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A lightly roasted coffee extract improves blood and tissue redox status in rats through enhancement of GSH biosynthesis

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ABSTRACT

Coffee is a highly consumed beverage with many putative beneficial health effects, however these often come from observational studies. In the current work, a lightly roasted coffee extract that has previously been reported to exhibit potent antioxidant properties was administered for two weeks in rats to examine the potential improvement of blood and tissue redox status. The dose was equivalent to a moderate human daily consumption. According to our results, coffee exerted beneficial effects in all tissues mainly by increasing reduced glutathione (GSH) levels. Interestingly, the brain was the most significantly affected tissue, while the gastrointestinal tract, the main metabolic organs and the quadriceps were also benefited. In addition, protein and lipid oxidation was reduced in several tissues. The observed increase in GSH was attributed to increased levels of the rate-limiting enzyme in its biosynthesis pathway, namely γ-glutamylcysteine ligase both in the protein and gene levels. Overall, moderate coffee consumption showed beneficial short term effects in rat tissues by stimulating parts of the endogenous antioxidant mechanisms.

1. Introduction

Coffee is one of the most highly consumed beverages throughout the world. Apart from its pleasant taste, aroma and stimulating effect, coffee contains a variety of bioactive compounds including caffeine and polyphenols (Martini et al., 2016). Polyphenols and especially chlorogenic acid isomers (CGAs) -the most abundant constituents-have gained scientific interest lately because the major beneficial health effects observed after coffee consumption have been attributed to them (Liang and Kitts, 2015; Poole et al., 2017; Sirota et al., 2013).

The amount and type of ingested compounds depend on coffee blend variety, the roasting process, the brewing method as well as the serving size (Vitaglione et al., 2012). For instance, a cup of coffee may contain 15–325 mg CGAs (Richelle et al., 2001). Interestingly, CGA absorption from the gastrointestinal tract amounts up to 30%, highlighting the importance of coffee as a source of dietary antioxidants (Farah et al., 2008). In general, coffee polyphenolic content has been associated with antioxidant activity as shown in previous *in vitro* studies, in which the currently tested lightly roasted extract displayed distinctive antioxidant properties (Priftis et al., 2018b, 2015). However, studies regarding the effects of coffee on redox status *in vivo* are often either observational, or lack in-depth analysis in order to provide an overall picture of the interaction between coffee and tissues (Panchal et al., 2012; Tajik et al., 2017).

Plant polyphenols are considered as potential dietary antioxidant supplements due to their well-studied favourable action against oxidative stimuli (Pandey and Rizvi, 2009), which lead to the formation of potentially harmful reactive species (Weidinger and Kozlov, 2015; Winterbourn, 2015). All aerobic organisms have developed numerous diverse antioxidant mechanisms against oxidative stress, including enzymes such as superoxide dismutase (SOD1) and catalase (CAT), as well as non-enzymatic compounds like reduced glutathione (GSH) and uric acid (Sies, 1993; Sies et al., 2017). Nevertheless, apart from endogenous antioxidants, dietary antioxidants may also act protectively against reactive species with polyphenols being the most abundant compounds (Fang et al., 2002; Landete, 2013, 2012). Polyphenols are intriguing biological entities since they demonstrate a dual mode of action (Castañeda-Arriaga et al., 2018). Interestingly, they exert antioxidant

Abbreviations: CGAs, Chlorogenic acids; GSH, Reduced glutathione; SOD1, Superoxide dismutase; CAT, Catalase; Nrf2, nuclear factor (erythroid-derived 2)-like 2; CQA, monocaffeoylquinic acid; TAC, Total Antioxidant Capacity; TBARS, Thiobarbituric Reactive Substances; CARB, protein carbonyls; RBCL, Red Blood Cell Lysate; ROS, Reactive Oxygen Species; TPC, Total Polyphenolic Content

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action at low concentrations, whereas above a threshold (that is yet unknown and different for every polyphenolic compound or mixture) they act as prooxidants (Bouayed and Bohn, 2010). Regarding coffee extracts, previous studies of our group have shown that they potentially stimulate endogenous mechanisms like the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) pathway (Priftis et al., 2018a, 2017).

Following a series of *in vitro* screening studies (Priftis et al., 2018a, 2018c; 2018b, 2017, 2015), the most potent coffee extract was selected for the current *in vivo* experiment to shed light on its effect as well as the mechanism of action in each tissue. Whether *in vitro* findings correspond to *in vivo* is an active field of research of particular interest to our research group (Veskoukis et al., 2012). To this end, in the current study a slightly roasted *Coffea arabica* extract was administered for 14 days to Wistar rats at a dose corresponding to 2 filtered coffee or 3 double espresso cups per day. Sequentially, blood and eleven tissues were collected and analyzed to allow a wide range screening regarding the effect of coffee consumption on rat redox status.

