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Original article

CYP1A2 polymorphism and caffeine ingestion in relation to apoptosis markers after a resistance exercise in trained men: a randomized, double-blind, placebo-controlled, crossover study

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Summary. The objective of this study was to determine the effects of caffeine ingestion and CYP1A2 polymorphism on Bax and Bcl-2 levels, as apoptosis markers, in acute resistance exercise (RE). In a randomized, double-blind, placebo-controlled, crossover study, 15 trained men completed acute RE at 85% of 1RM. The subjects ingested either caffeine (CAF, 6 mg.kg^{-1} body mass) or placebo (PLA) 1 h prior to the exercise. Blood samples were taken pre-exercise (PRE), immediately post (POST), and 15 min (15 min-POST) post RE for measuring the serum concentrations of Bax and Bcl-2 biomarkers. The *CYP1A2* -163C>A polymorphisms were analyzed by amplification refractory mutation system–polymerase chain reaction (ARMS–PCR) in genomic DNA samples which were isolated from the whole blood samples. The subjects were classified as either AA (n= 8) or AC/CC genotypes (n=7). At POST, Bax concentrations were significantly higher in PLA AC than PRE (p=0.014), CAF AA (p=0.003), CAF AC/CC (p=0.039), and PLA AA (p=0.034). No significant changes were observed in Bcl-2 levels at POST compared to PRE in both groups of CAF or PLA (p>0.05). However, Bcl-2 levels in 15 min POST were significantly higher in CAF AA than in CAF AC/CC (p=0.003). Changes in the percentage of Bax/Bcl-2 ratio were significantly higher in PLA AC/CC at POST when compared with CAF AA (p=0.002) or CAF AC/CC (p=0.007), and were significantly lower in CAF AA at 15min POST than in PLA AA (p=0.039). The findings suggested that exercise alone could accelerate apoptosis in the AC/CC group, while caffeine appears to attenuate susceptibility of cells to apoptosis.

Key Words: Caffeine, strength exercise, programed cell death, *CYP1A2* polymorphism

Introduction

A single bout of vigorous exercise as a form of cellular stress is known to induce immune dysfunction and apoptosis (programed cell death) in a variety of tissues (1). Vigorous exercise may influence circulation hormones, cytokines, reactive oxygen species (ROS), intracellular signaling, and transcription factors, all of which can influence apoptotic signaling (2). Depending on the type of stimulus, the intrinsic (formation of mitochondrial pores) and extrinsic (death receptors-dependent pathway) pathways can be involved in the apoptotic cascades. The mitochondrial-mediated pathway, also called the Bcl-2-regulated, is believed to be critical in regulating apoptosis (3,4). The mitochondrial pathway involves mitochondrial outer membrane permeabilization following internal stimuli such as irreparable genetic damage, growth-factor deprivation,

hypoxia, and high cytosolic Ca^{2+} or ROS. It appears that the Bax protein is crucial for inducing permeabilization of the outer mitochondrial membrane and the subsequent release of apoptogenic molecules (such as cytochrome *c*), leading caspases' effector activation (i.e., caspase-3, caspase-6, and caspase-7). Conversely, the Bcl-2 protein regulate apoptosis by blocking the mitochondrial release of cytochrome *c* (5). Additionally, Bcl-2 and Bax regulate Ca^{2+} homeostasis in the endoplasmic reticulum–sarcoplasmic reticulum (6). Increased Bax/Bcl-2 ratio upregulates caspase-3 and enhances apoptosis in cells (7). Therefore, the balance between the Bax and Bcl-2 determines initiation of apoptosis.

Among the natural compounds consumed on a daily basis, creatine and caffeine are largely consumed by athletes mainly due to their positive effects on fatigue resistance and improved exercise performance (8). Creatine supplementation protocols have been shown to decrease ROS and oxidative damage, (9) alleviate inflammatory biomarkers (10), and attenuate apoptosis in acute exercise (11,12). On the other hand, caffeine (1,3,7-trimethyxanthine) is considered an ergogenic compound due to its antioxidant and anti-inflammatory role (8). However, the effects of caffeine on apoptosis markers after acute exercise have not been fully elucidated. In one trial, caffeine reduced the expression level of Bcl-2 while leaving the expression of Bax intact (13). Nonetheless, caffeine did not alter the Bax/Bcl2 expression and so it did not induce apoptosis (14). These conflicting results may be related, in part, to inter-individual differences in caffeine metabolism or caffeine response.

