



# The Effects of Black Mulberry Supplementation on Plasma Interleukin-6 and Tumour Necrosis Factor- $\alpha$ Response to One Session Basketball Training in Female Basketball Players

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### ABSTRACT

**Background and Objectives:** This study aimed to evaluate the impact of mulberry supplementation on two proinflammatory markers response, Rate of Perceived Exertion (RPE) and performance after a session of strenuous exercise. Black Mulberry is a fruit rich in flavonoids and anthocyanins which have been proved to act as antioxidants in body.

Materials and Methods: Fifteen female basketball players participated in Basketball Exercise Simulation Test (BEST). Blood samples were taken in three turns including before BEST, immediately and one hour after to measure Interleukin-6 (IL-6) and Tumour Necrosis Factor (TNF- $\alpha$ ) concentrations. After 3 days of recovery, the same participants consumed 300 mL of pulped Black Mulberry Juice for 10 days and participated again in the same exercise protocol and their blood samples were collected in the same three turns. During both exercise sessions, performance variables were recorded considering the number of goals and RPE. ANOVA and Bonferroni's follow up tests were used for data analysis ( $\alpha$ =0.05).

**Results:** A significant increase in IL-6 concentration was seen immediately and one hour after BEST, before and after supplementation (P<0.05). The comparison of TNF- $\alpha$  concentration in six stages showed no significant difference (P>0.05). Analysis of Borg Test showed significant decrease after supplementation (P<0.05) but no significant results were shown in performance of participants (P<0.66).

**Conclusions:** Black Mulberry supplementation can reduce pro-inflammatory cytokines and PRE after exercise.

Keywords: Black mulberry, Pro-inflammatory, BEST, Performance, RPE

#### Introduction

Nowadays athletes non-steroidal antiuse inflammatory drugs (NSAIDS) to a wide extent in order to reduce the effects of tissue damage and pain caused by intensive physical activity (1), although their effectiveness has yet to be proven. Most consumers have been deterred from using NSAIDs, as they have side effects including upper gastrointestinal upset and renal side effects (2). Alternatives in current use include antioxidant supplements, massage therapy, cryotherapy, stretching, homeopathy, cooling the injured area and many more (3, 4). Among these options, the most regarded, is focused on consuming natural antioxidant sources rather than synthetic supplements, since the latter does not show the same benefits (5). It is believed that antioxidants can reduce damage in affected tissues by neutralizing Oxygen and Nitrogen free radicals (6). This group of supplements contains a vast range of vitamins, polyphenols and others.

Dietary phenolics or polyphenols are plant phytochemicals containing at least one aromatic ring as well as one or more hydroxyl groups that show antioxidant effects and are highly found in fruits and vegetables (7, 8). There are several sub-groups of polyphenols including phenolic acids, flavonoids, polyphenolic amides and others (9). The Black Mulberry (BM) (10) with Iranian origin (11), belongs to Moraceae family and Morus genus (12) which besides polyphenols like resveratrol, rutin and quercetin, contains Vitamin C and organic acids (13). This fruit has been reported to contain the highest phenolic content compared to other species of Morus

genus (14, 15). Previously, antioxidant effects of BM such as salvaging nitric oxide and balancing reactive oxygen species have been reported (16, 17). It is believed that synergistic actions of nutrients in whole foods make them more efficient in suppressing exercise related injuries.

It is well known that during strenuous physical activity, pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$  are released from contracting muscle (18). Mentioned cytokines are released into the blood; the phenomenon is determined by the increase in plasma catecholamines (19). This leads to a feeling of pain and reduction in range of motion throughout the days following exercise (20). IL-6 has both pro and anti-inflammatory effects (21) and increases the most following physical activity (22).