2. Materials and methods

2.1. Preparation of the coffee extract

The administered coffee extract was prepared as a 10% w/v solution in dH_2O as previously described (Priftis et al., 2015). Briefly, coffee beans were grounded using mortar and pestle, dH_2O was added up to 10% w/v and the solution was stirred for 20 min. After centrifugation (7000 × *g*, 10 min, 10 °C), the supernatant was stored in 200 μl aliquots at −80 °C. Its composition, which has been previously analyzed is as follows: 3.97 mg/g of caffeine and 30.23 mg/g of monocaffeoylquinic acid (CQAs) isomers (3-, 4- and 5-chlorogenic acid isomers) (Priftis et al., 2018c), while its total polyphenolic content equals to 42.55 mg/g (Priftis et al., 2015). In addition, apart from the three main CQAs it is rich in various chlorogenic acid isomers such as feruloylquinic acids, dicaffeoylquinic acids, caffeoyl-feruloylquinic acids and atractyloside (Priftis et al., 2018b).

2.2. Animals

Twelve male Wistar rats (3 months old, 192.1 ± 13.5 g) were housed in cages individually in the animal facility room with a 12 h light/dark cycle, controlled temperature (20–22 °C) and humidity (50–70%). The experiment was performed in the Veterinary Medicine School of Aristotle University of Thessaloniki in accordance to the Helsinki Declaration and National standards (Permission code EL54BIO10). The experimental protocol was approved by the National Veterinary Administration authorities [License No.: 220070(1331)]. All animals were treated in accordance with the guiding principles of the European Community Council Directive (89/609/EEC) for the care and use of laboratory animals.

2.3. Experimental design

Animals were randomly divided into 2 groups (control and experimental, 6 rats each) as follows: the rats of the control group were fed with standard commercial diet (containing corn, soybean meal, barley, bran, milk paste, molasses) purchased from Viozois S.A. (Ioannina, Greece) and the rats of the experimental group were fed with standard commercial diet plus the coffee extract (7.5 ml/kg body weight/day corresponding to 750 mg coffee/kg body weight/day) dissolved in their drinking water for 14 consecutive days. The condition and health of the animals were observed daily and their body weight was measured at days 1 and 14. Twenty four hours after the last administration the rats were anaesthetized with isoflurane (IsoFlo®, Abbot) and blood samples were drawn by cardiac puncture. Then, the stomach, the small and the large intestine, the liver, the pancreas, the spleen, the kidney, the lung, the heart, the quadriceps muscle and the brain were excised, snapped frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.4. Blood and tissue preparation

Blood samples were centrifuged immediately (1370 g, 10 min, 4 °C) and the plasma was collected and used for the measurement of total antioxidant capacity (TAC), thiobarbituric reactive substances (TBARS) and protein carbonyls (CARB). The packed erythrocytes were lysed with distilled water (1:1 v/v), inverted vigorously, centrifuged (4000 g, 15 min, 4 °C) and the red blood cell lysate (RBCL) was collected for the measurement of GSH and H_2O_2 decomposition activity. Plasma and RBCL were stored at −80 °C until further analysis. Tissue samples were thawed in ice and homogenized in 0.01 mM PBS (138 mM NaCl, 2.7 mM KCl, 1 mM EDTA, pH 7.4) containing a cocktail of protease inhibitors, namely phenylmethylsulfonyl fluoride (PMSF, 1 mM), EDTA (1 mM) and leupeptin (0.1 mM). Brief sonication (60 s, 70% amplitude, 0.7 s cycle) on ice followed and the homogenate was then centrifuged (10,000 g, 15 min, 4 °C), the supernatant (i.e., the tissue homogenate) was collected and stored at −80 °C until biochemical analysis.

2.5. Assays

A total of four biomarkers that reflect the redox state were measured, namely reduced glutathione (GSH), total antioxidant capacity (TAC), thiobarbituric acid reactive substances (TBARS) and protein carbonyls (CARB) (Veskoukis et al., 2018).

GSH was measured according to a slightly modified method of Reddy et al. (2004), as described by Gerasopoulos et al. (2015). Briefly, proteins in the erythrocyte lysate or the tissues were precipitated with 5% trichloroacetic acid (TCA) in order to eliminate protein-linked -SH groups. Briefly, 20 μl of erythrocyte lysate or tissue homogenate was mixed with 660 μl of sodium potassium phosphate buffer (67 mM, pH 8.0) and 330 μl of 5,5'-dithiobis-2 nitrobenzoate (DTNB, 1 mM). Samples were incubated in the dark at room temperature (RT) for 30 min and the absorbance was read at 412 nm (Veskoukis et al., 2016). Calculation of GSH concentration activity was based on the molar extinction coefficient of DTNB (13.6 $mM^{-1}cm^{-1}$). The GSH assay was carried out right after the homogenization protocol in order to avoid *in vitro* oxidation of the tripeptide.

TAC determination was based on the method of Janaszewska and Bartosz (2002) with slight modifications as previously described by Gerasopoulos (Gerasopoulos et al., 2015). Briefly, 20 μl of plasma or tissue homogenate was added to 480 μl of sodium potassium phosphate (10 mM, pH 7.4) and 500 μl of DPPH (2,2-diphenyl-1-picrylhydrazyl, 0.1 mM), followed by incubation in the dark for 45 min at RT. Samples were centrifuged (20,000 *g*, 3 min, 25 °C) and the absorbance was read at 520 nm. TAC is presented as mmol of DPPH reduced to 2,2-diphenyl-1-picrylhydrazine (DPPH:H) by plasma or tissue antioxidants.

