

Cell-specific and roasting-dependent regulation of the Keap1/Nrf2 pathway by coffee extracts

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Abstract. Coffee is a popular beverage that contains various bioactive compounds. However, its molecular mechanism of action is not fully elucidated. In this context, two previously characterized coffee extracts, a lightly roasted and the corresponding green one, were investigated for their effect on nuclear factor erythroid 2-related factor 2 (Nrf2) target gene expression in myoblasts and endothelial cells using quantitative PCR. The tested concentrations were non-cytotoxic and led to improved redox cell status, as was evident by increased reduced glutathione (GSH) levels. In both cell lines, the roasted extract upregulated gene expression more readily than its green counterpart leading to increased NAD(P)H quinone dehydrogenase 1 and γ -glutamyl cysteine ligase catalytic subunit, among others. The green extract had a mixed effect on the endothelial cells, while, as regards the myoblasts it caused the downregulation of some Nrf-target genes. Therefore, a potential dose- and roasting-dependent mechanism is proposed in the current study, accounting for coffee's antioxidant activity.

Introduction

Reactive oxygen species (ROS) are produced normally by metabolism, inflammation, phagocytosis and other physiological biological processes. ROS can be harmful when in excess; however, some levels are required to maintain cellular homeostasis through redox cell signalling (1). When the intracellular concentration of free radicals is excessive, they

interact with and cause oxidative damage to proteins, lipids and DNA (2). Oxidative stress has been associated with a variety of pathological conditions, including cancer, diabetes, obesity and neurodegenerative diseases (3,4). In addition, it occurs frequently in muscle tissue and especially during intense exercise (5,6). Furthermore, oxidative stress-induced damage of the vascular endothelium is one of the most important factors regulating aetiology of cardiovascular diseases (7,8). Indeed, oxidative stress in endothelial cells induces acute and chronic phases of leukocyte adhesion to the endothelium (9). The interaction between ROS and nitric oxide sets off a vicious cycle, which results in inflammation (7). Furthermore, ROS, such as H₂O₂, can be diffused throughout endothelial cells and react with cysteine groups of proteins modifying their function (10). Thus, in an oxidative stress context, endothelial cells may lose integrity, progress to senescence and detach into the circulation (11).

Every organism that dwells in the presence of oxygen, contains a variety of endogenous antioxidant mechanisms including both enzymes and non-enzymatic metabolites (12). The most significant intracellular antioxidant compound is reduced glutathione (GSH), a tripeptide consisting of glutamic acid, glycine and cysteine, with the latter containing a sulphhydryl group responsible for its antioxidant properties (13). Apart from the endogenous mechanisms, diet is a frequent supplier of antioxidant compounds, including polyphenols present in plants (14). Polyphenolic compounds are products of plant secondary metabolism playing an important role in cellular functions. When plant foods are consumed, the absorbed polyphenols may elicit a variety of important bioactivities having beneficial effects on human health (15,16). Polyphenolic compounds can also be found in coffee, one of the most popular beverage throughout the world due to its pleasant taste and aroma as well as its stimulating effect (17). Coffee annual production exceeds 8 Mt and the average daily consumption is approximately 2.3 billion cups (18). Traditionally, the beneficial effects of coffee consumption on human health have been mainly attributed to caffeine, which is its most widely investigated ingredient. Nevertheless, there also other components, such as chlorogenic acids (CGA) that contribute to its valuable antioxidant properties (19). Several studies investigated the quantity, and have reported the potent antioxidant and disease-related beneficial properties of CGAs (20-22).

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Abbreviations: ROS, reactive oxygen species; GSH, reduced form of glutathione; TPC, total polyphenolic content; Nrf2, nuclear factor erythroid 2-related factor 2

Key words: coffee, roasting, polyphenols, nuclear factor erythroid 2-related factor 2, glutathione

However, despite the fact that coffee beans undergo roasting before their consumption, there is not much evidence on the effects of roasting on coffee composition, nor the differences between green and roasted coffee beans regarding their antioxidant activity (23-25). Indeed, it is known that the roasting procedure, which differs between coffee varieties, greatly affects CGAs leading to their breakdown and the formation of products, which may alter the antioxidant capacity of coffee beans (23,26,27). Therefore, the aim of the present study was to examine the effects of a green and a roasted *C. arabica* extract on nuclear factor erythroid 2-related factor 2 (Nrf2)-target gene expression in two cell lines.

