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A pilot study investigating reactive oxygen species production in capillary blood after a marathon and the influence of an antioxidant-rich beetroot juice

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Abstract

We report that reactive oxygen species (ROS), as measured in capillary blood taken from the finger-tip, increased after a marathon (+128% $P < 0.01$; ES = 1.17), indicating that this collection method might be useful for measuring ROS in field settings. However, mitochondrial DNA damage remained unchanged. Beetroot juice, taken before and after exercise, was unable to mitigate exercise induced-ROS production, questioning its use an antioxidant rich-food.

Key words: Reactive oxygen species; running; beetroot; exercise

Introduction

To measure exercise-induced ROS generation rand the effectiveness of antioxidant (AOX) supplements, a number of biomarkers have been developed. These biomarkers are most commonly measured in blood matrices, given that muscle tissue can only be obtained with highly invasive biopsy procedures (Nikolaidis et al., 2012). Collecting venous blood samples can also be problematic, however; as well as being time consuming, it requires specialist expertise, meaning that monitoring ROS outside of the lab, in clinics or applied sport settings for instance, is very limited (Twist and Highton, 2013). One potential solution to this problem is the use of less invasive techniques. In this regard, we have developed methods that estimate physiological stress from a finger-tip capillary blood sample that measures either ROS production or damage to mitochondrial DNA (mtDNA). Collecting blood from the finger-tip is quick, minimally invasive, and requires little equipment, and therefore offers several advantages in applied or clinical point of care research settings. Thus, one of the aims of this study was to investigate the suitability of these measures for estimating physiological stress following strenuous exercise.

Another aim of this study was to assess the effectiveness of an AOX-rich food (beetroot juice; BTJ) for attenuating the ROS production. Numerous studies have shown that the constituents in beetroot, chiefly the betalain pigments, are potent AOX that have the potential to attenuate ROS generation (Clifford et al., 2015). However, most of these observations have been carried out either *in vitro* or in animal studies so these findings might not be directly transferable to the *in vivo* environment in humans. Accordingly, in this pilot study we wanted to; 1) to examine the effects of a ROS production and mtDNA damage in capillary blood after a marathon and; 2) to establish whether post-exercise these markers can be attenuated with BTJ. We selected a marathon because the high physiological stress imposed has been shown to serve as a good model to provoke a robust increase in ROS production and inflammatory related events (Gomez-Cabrera et al., 2006; Nieman et al., 2002).

Methods

Participants

Fourteen, healthy marathon runners volunteered to participate in this study. A summary of their physical characteristics and training history are presented in Table 1. The protocol received ethical approval from Newcastle University (16LIE016) and all participants provided informed consent.

Experimental design

This study was a double blind, placebo-controlled, independent group's trial with two experimental treatment arms: BTJ (n = 7) or a placebo (PLA) (n = 7). Capillary puncture samples from the finger-tip were obtained pre-supplementation, pre-race, 30 mins post-race and the morning following the race. Online dietary recall software (Intake24, Newcastle University, UK) was used to record participant's dietary intake the day before and on the day of the race.

Supplementation

The BTJ used in this study was Love Beets Beetroot Juice (Gs Fresh Ltd, Cambridgeshire, UK) and the PLA used in this study was water mixed with maltodextrin (Myprotein, Manchester, UK) and a fruit squash (Kia Ora, Coca Cola, UK) to match the BTJ for carbohydrate and energy content. Details on the polyphenol and AOX content of these two drinks— as well, as how they compare to other antioxidant-rich beverages, can be found in Clifford et al., (2016). As in a similarly designed, study (Howatson et al., 2010), participants consumed their respective supplements twice daily (250 ml per serving) at 08:00 and 20:00 on the 4 days prior to the marathon, on the day of the marathon and at 08:00 the morning after the marathon (11 servings in total). To ensure the drinks were provided in a double-blind fashion, both supplements were prepared in masked bottles that were identical in size and appearance.