For the determination of TBARS, (a biomarker of lipid peroxidation) a slightly modified assay of Keles et al. (2001) was used according to (Priftis et al., 2018c). Briefly, 20 μl of plasma or tissue homogenate was mixed with 500 μl of Tris–HCl (200 mM; pH 7.4) and 500 μl of 35% TCA and incubated for 10 min at RT. One milliliter of Na_2SO_4 (2 M) and thiobarbituric acid (55 mM) solution was added and the samples were incubated at 95 °C for 45 min. Sequentially, the samples were cooled on ice for 5 min and vortexed following the addition of 1 ml 70% TCA. The samples were centrifuged (15,000 g,3 min, 25 °C) and the absorbance of the supernatant was read at 530 nm. A blank, lacking the blood or tissue sample, was also measured. TBARS are expressed in terms of malondialdehyde (MDA) equivalents. The molar extinction coefficient of MDA is 155 × 10^3 $M^{-1}cm^{-1}$.

Protein carbonyl (a protein oxidation biomarker) determination was based on a slightly modified method of Patsoukis et al. (2004), as previously described by (Priftis et al., 2018c). In this assay, 20 μl plasma or tissue homogenate was mixed with 480 μl of PBS and 500 μl of 2,4-dinitrophenylhydrazine (10 mM in 2.5 N HCl) per sample (500 μl of 2.5 N HCl for the blank). The samples were incubated in the dark at RT for 1 h with intermittent vortexing every 15 min and were centrifuged

(15,000 g, 5 min, 4 °C). Proteins were then precipitated with 100 μl of 100% TCA and washed three times with ethanol-ethyl acetate (1:1 v/v). The supernatant was discarded and 1 ml of urea (5M, pH 2.3) was added, vortexed and incubated at 37 °C for 15 min. The samples were centrifuged (15,000 g, 5 min, 4 °C) and the absorbance of the supernatant was measured at 375 nm (Veskoukis et al., 2016). Calculation of protein carbonyl concentration was based on the molar extinction coefficient of 2,4-dinitrophenylhydrazine ($22 \times 10^3\ M^{-1}cm^{-1}$). Total protein was assayed using Bradford reagent (Sigma-Aldrich, Munich, Germany).

2.6. Measurement of γ-glutamylcysteine ligase, catalytic subunit (gclc), catalase (cat) and superoxide dismutase 1 (sod1) gene expression

The small and large intestines, as well as the liver, the kidney and the brain were selected for further analysis regarding the expression levels of γ-glutamylcysteine ligase, catalase and superoxide dismutase genes using real-time PCR. The redox status of these tissues was significantly affected by the administered coffee extract and we hypothesized that this response could be attributed to altered gene expression levels of crucial antioxidant defense enzymes such as the three tested herein. RNA was extracted from all tissues using an RNA isolation kit (PureLink™ RNA kit, Invitrogen, USA) according to the manufacturer's instructions. RNA was quantified and its purity was confirmed by measuring the $OD_{260/280}$ with a value > 1.8 indicating lack of protein contamination. Approximately, 10 μg of the extracted RNA were treated with DNase (RQ1 RNase-Free DNase, 1U/μl, Promega, USA). DNA-free RNA was then reverse transcribed to obtain cDNA (Superscript II Reverse Transcriptase, Invitrogen, USA) using oligo (dT) 12–18 primers (Invitrogen, USA). Amplification of cDNAs for *cat, sod1* and *gclc* as well as the *actin* gene was performed in 10 μl reactions containing SYBR® Select Master Mix (2 ×, applied biosystems, USA), 0.25 μM of each primer, 50 nM ROX Low and 25 ng cDNA for the amplification of all tested genes. The utilized primers were based on the literature and are shown in Table 1 (Al-Rejaie et al., 2013; Vnukov et al., 2015). The thermocycling conditions used for the amplification of the aforementioned genes were the following: 3 min at 95 °C; 45 cycles of 15 s at 95 °C, 30 s at 55 °C, followed by 30 s at 72 °C. Finally, a melting curve was carried out from 55 °C to 95 °C to check the specificity of the products. All qPCR were performed on a μx3005P system (Stratagene, UK). Amplification efficiencies were > 89% with r^2 values > 0.987 for all genes.