Among all the studies, evaluating the effects of different berry fruits, conflicting results have been shown. For example, in a study on acai berry, there were no advantages in performance of junior hurdlers; however; muscle damage markers decreased after supplementation (23). In other studies, blueberry consumption has been shown to accelerate the recovery of muscles after isometric, concentric and eccentric contractions and reduce oxidative stress caused by exercise when used before and after the exercise session (24) or resulted in an increase in antiinflammatory cytokines like IL-6 and Natural Killers (NK) (25). Nevertheless in another study which compared the effects of blueberry and vitamin C supplementation in participants who ran with 70% of VO<sub>2max</sub> for 44 minutes, blueberry supplementation made no significant changes in the amount of cytokines (26).

In light of current evidence, the question therefore is whether BM supplementation with its unique polyphenol profile is able to reduce exercise induced inflammation. This study aims to investigate the preventive effects of BM supplementation on IL-6, TNF- $\alpha$ , RPE and performance after the BEST.

#### Materials and Methods

**Participants:** Thirty female basketball players were volunteered to take part in this semi-experimental study. After completion of the Physical Activity Readiness Questionnaire (PAR-Q) and performing Body Analysis Tests, sixteen subjects with an average age of 23.73  $\pm 3.75$  years, height of 1.63  $\pm 0.07$  meters, weight of 62.71  $\pm 7.38$  kilograms, BMI of 23.68  $\pm 2.82$  kg/m<sup>2</sup> and Fat percentage of 29.06  $\pm$ 

3.92 who were non-smokers with at least 2 years of professional basketball training, who did not have any endocrine disorders. diabetes. or chronic cardiovascular problems; with a similar range of daily calorie intake, without any exercise induced injury and antioxidant supplement consumption during the last three months were selected for the study. One of the participants did not take part in the second exercise session and was eliminated from the study. All the participants received adequate information about the study protocol and possible positive and negative effects of training. They arbitrarily entered the study and were free to leave the protocol upon their request.

Participants completed a 24 hour recall and a Health History Questionnaire. Subjects were asked not to use any vitamin and antioxidant supplements during the study. Daily intake of macronutrients including carbohydrates (55%), protein (15%) and fat (30%) were recommended.

**Procedure:** Participants were asked to fast for 12-14 hours for the first blood sample collection. Following the blood sampling at 9 am, a breakfast containing approximately 200 calories from white bread and low salt cheese was used by participants. Afterwards, the 87 minute BEST was completed. During the exercise the number of goals was recorded. By the end of each quarter, participants also were asked to report their rate of perceived exertion (RPE), using Borg's 1-10 modified scale (27). Blood samples were collected immediately and one hour after the BEST was performed. The three subsequent days were assumed as a recovery period and subjects started drinking 300 mL of pulped BM juice for the following 10 days. Dosage of BM juice was chosen with reference to one and only study supplementing BM pulped juice to participants (28). We decided on 10 days supplementation since it was hard to keep our selected participants in the study, mostly due to their participation in different basketball Moreover, in the study by Tapiero et al, it is pointed out that the half-life of flavonoids is around 1-24 hours, depending on the type of flavonoid (29). Considering these facts, 10 days supplementation seemed to be enough.

After completion of the supplementation period, a second exercise session was held. Blood samples were taken again in the same three turns to measure pro-inflammatory factors.

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Blood samples were taken from basilic vein. For serum division, samples were collected in tubes containing no anticoagulant agent and incubated for 15 minutes at room temperature and then they were centrifuged with 6000 rpm for 10 minutes. Serum was separated with Sampler 1000. Serum samples were moved to -70 centigrade degree refrigerators in a cool box and remained there until further analysis.