Indeed, some studies have indicated that the rate of caffeine metabolism could also have implications for sports performance (15). Individuals are divided into fast and slow metabolizers of caffeine based on their genotypes (16). Several enzymes are involved in the metabolism of caffeine, but over 95% of caffeine is metabolized by the P450 1A2 (CYP1A2) enzyme, which is encoded by the *CYP1A2* gene (17). The polymorphism of the *CYP1A2* gene has been shown to affect the levels of enzymatic activity and has been used to categorize individuals as fast or slow metabolizers of caffeine (17). In theory, rs762551 C allele carriers metabolize CAF more slowly than those with the AA genotype do (18). People with the AC or CC genotype (slow metabolizers) who increasingly consume caffeinated coffee are at a higher risk of diseases such as myocardial infarction, hypertension and pre-diabetes, while those with the AA genotype show no such a risk (17,16). Some studies investigated the effects of this polymorphism on exercise performance with caffeine. Rahimi (2018) recently reported that acute ingestion of caffeine enhanced resistance exercise performance in resistance-trained men who were homozygous for the A allele, but not for C allele carriers (15). In addition, caffeine consumption reduced 40-km time trial to a greater extent in AA homozygotes compared to the C allele carriers in the trained male cyclists (19). However, to the best of our knowledge, no study has yet investigated how caffeine consumption could affect circulation apoptosis markers after resistance exercise in relation to the genetic polymorphism. Therefore, the principal purpose of this study was to determine the effects of caffeine supplementation on apoptosis markers during a resistance exercise session. A secondary goal was to study whether the possible relationship between consumption of caffeine and apoptosis markers in the resistance exercise is influenced by genetic polymorphism in the *CYP1A2* gene.

Material and Methods

Participants

According to entry criteria, 15 eligible volunteers were selected using simple random sampling method. The physical characteristics of the participants are re-

Table 1. Physiological characteristics of the subjects in homozygote AA (n= 8) and C allele (n=7) carriers (CA/CC)

Genotype group	AA (n=8)	AC/CC (n=7)	P value
	mean ± SD		
Age (y)	20.8±2.2	20.3±2.6	0.68
Weight (kg)	74±7.9	71.2±11.2	0.78
Height (cm)	178.5±4.8	179.1±3.9	0.59
Soft lean mass (kg)	62.3±5.5	57.9±7	0.21
Body fat mass (kg)	8.3±3.6	10.1±4.3	0.41
Body fat (%)	10.9±4	13.7±3.9	0.22
Body mass index (kg/m^2)	23.2±2.1	22.2±4	0.58
Basal metabolic rate	2095.2±185.4	1983.1±177	0.27

ported in Table 1. Inclusion criteria comprised age, gender, and regular exercise training for at least 1 year. Exclusion criteria included use of androgens or nutritional supplements within the previous 6 months, smoking, use of drugs or supplements containing heavy caffeine (≥70 mg day–1) in the previous 2 weeks, and any diseases or absolute contraindications to acute exercise. The participants were fully informed of the procedures and possible risks of the investigation, and gave their informed written consent for participation. The study was approved by the Department of Exercise Physiology at University of Kurdistan and carried out in agreement with the latest version of the Declaration of Helsinki (2013).

Experimental design

A double-blind, placebo (PLA)-controlled, crossover design and randomized experimental procedure was used in this study. The participants were randomly assigned to CAF (C0750 Sigma-Aldrich, Germany) or PLA treatments using an online research randomizer (https://www.randomizer. org/). Two sessions were used to familiarize them with the study one week prior to the study. During these sessions, following a standardized warm-up exercises, including 5 min of jogging, static stretches, and joint mobilization exercises, and stretching exercise for 5 min, one repetition maximum (1RM) in bench press, leg press, seated cable row, and shoulder press was determined.