2.7. Measurement of γ-GCLc, CAT and SOD1 protein levels

The protein levels of γ-GCLc, CAT and SOD1 were measured in the small and large intestines, the liver, the kidney and the brain using western blot analysis. Tissue homogenate containing 30 μg of protein was prepared (see section 2.4) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using an 8% polyacrylamide gel. Proteins were then transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The membranes were blocked overnight with 5% non-fat milk in 13 mM Tris/150 mM NaCl, pH 7.5, 0.2% Tween-20. They were then probed with polyclonal goat anti-rat SOD1 (1:600; Cat. no. sc-8637) or polyclonal rabbit anti-rat γ-GCLc (1:600; Cat. no. sc-28965; both from Santa Cruz Biotechnology Inc., Dallas, TX, USA or polyclonal goat anti-rat CAT (1:400; Cat. no. AF3398; from R&D Systems, Minneapolis, MN, USA) primary antibodies for 1 h at RT. According to their manufacturers, these antibodies are suitable for rats. Following five 5 min washing steps the membranes were incubated with horseradish peroxidase-conjugated polyclonal goat anti-rabbit (1:5000; Cat. no. 31462) or polyclonal donkey anti-goat (1:3000; Cat. no. PA1-28659; both from Thermo Scientific, Rockford, IL, USA) secondary antibodies for 30 min at RT. All membranes were re-probed with polyclonal rabbit anti-human (anti-mouse) glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1000; Cat. no. PA1-988; Thermo Scientific) for normalization. The optical density of the protein bands was measured using Alpha View quantification software (Alpha Innotech, San Leandro, CA, USA). Each experiment was repeated 3 times.

Table 1
Primer sequences.

Gene	Gene ID	Primer (5'-3')
cat	24248	Forward: *TTCTACACTGAAGATGGTAACTG* Reverse: *GAAAGTAACCTGATGGAGAGAC*
sod1	24786	Forward: *AACCAGTTGTGGTGTCAGGA* Reverse: *CTCCTGAGAGTGAGATCACA*
gclc	25283	Forward: *CAGAGTATGGGAGTTACATGATTGAAG* Reverse: *TGTGTTGAACTCGGACATCGTT*
actin	81822	Forward: *AGCCATGTACGTAGCCATCC* Reverse: *TCGGAACCGCTCATTGCCG*

2.8. Statistical analysis

One-way ANOVA followed by Tukey's test was applied to compare the means between the two groups. Differences were considered significant at $p < 0.05$. The results are expressed as mean ± SD. Statistical analyses were performed using the SPSS software (version 20.0; SPSS, Inc., Chicago, IL, USA).

3. Results

3.1. Redox biomarkers

According to the results, consumption of the coffee extract improved redox status of blood and all examined tissues. Specifically, GSH concentration in RBCL was increased in the coffee treated group compared to the control group (Fig. 1A). Regarding plasma TAC, TBARS and CARB, no significant differences were observed between the two groups (Fig. 1B–D). The administration of the coffee extract improved tissue redox status as well. With respect to GSH concentration, it was elevated in all the examined tissues (Fig. 2). TAC was increased in the large intestine, kidney and brain (Fig. 3). As for protein and lipid oxidation, decreased levels were observed in various tissues. CARB levels were reduced in the stomach, the small intestine, the kidney, the quadriceps and the brain (Fig. 4). On the other hand, TBARS levels were decreased in the liver, the kidney the lung, the quadriceps and the brain (Fig. 5). It is also noteworthy that the weight was not significantly altered between the two groups at the end of the experimental procedure (in the control group the starting and final weight were 192.2 ± 8.6 g and 221.7 ± 10.9 while the respective weights in the coffee group were 192.0 ± 17.0 and 219.7 ± 13.9).

3.2. Gene and protein expression

According to our findings, the mRNA levels of γ-glutamylcysteine ligase were increased in all tissues tested in a range between 2.2 and 3.5-fold. Superoxide dismutase levels were statistically significantly increased only in the brain (i.e., 2.0-fold, Fig. 6E), while catalase levels were not altered in any tissue (Fig. 6A–E). As for the respective protein levels, γ-glutamylcysteine ligase was increased in all tissues tested in a range between 29.1% and 50.4% (Fig. 7A–F). Superoxide dismutase was only increased in the brain (i.e., 119%, Fig. 7 E&F), while catalase levels were not significantly altered in any tissue (Fig. 7A–F).

4. Discussion

According to our results, moderate coffee consumption for two weeks reinforced redox status in the blood and all tested tissues of rats. This is reflected not only through the increases in the antioxidant