The study was registered by the Iranian Registry of Clinical trials; the allocated unique code is IRCT2015100524358N1. All procedures performed in this study involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

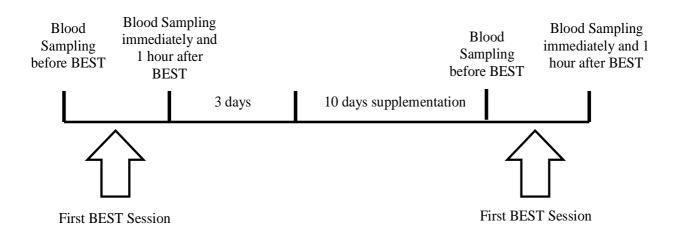
Nutritional Supplement: Fifty kilograms of BM were bought from a single garden in Kan (a village located in North West Tehran) and were immediately transferred to A-One Food Products Factory in Qazvin. Black Mulberries were kept in standard conditions in a refrigerating room during the night, before entering the production line the next day. The process of pasteurizing the juice was considered to increase its shelf time. No preservatives were used. The final product was the pulped BM juice, which

was poured into bottles. Bottles were kept in a refrigerator at +5 centigrade degrees until usage. During intervention, bottles were given to participants and they were asked to keep them in a refrigerator.

Participants were recommended to use the BM juice before and during regular exercise sessions or between meals. Supplement consumption aside dairy products was prohibited. Research process is summarized in figure 1.

Physical activity protocol: Physical activity protocol was based on the Basketball Exercise Simulation Test (BEST) which includes various activities during a basketball game, such as high and low intensity activities, shuffles, free throws and resting periods. This test is held in one half of a basketball court (30). The circuit base design of BEST is shown in the figure 2. Complete exercise protocol is shown in figure 3.

**Biochemical Analysis:** Measurement of TNF- $\alpha$  serum concentration was done by ELISA kits (Diaclone Company, France, Range: 25pg/ml- 800 pg/ml Sensitivity: 8 pg/ml) and IL-6 serum concentration was analysed by high sensitivity ELISA kits (Diaclone Company, France, Range: 1.56 pg/ml-50 pg/ml, Sensitivity < 0.8 pg/ml).



**Figure 1.** Research Process: This study is conducted on two BEST sessions which have been held in a 14 day period. During this period subjects were supplemented with 300 mL pulped Black Mulberry juice.

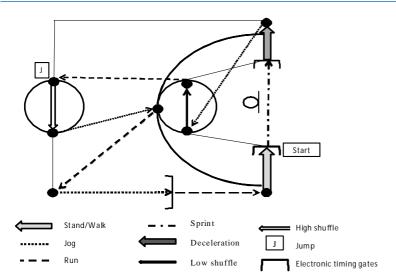


Figure 2. Circuit base design of BEST (45).

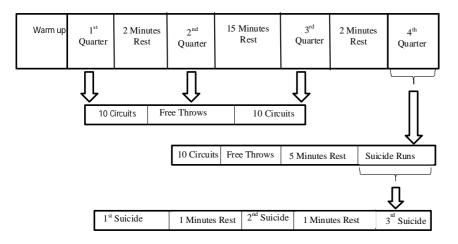


Figure 3. Complete exercise protocol (46).

Black Mulberry Analysis: Folin-Ciocalteu was used as reagent and rutin was our Phenolic Standard Compound. One ml of rutin with different concentrations of 10,20,40 µg/ml was mixed with 5 ml of diluted Folin-Ciocalteu (1 to 10) and then incubated in room temperature. After 10 minutes, 4 ml of sodium carbonate (7.5 mg/ml) was added. The final mixture was incubated at room temperature and away from light for 30 minutes. Finally, each rutin sample was measured in 765 nm wavelengths. This experiment was repeated for each rutin composition three times and then according to achieved absorption data, rutin standard calibration curve and a calibration curve of absorbance vs. concentration linear curve was plotted. This method was also used for BM fruit, 5 gr of final product used for supplementation was dissolved in water, filtrated and its volume was increased up to 100 mL. the concentration was 50 mg/l. The only difference was that instead of 1 ml of rutin, 1 ml of BM extract with concentration of 1 mg/ml was used and total phenolic content was calculated using rutin standard curve. The results are shown in table 1.