Materials and methods

Coffee beans and roasting conditions. Both the green and roasted coffee bean varieties were provided from our collaborators (Coffee Island SA, Athens, Greece). The roasted beans were roasted for 4 min at 215°C (corresponding to a light roast).

Preparation of the extracts from coffee beans. For both the green and roasted beans a 10% w/v extract in H₂O was prepared. The beans were grounded using mortar and pestle, then a brief sonication treatment was applied for 20 min (70% amplitude, 0.7 sec cycle) and finally they were stirred for 20 min in moderate heat (35°C). After centrifugation (7,000 x g, 10 min, 10°C), each extract was stored in aliquots and kept at -80°C for further analysis.

Cell culture conditions. The endothelial EA.hy926 cells were donated by Professor Koukoulis (University of Thessaly, Larissa, Greece), while the C2C12 muscle cells were donated by Professor Koutsilieris (National and Kapodistrian University of Athens, Athens, Greece). The cells were cultured in normal Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/ml of penicillin and 100 U/ml of streptomycin (all purchased from Gibco, Paisley, UK) in plastic disposable tissue culture flasks at 37°C in 5% carbon dioxide.

Treatment of the cells with the coffee extracts. Cells were seeded in culture flasks and incubated for 24 h. The medium was then removed and replaced with serum-free medium containing the coffee extracts at non-cytotoxic concentrations. The cells were treated with the extracts (or just with serum-free medium for the control flasks) for 24 h and were then trypsinised, collected and centrifuged twice (300 x g, 5 min, 5°C). At the end of the first centrifugation, the supernatant fluid was discarded and the cellular pellet was kept at -80°C until further analysis.

qPCR of Nrf2 target genes. RNA was extracted from the cell pellet as mentioned earlier using an RNA isolation kit (PureLink™ RNA kit; Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). RNA was quantified and its purity was confirmed by measuring the OD_{260/280}, with a value >1.8 indicating lack of protein contamination. The extracted RNA (~10 µg) was treated with DNase (RQ1 RNase-Free DNase, 1 U/µl; Promega Corporation, Madison, WI, USA). DNA-free RNA was then reverse transcribed to obtain cDNA (Superscript II Reverse Transcriptase) using oligo (dT) 12-18 primers (both from

Invitrogen; Thermo Fisher Scientific, Inc.). Amplification of cDNAs for the Nrf2 target genes (*cat*, *sod1*, *txn*, *hmx1*, *nrf2*, *nqo1*, *gclc*, *gsr*, *gpx1* and *gsta2*) and for the *gapdh* gene was performed in 10 µl reactions containing SYBR® Select Master mix (2X; Applied Biosystems; Thermo Fisher Scientific, Inc.), 0.25 µM of each primer, 50 nM ROX Low and 25 ng cDNA for the amplification of all tested genes. The utilised primers were based on the literature (Tables I and II). The thermocycling conditions used for the amplification of the aforementioned genes were: 3 min at 95°C; 45 cycles of 15 sec at 95°C, 30 sec at 55°C for all the genes in the myoblasts and the majority of genes in the endothelial cells with the exception of *cat*, and *gsta2* for which 30 sec at 53°C were used, followed by 30 sec at 72°C. Finally, a melting curve was carried out from 53 to 95°C to check the specificity of the products. qPCR was performed on a MX3005P system (Stratagene, UK). Amplification efficiencies were >87% with r² values >0.982 for the genes.

Statistical analysis. Results were analysed by one-way ANOVA followed by Tukey's test for multiple pair-wise comparisons. All the results were expressed as mean ± SD. Differences were considered significant at P<0.05. Data were analysed using SPSS, version 20.0 (SPSS, Inc., Chicago, IL, USA).

Results and Discussion

This is a follow-up study from a previous one where nine coffee extracts were analyzed regarding their composition and effect on cellular redox status (28). According to the results, three mono-caffeoylquinic acid isomers (CQAs), namely 3-, 4- and 5-CQA were identified as the main polyphenolic compounds of the extracts, accounting for up to 30.23 mg/g of coffee with 3-CQA being the most abundant. Furthermore, upon administration to myoblasts and endothelial cells, differential toxicity was observed since green coffee extracts demonstrated higher toxicity towards the myoblasts, while in the endothelial cell line the opposite was observed. Nevertheless, all the extracts resulted in increased reduced GSH levels, an important endogenous antioxidant tripeptide due to its reactive sulfhydryl group (29). Coffee affected GSH levels differently in the two cell lines as in the myoblasts GSH was increased up to approximately 70%, while in the endothelial cells it was increased up to approximately 30%. An interesting observation was that GSH reached a peak concentration at an intermediate coffee extract concentration, while at higher extract concentration GSH was decreased. This finding could be probably attributed to the fact that polyphenols, depending on their concentration, induce a shift of their antioxidant activity towards prooxidant effects (30). Endogenous ROS levels were not affected by the tested extracts, while lipid and protein oxidation levels were slightly reduced, especially in the myoblasts (26).