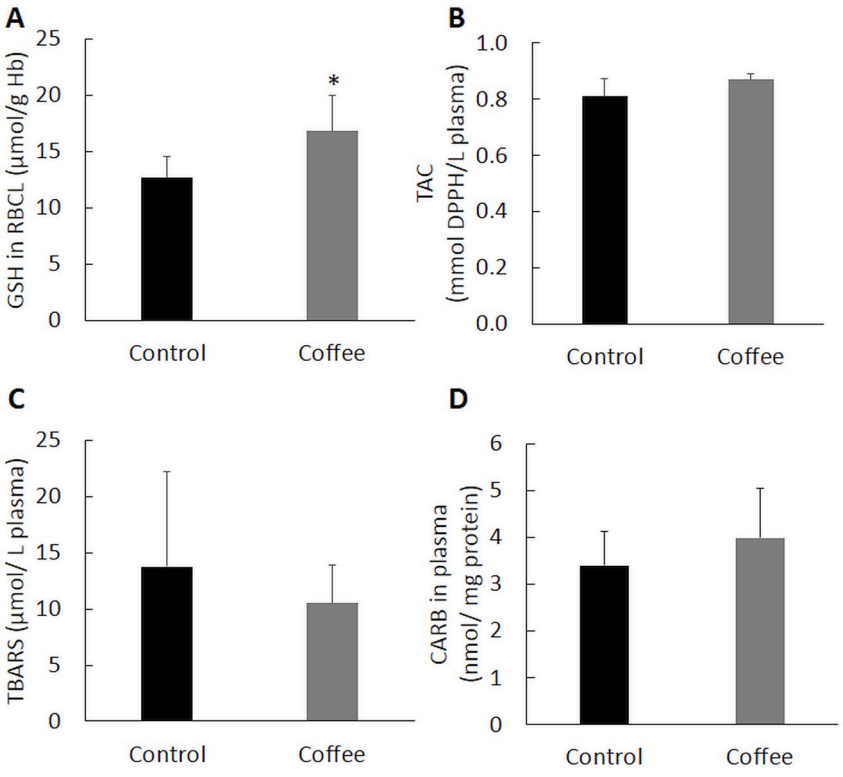

Fig. 1. The effects of the coffee extract on redox biomarkers of blood. (A) GSH in RBCL, (B) TAC in plasma, (C) TBARS in plasma and (D) CARB in plasma. *($p < 0.05$): Statistically significant compared to the control group. GSH: reduced glutathione; TAC: total antioxidant capacity; TBARS: thiobarbituric reactive substances; CARB: protein carbonyls.

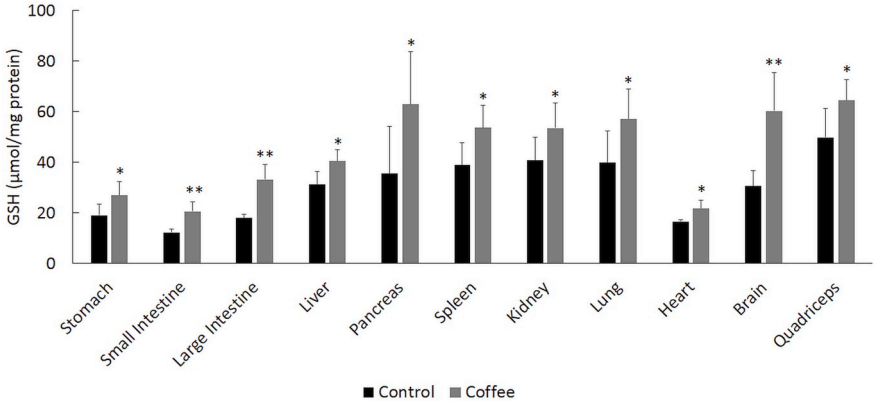

Fig. 2. The effects of the coffee extract on GSH levels of the rat tissues. *($p < 0.05$); **($p < 0.01$): Statistically significant compared to the control group. GSH: reduced glutathione.

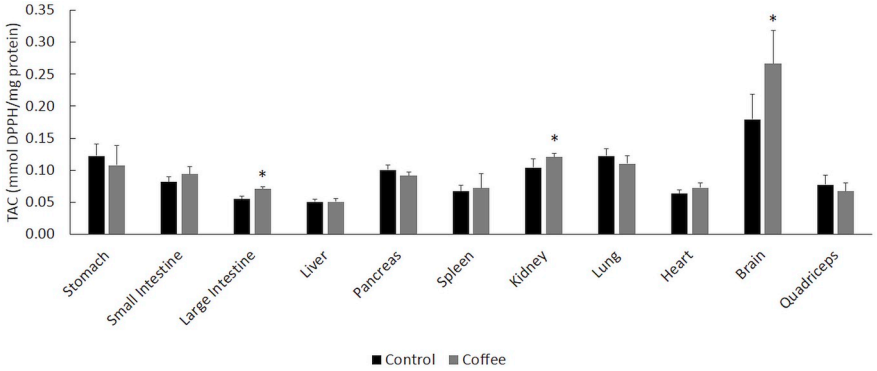

Fig. 3. The effects of the coffee extract on TAC of the rat tissues. *($p < 0.05$): Statistically significant compared to the control group. TAC: total antioxidant capacity.

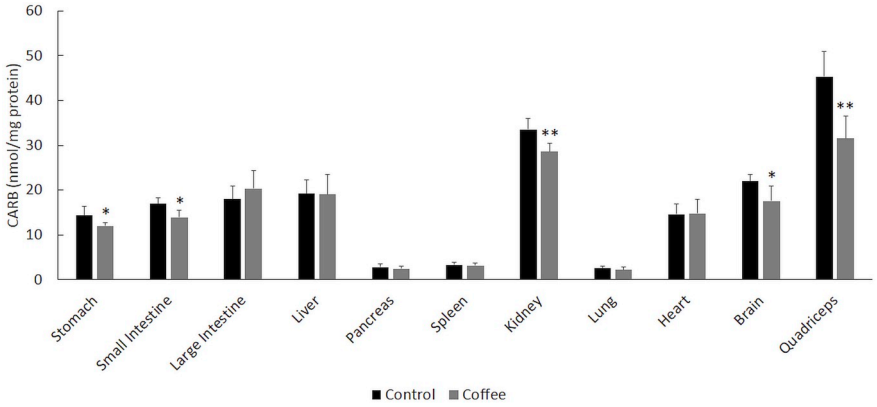

Fig. 4. The effects of the coffee extract on CARB levels of rat tissues. *($p < 0.05$); **($p < 0.01$): Statistically significant compared to the control group. CARB: protein carbonyls.