During the experimental sessions, separated by a 3-day washout period (20), participants consumed gelatin capsules containing 6 $mg.kg^{-1}$ body mass of caffeine (CA, C0750 Sigma-Aldrich, Germany) or Maltodextrin as a placebo (6 mg.kg–1 body mass) with 250 mL of water. After 60 min (21), the participants performed acute resistance exercise protocol with a standardized warm-up. The participants completed three sets in the order of bench press, leg press, seated cable row, and shoulder press up to failure (unable to complete repetition with the proper technique) at 85% of 1RM with an interval of 60s between sets and 2 min between the exercises for both the CAF and PLA conditions. The participants were verbally encouraged to perform all sets to exhaustion. Exercise trials were conducted in the early afternoon to avoid circadian variance (22).

Genotyping

During the first familiarization session, a venous blood sample was collected using antecubital venipuncture for genotyping analysis. The human genomic DNA was extracted from 2 mL of peripheral blood, using the TIANamp Genomic DNA Kit (Cat. No. DP304). The single-nucleotide polymorphism (SNP) in the intron 1 of the human CYP1A2 gene (rs 762551) was analyzed by amplifying the refractory mutation system–polymerase chain reaction (ARMS–PCR) (23), wherein the SNP-163A>C was amplified using allele A primer (forward): 5′-CAAAGGGTGAGCTCTGTGGACA-3′, Allele C primer (forward): 5′-CAAAGGGTGAGCTCTGTGGTCC-3′, and reverse primer: 5′- GAGGCGATGGAGAAGGTGTTGA-3′ (Macrogen Korea). The PCR reactions were performed in a 25μL volume containing 3μLDNA, 1μLof each primer, and 18μL of PCR Master Mix (Thermo Fisher Scientific).

The PCR condition consisted of an initial denaturation at 94 °C for 10 min, 32 cycles of 94 °C for 1 min, 65 °C for 1 min, and 72 °C for 1 min, followed by 72 °C for 5 min as a final elongation step (Thermal Cycler, Analytik Jena, Germany). The PCR products were analyzed by 2% agarose gel electrophoresis and staining with ethidium bromide. For each sample, two reactions were studied for both alleles of A and C. The presence of a 636-bp fragment in both reactions identified the A/C genotype, while the presence of a 636-bp fragment in alleles C and A reaction identified the C/C and A/A genotypes, respectively. The internal control was used in all samples to verify the reaction, in which two primers of mitochondrial genome—namely L strand: 5′-CTCC ACCATTAGCACCCAAAGC-3′ and H strand: 5′-CCTA TTTGTTTATGGGGTGATG-3′—were used to produce a 250-bp fragment (15). All samples were run in duplicate with two negative controls.

During the exercise trials, blood samples (10 mL) were obtained via venipuncture at pre-exercise (PRE), immediately post (POST), and 15 min post exercise (15 min-POST) for determination of serum Bax and Bcl-2 levels, with regards to a potential drop in blood apoptosis marker levels after acute exercise (24,25). The tubes were kept in the dark and refrigerated on ice until the end of the test. The blood was processed

and centrifuged at 3,000 rpm for 20 min. The Bax and Bcl-2 serum levels were determined using commercial kits by sandwich ELISA (Human ELISA, Bioassay Technology laboratory, China) according to the manufacturer's protocol. The serum Bax sensitivity was 0.15 ng/ml with the inter- and intra-assay variability being <10% and <8%, respectively (26). The serum Bcl-2 sensitivity was 1.15 U/ml and the inter- and intra-assay variability was <10% and <8%, respectively (26).

Statistical analysis

As the AC and CC genotypes have similar effects on caffeine metabolism, all C allele carriers were grouped together for the statistical analysis (19). Data were presented as means, standard deviations, and percentage changes. The normality of distribution was calculated by the Shapiro–Wilk test. The homogeneity of variance was calculated by the Levene test. For all statistical analyses, significance was accepted at $p < 0.05$. Differences between the AA genotype and AC/CC genotype in the physical characteristics (age, height, weight, soft lean mass, body fat mass, percent body fat, body mass index, and basal metabolic rate) were examined using unpaired *t* test. Genotype (AA, AC/CC), conditions (CAF, PLA), and time (PRE, POST, 15 min-POST) comparisons were analyzed using the two-way ANOVA with repeated measures and post-hoc *Tukey* test. Statistical significance was accepted for values of $p < 0.05$. Data were analyzed using the IBM SPSS software for Windows, version 22.0.