In the current study, two extracts were selected for further analysis in order to shed light on coffee's mechanism of action. According to the literature, coffee constituents are able to cause Nrf2 derepression (i.e., translocation to the nucleus following detachment from the cytosol-localized Keap1) and concomitantly antioxidant gene expression (31-33). Therefore, the lightly (i.e., for 4 min) roasted *C. arabica* coffee extract was selected since it has previously demonstrated the highest antioxidant potency in various assays (34). In addition, its

Table I. Primers for nuclear factor erythroid 2-related factor 2 target genes in myoblasts.

Gene	Gene ID	Primer (5'-3')
<i>cat</i>	12359	Forward: TGAGAAGCCTAAGAACGCAATTC Reverse: CCCTTCGCAGCCATGTG
<i>sod1</i>	20655	Forward: GTGATTGGGATTGCGCAGTA Reverse: TGGTTTGAGGGTAGCAGATGAGT
<i>txn</i>	22166	Forward: CCGCGGGAGACAAGCTT Reverse: GGAATGGAAGAAGGGCTTGATC
<i>hmox1</i>	15368	Forward: CACGCATATACCCGCTACCT Reverse: CCAGAGTGTTCATTCGAGCA
<i>nrf2</i>	18024	Forward: CGAGATATACGCAGGAGAGGTAAGA Reverse: GCTCGACAATGTTCTCCAGCTT
<i>nqo1</i>	18104	Forward: TATCCTTCCGAGTCATCTCTAGCA Reverse: TCTGCAGCTTCCAGCTTCTTG
<i>gclc</i>	14629	Forward: ATCTGCCAAGGCGGCAAC Reverse: ACTCCTCTGCAGCTGGCTC
<i>gsr</i>	14782	Forward: GCTATGCAACATTCGCAGATG Reverse: AGCGGTAAACTTTTTCCCATTTG
<i>gpx1</i>	14775	Forward: GAAGAAGCTTGGGCCATTTGG Reverse: TCTCGCTGGCTCCTGTTT
<i>gsta2</i>	14858	Forward: CGTCCACCTGCTGGAAGCTTC Reverse: GCCTTCAGCAGAGGGAAAGG
<i>gapdh</i>	14433	Forward: AACGACCCCTTCATTGAC Reverse: TCCACGACATACTCAGCAC

Table II. Primers for nuclear factor erythroid 2-related factor 2 target genes in endothelial cells.

Gene	Access no.	Primer (5'-3')
<i>cat</i>	847	Forward: CCAGAAGAAAGCGGTCAAGAA Reverse: TGGATGTGGCTCCCGTAGTC
<i>sod1</i>	6647	Forward: AGGGCATCA TCAATTTTCGAG Reverse: GGGCCTCAGACTACATCCAA
<i>txn</i>	7295	Forward: TTTCCATCGGTCTTACAGC Reverse: TTGGCTCCAGAAAATTCACC
<i>hmox1</i>	3162	Forward: GGCCTGGCCTTCTTACCTT Reverse: GAGGGGCTCTGGTCTTGGT
<i>nrf2</i>	4780	Forward: ATGCCTGTAAGTCTGGTCA Reverse: ACTGCTCTTTGGACATCATTTCG
<i>nqo1</i>	1728	Forward: GGGCAAGTCCATCCCAACTG Reverse: GCAAGTCAGGGAAGCCTGGA
<i>gclc</i>	2729	Forward: GAAGAAGATATTTTTCTGTCATTGAT Reverse: CCATTCATGTATTGAAGAGTGAATTT
<i>gsr</i>	2936	Forward: CCAGCTTAGGAATAACCAGCGATGG Reverse: GTCTTTTTAACCTCCTTGACCTGGGAGAAC
<i>gpx1</i>	2876	Forward: CGCTTCCAGACCATTGACATC Reverse: CGAGGTGGTATTTTTCTGTAAGATCA
<i>gapdh</i>	2597	Forward: TGCACCACCAACTGCTTAG Reverse: GATGCAGGGATGATGTTC

corresponding green extract was also examined due to its peculiar toxicity pattern (i.e., 320-fold more toxic in the myoblasts compared with the roasted one).