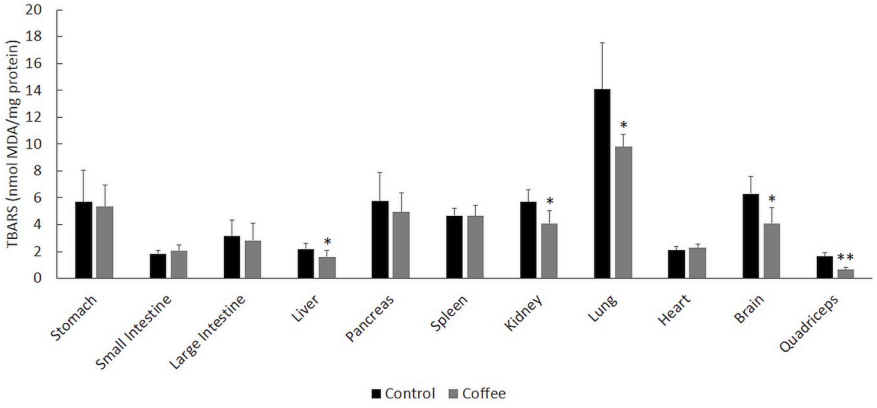

Fig. 5. The effects of the coffee extract on TBARS levels of the rat tissues. *($p < 0.05$); **($p < 0.01$): Statistically significant compared to the control group. TBARS: thiobarbituric acid reactive substances.

biomarkers (i.e., GSH, and TAC) but also from the decreases in the biomarkers of macromolecule oxidation (namely TBARS and CARB). GSH levels in particular were elevated in all tissues, perhaps due to the increased biosynthetic rate. Indeed, the expression of the gene coding for the enzyme responsible for the rate-limiting step in GSH biosynthesis (i.e., γ-GCLc) was upregulated in all examined tissues. Therefore, coffee consumption fortified the endogenous antioxidant defense. In the current study, the tested coffee extract was selected following previous screening experiments of our research group (Priftis et al., 2018a, 2018c; 2018b, 2015). According to them, it exhibited the highest potency in a variety of *in vitro* free radical scavenging and antimutagenic assays. Therefore, with the *in vivo* experiment described herein, we tried to shed light on whether *in vitro* observations actually correspond to *in vivo* systems. This concept is of high importance regarding polyphenol studies, since a major trait of these compounds is their low level of absorption and bioavailability (D'Archivio et al., 2010). In addition, the promising properties of plant extracts observed *in vitro* often do not correspond to *in vivo* settings (Veskoukis et al., 2012).

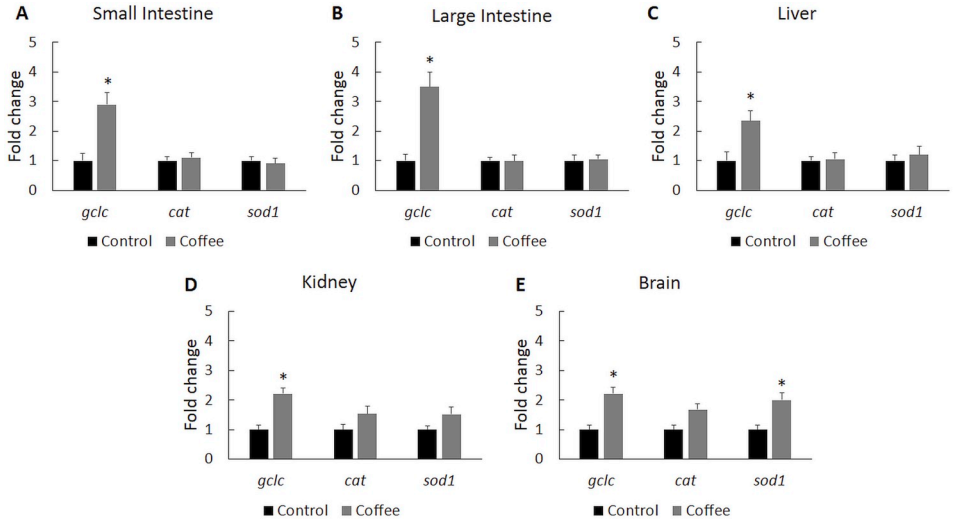

Fig. 6. The effects of the coffee extract on the expression levels of genes associated with the endogenous antioxidant defense mechanisms in selected rat tissues. *($p < 0.05$): Statistically significant compared to the control group. Results are presented as mean fold change ± SD following normalization with the *actin* gene. *gclc:* γ-glutamylcysteine ligase, catalytic subunit; *cat:* catalase; *sod1:* superoxide dismutase.