Blood sampling and analysis

Capillary blood samples were analysed for haemoglobin (Hb) concentrations (Hemocue 201+; HemoCue AB, Angelholm, Sweden), ROS production, and mtDNA damage. ROS production was measured in whole blood using a luminescence assay according to the methods described by Yamazaki et al., (2011). 2µl of blood was immediately added to a buffer at a dilution of 1:50 blood to buffer. Phorbol myristate acetate (PMA) was later added to whole blood to stimulate ROS production in blood leukocytes. The pre and post-race samples were kept on ice until transported by car to the University, where they were analysed within 24 h of race completion. The assay

established by PB Biosciences Ltd (Newcastle, UK) is based on the principle that luminol is oxidised by ROS to produce light, which is measured over 22 minutes in real time, and the area under the curve determined. The inter-assay coefficient of variation (CV) for this technique was established as 9.9%. It is important to acknowledge that because this assay is performed at an ambient PO_2, ROS production might be overestimated compared to *in vivo* where PO_2 is lower.

MtDNA damage was measured via real-time quantitative PCR (qPCR). DNA was extracted from a blood spot on an FTA card (GE Healthcare Life Sciences), using a QIAamp DNA Mini Kit (Qiagen, UK) with the manufacturer's protocol for extracting DNA from dried blood spots modified. Six punched-out circles from a dried blood spot were placed in a 1.5 ml microcentrifuge tube, for each time point of each volunteer. To these tubes, 190 µl Buffer ATL was added and the tubes incubated at 85°C for 10 minutes. Following incubation, 10 µl proteinase K was added and samples were incubated at 56°C for 1 hour, with pulse-vortexing every 15 minutes for 15 seconds. Samples were incubated at 95°C for 5 minutes, 200 µl Buffer AL added, and samples incubated at 70°C for 10 minutes. Extraction was then continued according to the manufacturer's protocol (Qiagen, UK), and samples were stored at 4°C until analysis. DNA concentrations were determined using an ND-1000 Nanodrop Spectrophotometer (Thermo Scientific, UK) at a wavelength of 260 nm. To perform the qPCR reaction, the following components were assembled on ice to a final volume of 20 µl per well of a MicroAmp Fast Optical 96-Well Reaction Plate (Applied Biosystems, UK): dH20, 1x SensiMix SYBR Hi-ROX (Bioline, UK), 0.25 µM each of the forward primer (AL4_F, CTGTTCTTTCATGGGGAAGC) and the reverse primer (AS1_R AAAGTGCATACCGCCAAAAG) (Eurofins MWG Operons, Germany), and 12 ng DNA. QPCR was performed using a StepOnePlus Real-Time PCR System (Applied Biosystems, UK) with the results viewed using StepOne Software V2.1 (Applied Biosystems, UK). The following conditions were used for the qPCR run: 95°C for 10 minutes; 40 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 55 seconds; and a final stage of 72°C for 7 minutes. A melt curve was added immediately after the reaction with the conditions of: 95°C for 15 seconds; 60°C for 1 minute, followed by a plate read at every 0.3°C temperature increase; ending at 95°C for 15 seconds. The CV for this technique is <1%.

Data Analysis

All data are expressed as mean ± SD and statistical significance was set at $P < 0.05$. Differences in participant group characteristics, training history and dietary intake were analysed with multiple student t-tests. Prior to analysis, ROS production was adjusted for changes in plasma volume using the methods of Dill and Costill (1974). A mixed model ANOVA with 2 independent group levels (BTJ vs. PLA) and 4 repeated measures time points (pre-supplementation, pre-race, post-race and day 1 post) was used to analyse for group differences in ROS production and mtDNA damage. Bonferroni *post hoc* analysis was performed to locate any significant differences. Data were analysed using GraphPad (GraphPad, Prism, CA, US).