**Table 1.** Polyphenol content of BM juice consumed by participants.

Extract	Concentration (mg/ml)	Total phenolic/rutin (µg/ml)	% Total phenol
BM fruit	1	18.8	1.88

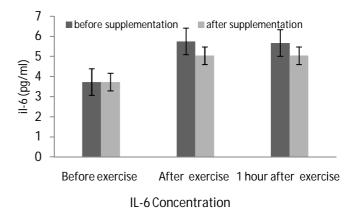
Statistical Analysis: The data regarding IL-6 and TNF $\alpha$  concentration was analysed using arithmetic mean and standard deviation. Since Smirnov-Kolmogorov showed no significant results, analysis

of variance with repeated measurements and Bonferroni's post-hoc tests were performed ( $\alpha$ =0.05). We used One-Sample repeated measures ANOVA test to examine the significance of results for goals and RPE showing the changes in performance during exercise and also dependent samples *t*-test to assess supplementation effects ( $\alpha$ =0.05). For analysing data SPSS version 22 was used. Results

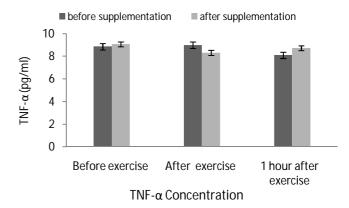
**Pro-inflammatory cytokines:** Data analysis suggests that BEST effectively developed inflammation before and after supplementation which is shown by the significant increase in IL-6 immediately and one hour after the BEST. Although no significant changes were indicated in TNF- $\alpha$  plasma concentration, its changes were descending after supplementation.

Figures 4, 5 and Table 2 show the changes for IL-6 and TNF- $\alpha$  in six steps:

**RPE and Performance:** T-Test shows that mean  $\pm$  SD of RPE after supplementation (4.11  $\pm$  1.30) was significantly lower than before supplementation (5.73  $\pm$  1.39) (P<0.0001, df= 14, t= 5.44). No significances were shown in the number of goals when comparing before (2.60  $\pm$  0.92) and after (2.48  $\pm$  0.88) results (P<0.066, df= 14, t. 0.44).



**Figure 4.** IL-6 trend. This figure shows the changes in IL-6 before, immediately and after BEST before and after supplementation.



**Figure 5.** TNF- $\alpha$  trend. This figure shows the changes in TNF- $\alpha$  before, immediately and after BEST before and after supplementation.

**Table 2.** Descriptive statistics and summary of analysis of variance with repeated measurements results for IL-6 and TNF- $\alpha$  in six steps.

	Before BEST	Immediately After BEST	1 hour after BEST	
	IL-6 (pg/ml) F $_{5,70} = 13.750$ , Sig = 0.001, Eta = 0.495			
Before Supplementation	$3.73 \pm 0.84$	$5.75 \pm 1.72*$ P = 0.001	$5.67 \pm 1.53*$ P = 0.001	
After Supplementation	$3.73 \pm 0.78$	$5.04 \pm 1.21$ † $P = 0.023$	$4.93 \pm 1.01 \dagger$ P = 0.007	
		TNF-α (pg/ml) F <sub>2.92, 40.84</sub> = 1.658, Sig = 0.192, Eta =	: 0.106	
Before Supplementation	$8.86 \pm 1.98$	$8.99 \pm 1.79$ P > 0.05	$8.10 \pm 2.17$ P > 0.05	
After Supplementation	$9.06 \pm 1.98$	$8.32 \pm 2.06$ P > 0.05	$8.73 \pm 1.88$ P > 0.05	

Notes: \*Significant difference in comparison with before activity and before supplementation (P<0.05). †Significant difference in comparison with before activity and after supplementation (P<0.05).

#### Discussion

The BEST resulted in a significant increase in IL-6 immediately after exercise which remained high for an hour following physical activity. Supplementation slowed down the increase of IL-6 caused by physical activity. TNF-α trends before and after supplementation were upward and downward respectively. RPE scores were significantly lower after supplementation. The number of goals (our indicator for performance) did not change considerably.