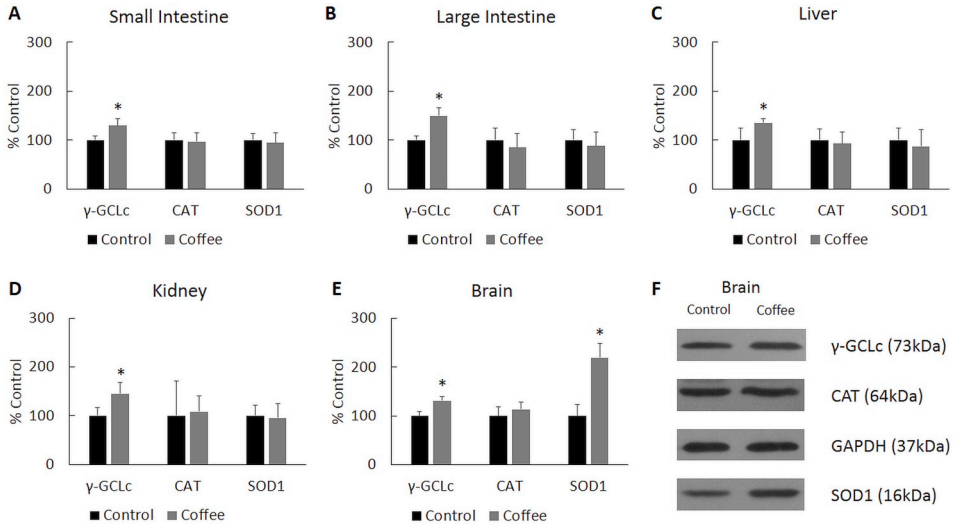

Fig. 7. The effects of the coffee extract on the protein levels of antioxidant enzymes in selected rat tissues. *($p < 0.05$): Statistically significant compared to the control group. Results are presented as a percent change of the levels of the respective protein in the control group ± SD following normalization with the GAPDH protein. A–E: The results from the five tested tissues. F: A representative Western blot from the brain tissue. γ-*GCLc*: γ-glutamylcysteine ligase, catalytic subunit; CAT: catalase; SOD1: superoxide dismutase.

4.1. Redox biomarkers

On the one hand, the tested coffee extract exerted a positive effect on antioxidant biomarkers (GSH and TAC). GSH levels were statistically significantly increased in all examined tissues as well as in the erythrocytes, from 29.2 5 up to 97.1% compared to the control. Interestingly, the highest increase was observed in the brain tissue. Data regarding the ability of coffee metabolites to cross the blood-brain barrier (BBB) are scarce and contradictory (Arendash and Cao, 2010; Lardeau and Poquet, 2013; Lee et al., 2015), however our data suggest that, if not coffee compounds *per se* (i.e. caffeine or CGA metabolites), at least some endogenous antioxidant compounds crossed the BBB eliciting an intriguing response evident by the fortification of the brain's antioxidant system. For instance, N-acetylcysteine (NAC), a GSH precursor is able to cross the BBB in a dose-dependent manner (Katz et al., 2015). Coffee constituents have demonstrated the ability to increase GSH levels in previous studies in both humans and rats (Bakuradze et al., 2011; Huber et al., 2002; Vitaglione et al., 2010), however the current work is the first to investigate the majority of tissues and not just blood or liver. Interestingly, blood seems to reflect the effect of a stimuli or an administered extract on tissue redox status (Margaritelis et al., 2015; Veskoukis et al., 2009) and this is partly evident in our experiment since GSH was increased not only in the blood but also in all examined tissues. Being the most important non-protein intracellular antioxidant compound, GSH is crucial against oxidative insults. Its increased levels can be attributed to either a higher recycling rate facilitated by enzymes such as glutathione peroxidase (GPx) and glutathione reductase (GR), or a higher biosynthetic rate induced by γ-glutamylcysteine ligase (γ-GCL) and glutathione synthetase (GS). We have previously demonstrated that the currently used coffee extract increases the levels of both GSH and the mRNA from the γ-GCL catalytic subunit (γ-GCLc) and GR in endothelial and myoblast cell lines (Priftis et al., 2018a, 2018c). Furthermore, the altered enzyme expression levels were ascribed to the derepression of Nrf2, since coffee compounds are known Nrf2 modulators (Boettler et al., 2011). Specifically, the Nrf2-Keap1 pathway drives the expression of a number of antioxidant and metabolic enzymes (Cavin et al., 2008). When no oxidative stressor is present, Nrf2 localizes in the cytoplasm attached to Keap1, an association which quickly leads to its ubiquitination and proteasomal degradation. The activation/derepression of Nrf2 may occur through a variety of mechanisms including the oxidation of Keap1 cysteine residues or the activation protein kinases that in turn phosphorylate Nrf2 (Tebay et al., 2015). Sequentially, Nrf2 translocates to the nucleus, where it binds to the antioxidant response elements (ARE), inducing the expression of various antioxidant enzymes (Itoh et al., 1999). Therefore, we hypothesized that the current observations regarding GSH could be attributed to an enhanced biosynthetic rate.