Results

Eight (53%) subjects were homozygous for the A allele and seven (46%) were C allele carriers. No differences were found in physical characteristics between genotypes ($p>0.05$). Based on one-way ANOVA, circulating levels of Bax did not differ significantly between genotypes and conditions at pre-exercise (CAF AA, 4.95±0.82; CAF AC/CC 5.48±0.55, PLA AA, 5.12±0.74; PLA AC/CC 5.14±1.25 $ng.mL^{-1}$; $p=0.532$). At POST, Bax concentrations were significantly higher in PLA AC/CC ($p=0.014$; Fig. 1) compared to PRE. In addition, Bax in PLA AC/CC was significantly higher than in CAF AA, $p=0.003$; CAF AC/CC, $p=0.039$, and PLA AA, $p=0.034$ at POST. No significant main effect for caffeine condition (CAF AA, CAF AC/CC) was found ($p=0.656$), indicating that Bax levels were not impacted by the exercise in the caffeine condition.

Figure 2 illustrates responses of Bcl-2 levels to resistance exercise after CAF or PLA supplementation in the AA and AC/CC genotypes. No significant changes were observed in Bcl-2 levels after-exercise compared to pre-exercise in both groups of CAF or PLA ($p > 0.05$). However, Bcl-2 concentrations in 15 min POST of CAF were significantly different between the AA and AC/CC genotypes (92.23±14.86 *vs* 75.32±18.49, $p=0.003$).

Changes in the percentage of Bax/Bcl-2 ratio were significantly higher in PLA AC/CC at POST when compared with CAF AA ($p=0.002$) or CAF AC/

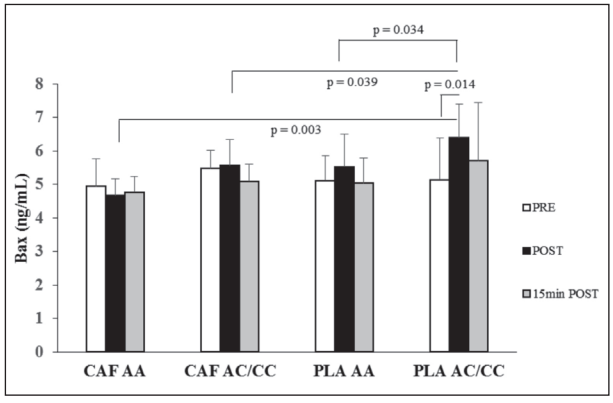

Figure 1. Responses of Bax level to a bout of resistance exercise after CAF or PLA supplementation in the AA and AC/CC genotypes ($p<0.05$).

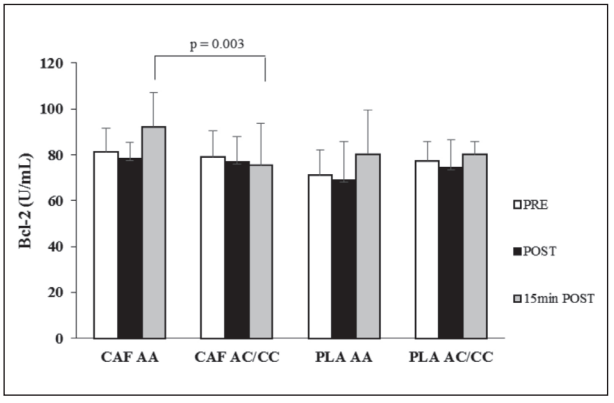

Figure 2. Responses of Bcl-2 level to a bout of resistance exercise after CAF or PLA supplementation in the AA and AC/CC genotypes ($p<0.05$).

CC (p=0.007), and significantly lower in CAF AA at 15 min POST than in PLA AA (p=0.039; Figure 3).

