10; TNF-α, tumor necrosis factor-alpha; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; INF-γ, interferon-gamma; MCP-1, monocyte chemoattractant protein 1.

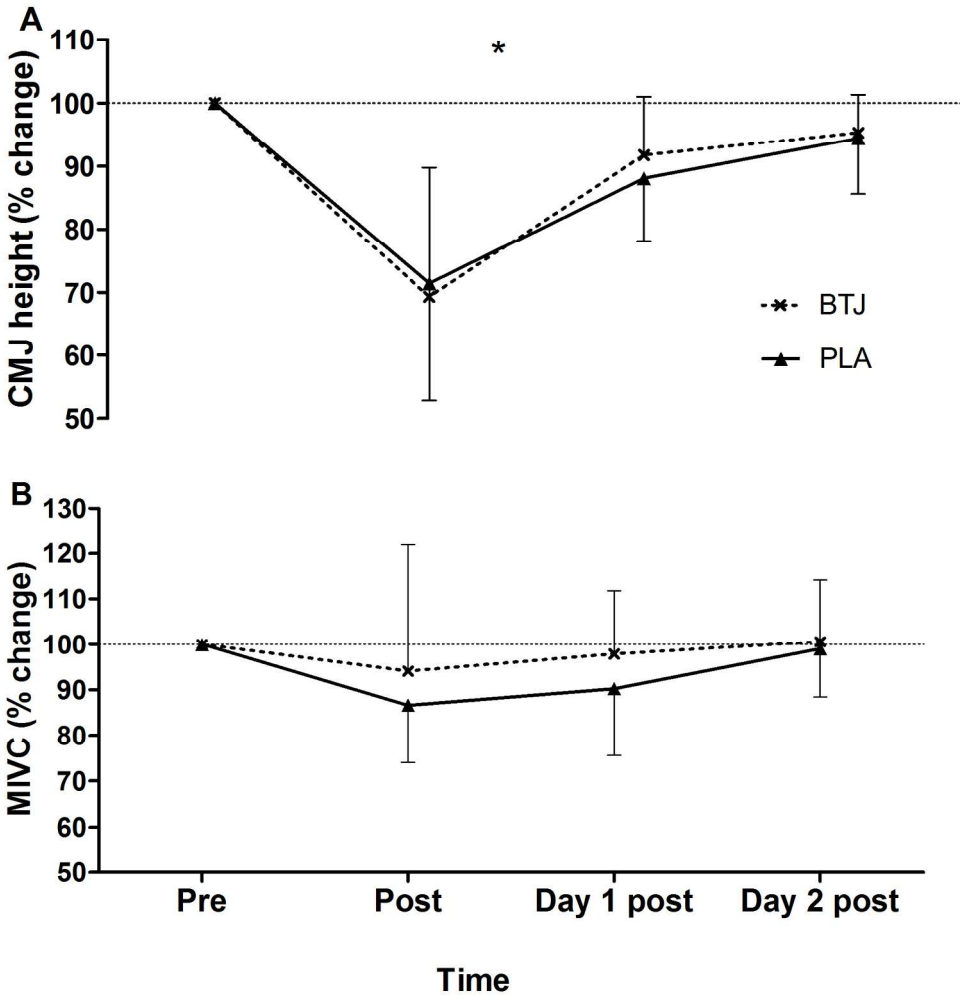

Figure 1 – (A) Percentage change from baseline in counter movement jump (CMJ) height before and after the marathon. (B) Percentage change from baseline in maximal isometric voluntary contractions (MIVC) before and after the marathon. *Represents time effect ($P < 0.05$). Values are mean ± SD (n = 17 per group).

178x196mm (300 x 300 DPI)

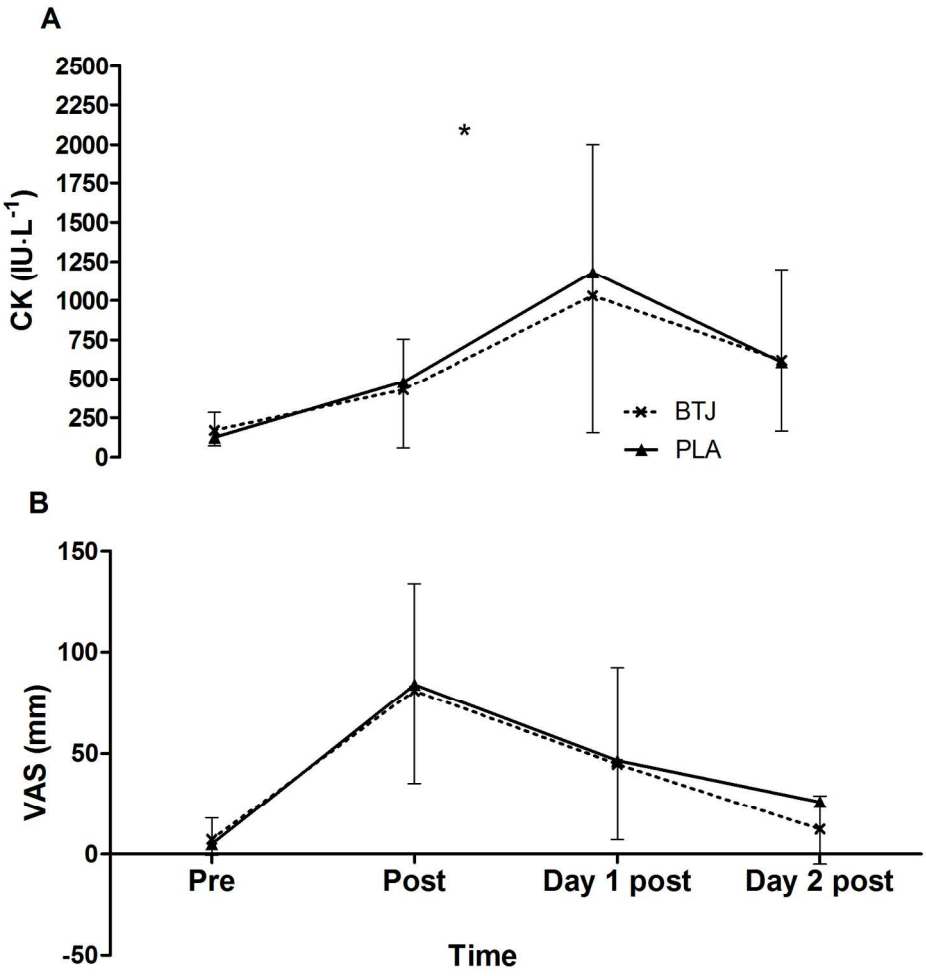

Figure 2 – (A) Serum creatine kinase (CK) concentrations before and after the marathon. (B) Muscle soreness (VAS) before and after the marathon. *Represents time effect ($P < 0.05$). Values are mean ± SD (n = 17 per group).

163x172mm (300 x 300 DPI)