With respect to TAC, it was slightly affected by coffee consumption as increases were observed only in the large intestine, kidney and brain. Taken together, these findings support the notion that coffee contributes to the overall enhancement of rat tissue redox status. In addition, variations between tissues in these two biomarkers are potential indicators that the coffee extract shows tissue-specificity. Hence it is imperative to assess biomarkers that have structurally and functionally been grouped in order to increase their translational potency (Margaritelis et al., 2016; Veskoukis et al., 2018).

In the same line, the coffee extract exerted a protective activity regarding macromolecular oxidation as indicated by CARB and TBARS. Macromolecular oxidations are a result of free radical production, even at rest. Hydroxyl radicals ($HO^{\bullet}$), that are generated from H_2O_2 due to the reactions of Fenton and Haber-Weiss in the presence of free iron (Fe^{2+}) excess are highly reactive (Liochev, 2013). Therefore, compounds capable of alleviating their production and/or reactivity are sought as potential beneficial factors against several redox-pertinent pathological conditions. For instance, coffee compounds increased GSH levels, as well as glutathione S-transferase alpha 2 (GSTa2) and glutathione peroxidase 1 (GPx1) levels in myoblasts (Priftis et al., 2018a), the combination of which may reduce the formation of $HO^{\bullet}$. GSH neutralizes H_2O_2 through a GPx-catalyzed reaction, thus preventing $OH^{\bullet}$ formation (Brigelius-Flohé and Maiorino, 2013), whilst GST conjugates electrophilic compounds to GSH leading to their elimination and excretion from the body (Hayes et al., 2005). On the same grounds, according to a recent study coffee polyphenols displayed the ability to protect plasma protein from postprandial carbonylation (Sirota et al., 2013).

Out of eleven tissues and the blood, only in three tissues (i.e., kidney, brain and quadriceps) protein and lipid oxidation biomarkers were reduced consistently. To take all the above mentioned results together, the complexity of the interactions between the bioactive compounds present in the coffee extract and the examined tissues is apparent. Increased GSH levels may be considered responsible for the observed lower oxidation levels in certain tissues, however discrepancies still exist, since each tissue is different in terms of accessibility through the circulation and endogenous baseline antioxidant levels. Therefore, it is of paramount importance to pick the appropriate combination of biomarkers in an *in vivo* (or *in vitro*) experiment, in order to comprehend the effect of a tested extract (Margaritelis et al., 2016; Veskoukis et al., 2018).

4.2. Effect of coffee on gene and protein expression levels

To investigate whether coffee compounds affected antioxidant enzyme expression, the levels of three key enzymes were assessed. Specifically, superoxide dismutase (SOD1) which is responsible for the transformation of superoxide radicals (O_2^{-}•) to H_2O_2, catalase (CAT) which further reduces H_2O_2 to H_2O and O_2, as well as the catalytic subunit of γ-glutamylcysteine ligase (γ-GCLc), which catalyzes the rate limiting step in glutathione biosynthesis. These three enzymes contribute directly (the former two) or indirectly (the latter) to the endogenous antioxidant defense mechanisms and interestingly their expression is regulated by Nrf2 -among other transcription factors-, therefore coffee compounds could affect them.

Taking the results from section 4.1 under consideration, five tissues were selected, namely the small intestine, large intestine, liver, kidney and brain due to the fact that coffee seemed to affect the gastrointestinal tract (small and large intestine), main metabolic organs (liver and kidney) and the brain more significantly compared to the other organs. That was evident by the combination of a substantial increase in GSH levels as well as TAC along with reduced macromolecule oxidation levels. According to the results, on the one hand γ-GCLc expression was increased in all five tissues as observed both in mRNA and protein levels. On the other hand, CAT was not affected in any of the tested tissues, while SOD expression was upregulated only in the brain. The latter was the only observed difference between the tested tissues in terms of gene expression. Therefore, it can be postulated that coffee leads to the upregulation of crucial antioxidant enzymes. These results are in accordance with previous *in vitro* findings of our research group, pointing out that this coffee extract upregulated several antioxidant-related genes in myoblasts and endothelial cells (Priftis et al., 2018a). Overall, the increased GSH levels can be attributed to a higher biosynthetic rate since the rate limiting step can be catalyzed more readily. In addition, the increase in brain SOD1 further fortifies its antioxidant system with potential implications in neurodegenerative diseases in which oxidative stress plays a major role (Baillet et al., 2010).

5. Conclusions

The administration of a coffee extract for two weeks in a dose that corresponds to moderate every day coffee consumption led to the improvement of tissue redox status in rats mainly through increase of GSH levels. The enhanced biosynthetic rate of GSH via upregulation of γ-GCLc expression, as observed in the present study is one potential way. In general, the effects of the coffee extract on the *in vivo* antioxidant status of rats seem to adequately reflect its potent antioxidant action reported *in vitro* by previous studies of our group. However, we did not observe any uniformity between the tested tissues, following a wide ranged, crucial screening regarding the putative beneficial impact of coffee on systemic redox status. Our results support the notion that moderate coffee consumption boosts the endogenous defense system and, thus, it could potentially contribute to partial alleviation of oxidative stress-related pathological conditions.

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