Discussion

In this study, we focused on the interaction between acute exercise, caffeine supplementation, genetic variations, and cell apoptosis. To date, research within these areas has been limited to using caffeine supplementation and exercise performance, suggesting that no study to date has examined the interaction between caffeine supplementation and cell apoptosis in regard to the genetic polymorphism of the individuals. The main findings were: 1) resistance exercise performed at 85% 1RM evoked apoptosis, which was confirmed by increased serum Bax in the PLA AC/CC group; 2) caffeine supplementation inhibited increases in Bax levels unaffected by genetic polymorphism; and 3) elevated changes in the percentage of Bax/Bcl-2 ratio were significantly greater in PLA AC/CC than in CAF group.

Excessive exercise is a physiological stimulus which may lead to disturbance of the entire body homeostasis, including apoptosis not only in working skeletal muscles but also in many distant organs (2,27). Some studies have investigated circulating apoptotic biomarkers from the blood in relation to acute eccentric exercise (28,11,12,24,29,25). In humans, resistance or eccentric exercise has also been shown to elevate Bax protein levels (30-32,26). Our results

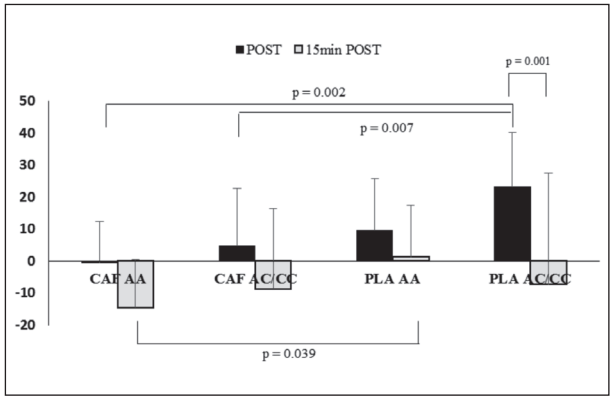

Figure 3. Change percent of Bax/Bcl-2 ratio to a bout of resistance exercise after CAF or PLA supplementation in the AA and AC/CC genotypes ($p<0.05$).

agree with these findings revealing that acute exercise evoked apoptosis, which was confirmed by increased serum Bax in the PLA AC/CC group. In theory, Bax is a transcriptional target for the p53, which induces apoptosis in response to DNA damage. Furthermore, ROS induces a rapid rise in Bax production (2). Bax protein dramatically induces release of cytochrome *c* into the cytosol and accelerates apoptosis (33).

While PLA AC/CC group had a significantly higher Bax serum compared to pre-exercise levels, there was no significant growth in Bax serum levels in the PLA AA subjects. In one study, circulating levels of caspase-3, -9, and p53 did not change following acute resistance exercise at 80% of 1RM in resistance-trained men (24). CYP1A2 metabolizes arachidonic acid to epoxyeicosatrienoic acids, which have anti-apoptotic functions (34). CYP1A2 in individuals with the A allele (AA genotype) has a higher enzymatic activity compared to that found in individuals who are homozygous CC (18). Therefore, diminished CYP1A2 activity in individuals with the AC/CC genotype may contribute to a decline in epoxyeicosatrienoic acids production. Furthermore, the adverse effects of ROS produced by CYP1A2, which would occur to a greater extent among those with the genotype AC/CC corresponding to AA genotype (35), may be another plausible mechanism. Further studies are clearly required to expand our understanding about possible mechanisms of interactions regarding CYP1A2 genotype and exercise induced-apoptosis. Collectively, our findings suggest evidence of genotype differences in apoptotic Bax marker in response to a bout of resistance exercise.