Results

There were no differences in physical characteristics, training history and marathon performance between the two groups (see Table 1; $P > 0.05$), and no differences in average dietary intake the day before and on the day of the marathon (supplementary table S1). ROS was unchanged from pre-supplementation to pre-race, irrespective of supplement ($P > 0.05$; ES = 1.96). Immediately after the marathon ROS production increased by 128% (average across groups; time effect; $P < 0.01$; ES = 1.17; Figure 1), but returned to pre-exercise levels the following day (ES = 0.57). At no time point did the level of ROS differ between the BTJ and PLA groups ($P > 0.05$). The marathon did not induce mtDNA damage (pre to post-marathon change; $P > 0.05$, ES = 0.36) and did not differ between BTJ and PLA post-marathon (ES = 0.51) (Figure 2).

Discussion

Systemic ROS production in whole blood samples was increased immediately after the marathon before returning to resting levels the following morning. These findings appear to be consistent with others (Gomez-Cabrera et al., 2006; Nieman et al., 2002), who employed indirect markers to measure ROS generation after long distance running (malondialdehyde and lipid hydroperoxides, F_2- isoprostanes, respectively). The clear advantage of this method for detecting ROS generation over those used in previous studies is that only a very small amount of blood is required from the finger-tip (2 µl). Therefore, it can be useful for estimating ROS in settings outside of the laboratory, when other methods of blood collection (i.e., venepuncture) might be time consuming and impractical. Another advantage of this method is that it gives a direct indication of radical production in the blood, as opposed to an estimation based on (presumably) radical-mediated damage to molecules such as proteins, fats or DNA as

other point of care methods tend to do. Future research testing the agreement of this method with the most current valid and reliable methods of exercise-induced ROS detection is warranted.

Interestingly, although not statistically significant, the ES for an increase in ROS production from pre-supplementation to pre-race was very large. We are not clear why this was the case, but it was evident in both groups, suggesting it was not related to the drinks. One possible explanation is that pre-race nerves imposed a degree of psychological stress sufficient to elevate oxidative stress above baseline levels. Such a possibility should be examined in future work.

To the best of our knowledge, the current study is the first study to measure mtDNA deletion in blood after endurance exercise, and use micro-invasive techniques; nevertheless, akin to the findings of Beiter et al., (2011), who measured mtDNA damage after an exhaustive treadmill run, we did not find any evidence of circulatory mtDNA damage immediately or the morning after a marathon. The exact source of circulating mtDNA after exercise is unclear (Nasi et al., 2016), but release from muscle is probable (Nasi et al., 2016). In this case, it is possible that ROS induced by the marathon was not sufficient to induce a large efflux of mtDNA into the circulation, and perhaps this could explain why we were unable to detect changes from pre to post-exercise.

BTJ was no more effective than a PLA for attenuating the rise in ROS generation or mtDNA damage after a marathon. Our findings are in contrast to the work in animals, which has consistently shown that BTJ, beetroot extracts, or its pigments, the betalains, reduce ROS generation in response to a xenobiotic challenge (Clifford et al., 2015). It is not clear exactly why our findings are inconsistent with others, but differences in species and participants, the type and dose of beetroot administered, oxidant stimulus, and ROS markers used—along with their method of assessment—could all provide plausible explanations. Yet, another possibility is that we were simply underpowered to detect small changes in ROS generation between the BTJ and the PLA groups, given the low number of participants per group (n=7). Indeed, to observe a 10% difference in our primary outcome measure, ROS production, it is estimated that we would need 16 participants per group (at 80% power and α of 0.05). We acknowledge that this is a key limitation of the study and stress that this was only a pilot study and, thus, these findings should be treated as preliminary.

In conclusion, we have shown that measuring ROS production in only 2 µl of blood from a finger-tip holds potential as a minimally invasive, quick, and simple method of detecting ROS generation in applied sport settings outside of the laboratory. In contrast, circulatory mtDNA damage was unaffected by a marathon. Finally, BTJ did not mitigate ROS generation after the marathon and, thus, its benefits as an exogenous antioxidant in humans remain questionable. Further research with larger samples sizes are warranted to clarify these findings.

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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