In previous scientific studies eccentric exercises (24), marathon runs (31) and High Intensity Interval Training (HIIT) (32) increased plasma IL-6 levels. In our study, BEST could increase IL-6 as well. IL-6 response to physical activity depends on the intensity and duration of exercise; however type of exercise has little effect on its concentrations. Moreover, decrease in glucose levels and increase of adrenaline leads to IL-6 secretion from muscle tissue which in turn increases its plasma levels (33).

Current results indicate that supplementation with BM pulped juice could reduce the rate of IL-6 increase immediately and one hour after the BEST similar to the study by McAnulty et al., suggesting that IL-6 decreased in participants who consumed 250 grams of blueberry for 6 weeks and 375 grams prior to exercise, but these changes were not statistically significant (25).

The theory behind these findings maybe that, berry polyphenols, namely quercetin and resveratrol, are capable of improving mitochondrial biogenesis, which activates the Sirtuin-1 pathway (34). This pathway decreases glucose consumption, resulting in fatty acids being used as fuel. Since IL-6 concentrations are directly associated with glycogen stores, BM polyphenols probably inhibits IL-6 growth (35). However, none of the human and clinical studies proved these mechanisms.

Contradictory results have been achieved in studies which examined the effects of exercise on TNF-α response. To illustrate, Bernecker et al. showed significant increases of IL-6 and TNF-α in male marathon runners (36). Konrad et al. also reported increase in many cytokines including TNF-α and IL-6 following 2 hours running on a treadmill with 70% of  $VO_{2 \text{ max}}$  (10). While, in a study done by Ghorbani et al. no significant increase in TNF-α was observed after a HIIT exercise protocol. Ghorbani et al.

assigned different TNF-α responses to exercise protocol, its intensity, duration and physical fitness (37).

It is well demonstrated that TNF-α is produced by macrophages. This contributes to the inflammatory response of muscles to damage, proteolysis and impairment of glucose uptake (18). In the onset of secretion, TNF-α leads to increases in other proinflammatory cytokine secretions like IL-6. IL-6 down-regulates further TNF-α secretion (38) which is one of the reasons why TNF-α does not increase immediately after physical activity.

TNF-α showed an upward trend supplementation while the opposite happened after supplementation; however, none of the above changes were significant.

It has been pointed out that berry anthocyanins anti-inflammatory effects different pathways including inhibition of NF-κB pathway and activator protein activation which in part results in a reduction of pro-inflammatory cytokines (39, 40). In the current study, BM could not completely prevent IL-6 and TNF-α release. These differences in results might be due to different polyphenolic compounds in different berry fruits.

In this study we assessed RPE scores reported by participants and rate of their goals before and after supplementation. The results showed that BM supplementation could reduce the RPE scores considerably. The results are inconsistent with the results of a study by Kim Labonte et al. In this study, no significant results were showed in athletes who drank 600 mg of cranberry and grape seed polyphenols right before they started their time trial on an ergo cycle (41). In another study acai beverage was displayed to have positive effects on RPE (42). In these studies results are attributed to the impact of polyphenols on vascular function. Polyphenols have been shown to have vaso-dilating effects which consequently improves aerobic capacity and time to exhaustion.

Contradictory results from current evidence may be due to high rates of oxidative stress and inflammation caused by intensive physical activity which eliminates the effect of supplements to some extent. Therefore, Food polyphenol consumption may only reduce oxidative stress and inflammation caused by chronic diseases (43). Furthermore, some studies express that

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plasma concentrations of berry anthocyanins are low and they reach a peak after 0.75-4 hours of consumption (44). Therefore; the BM pulped juice may bring about different results if used immediately before exercise.

In conclusion, BM supplementation is beneficial for athletes, since it is rich in resveratrol, quercetin, organic acids etc. and may possibly be able to reduce inflammatory reactions caused by intensive physical activity.

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