We observed that the Bax levels were significantly suppressed by oral caffeine supplementation immediately after resistance exercise similarly in both CAF groups. Although caffeine may stimulate the muscular apoptosis dose-dependently (36), we found no significant effect of genotype on attenuation of apoptosis after caffeine intake in combination with resistance exercise. There is still no clear understanding of the mechanisms by which caffeine exerts its effects on the Bax; however, it may be related to the suppression of p53 induced by caffeine (37). Furthermore, most studies have found a preventive effect of caffeine on ROS during a single bout of exercise (38,39). Altogether, these results suggest that caffeine may attenuate apoptosis

by inhibiting Bax in resistance exercise. Caffeine has a double-edged sword effect on apoptosis. Indeed, caffeine could induce a dose-dependent apoptosis in Bax-overexpressing normal cells (40). Caffeine may also stimulate the muscular apoptosis dose-dependently: low-dose caffeine intake prevents apoptosis, while high-dose caffeine intake stimulates it (36). We did not measure the different doses of caffeine on BAX, though our results revealed that low caffeine dose suppresses Bax after resistance exercise.

As mentioned earlier, intrinsic apoptotic pathway is regulated by anti-apoptotic Bcl-2 protein which regulates the integrity of mitochondrial membrane. When Bcl-2 is translocated to the outer membrane of the mitochondria, Bcl-2 is capable of forming a heterodimer by binding to Bax (41). The heterodimer formation may inhibit apoptosis by blocking the mitochondria pores, thereby preventing the release of cytochrome *c*, caspase-9, and caspase-3 (42,43). To date, there has been no data on the effects of genotype on anti-apoptotic markers at acute exercise. Acute caffeine administration caused a transient fall of anti-apoptotic Bcl-2 expression in the skeletal muscle (44,13). On the other hand, Bcl-2 after caffeine treatment did not change in the heart (14). However, our results demonstrated that the concentrations of the Bcl-2 after caffeine intake and exercise (15min POST) were significantly higher in resistance-trained AA genotype compared to the C allele ($p=0.003$), which may provide increased protection against apoptosis. It is unclear how enhanced CYP1A2 activity contributes to an increased anti-apoptotic marker after caffeine intake and resistance exercise. Note that reduced CYP1A2 activity (AC/CC genotypes) contributes to an increased risk of prostate cancer (45). The possible mechanisms behind these relationships is still unknown and further research is needed to understand how CYP1A2 polymorphisms metabolically influence anti-apoptotic proteins.

As indicated in previous studies, Bax/Bcl-2 ratio can act as a rheostat which determines cell susceptibility to apoptosis (41,46). Lower levels of this ratio may lead to resistance of human cells to apoptosis (41). We found that changes in the percentage of Bax/Bcl-2 ratio were higher in the PLA AC/CC than in other conditions at POST. Also, CAF AA had a lower percent change of Bax/Bcl-2 ratio compared to PLA AA at 15 min POST. Notably, the rise in the ratio of Bax/Bcl-2 matched the mechanism of apoptosis, as the process of apoptosis in the intrinsic pathway occurs because of the expression of Bax being higher than Bcl-2 (43). We posit that this finding indicates that individuals with the AA genotype have increased chance of cell survival after acute exercise with caffeine intake, compared with AC/CC genotype in trained men.

Some limitations need to be considered when interpreting the results of the current study. The analysis was limited by a relatively small sample size. We studied the impact of 6 mg/kg caffeine on the apoptosis markers. It is known that caffeine doses might influence apoptosis responses in certain situations (47,36). In addition, we examined only two of the several apoptosis markers. Therefore, further research is required to assess the effects of various other doses of caffeine on other systemic markers of apoptosis such as cytochrome *c,* p53, and caspases in the acute resistance exercise program.

In summary, we performed a study to determine the effects of caffeine supplementation on apoptosis markers during a resistance exercise session and the relationships between caffeine intake and specific polymorphisms in the CYP1A2 gene to resistance exercise in resistance-trained men. Based on our analysis, we found that acute exercise elevated Bax level in the PLA AC/CC group. Caffeine intake was associated with attenuated Bax after acute exercise, unaffected by genetic polymorphism. The concentration of the Bcl-2 after caffeine intake and exercise, at 15min POST, was significantly higher in the CAF AA compared to the CAF AC/CC group. In addition, we found that elevated percent changes of Bax/Bcl-2 ratio were significantly greater in PLA AC/CC than in CAF group. These findings suggest that caffeine appears to attenuate the susceptibility of cells to apoptosis, while exercise alone could accelerate apoptosis in the AC/CC group.

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