Effect of coffee on Nrf2-target gene expression. To test the hypothesis whether Nrf2 derepression is a potential molecular mechanism of extract action, the green and roasted extracts were

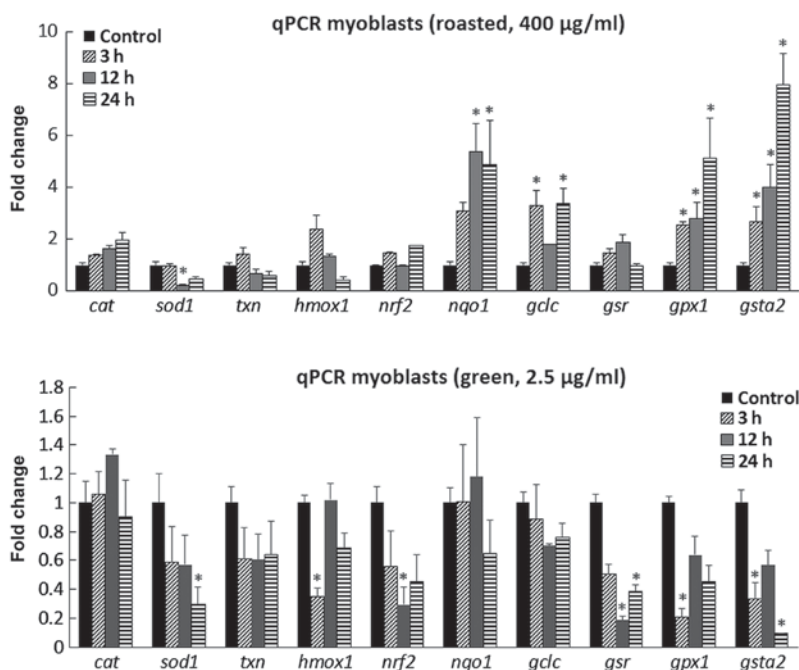


Figure 1. Effect of the tested coffee extracts on Nrf2 target-gene expression in C2C12 cells. qPCR results are depicted, following administration of coffee extracts on myoblasts for 3, 12 or 24 h. Gene expression has been normalised using *gapdh* expression and the fold-change in gene expression in comparison to the control cells (black bars) is displayed. The results are expressed as mean \pm SD and the asterisk (*) symbolises statistical significance at the $P < 0.05$ level. Nrf2, nuclear factor erythroid 2-related factor 2.

administered in the two cell lines at concentrations that caused the maximum increase in GSH levels and sequentially were assessed for their effect on gene expression levels of Nrf2 target genes by qPCR. These genes encode for catalase, superoxide dismutase 1, thioredoxin, heme oxygenase 1, Nrf2, NAD(P)H quinone dehydrogenase 1, γ -glutamyl cysteine ligase catalytic subunit, glutathione reductase, glutathione peroxidase 1 and glutathione S-transferase α 2. The expression of all these genes is driven by an antioxidant response element (ARE) in their promoter region, recognized by the nuclear localised Nrf2. These proteins are a part of the complex antioxidant system that protects cells from oxidative damage by neutralising free radicals and oxidising agents, therefore extracts or compounds that upregulate their expression may be used as potential antioxidant supplements. In a previous study, the roasted extract was administered in myoblasts and a microarray analysis was conducted, indicating that coffee constituents may act through the Nrf2 pathway (35). As for the used extract concentrations, the roasted extract was administered at 400 $\mu\text{g/ml}$ and the green at 2.5 $\mu\text{g/ml}$ in the myoblasts, while both extracts were administered at 100 $\mu\text{g/ml}$ in the endothelial cells.

According to the qPCR results, in the two cell lines the roasted extract had a more profound effect on gene expression, upregulating most genes compared to the green extract. Specifically, in the myoblasts the roasted extract caused the upregulation of *nqo1* 3.1-, 5.4- and 4.9-fold at 3, 12 and 24 h, respectively (Fig. 1), *gclc* expression was increased 3.3- and 3.4-fold at 3 and 24 h, respectively, *gpox1* was upregulated 2.5-, 2.8- and 5.1-fold at 3, 12 and 24 h, respectively, while *gsta2* increased mRNA levels by 2.7-, 3.0- and 8.0-fold at 3, 12 and 24 h, respectively. On the other hand, *sod1* levels were decreased by 78%. No significant differences were observed for *cat*, *txn*, *hmox1*, *nrf2* and *gsr*.

The *C. arabica* green extract caused the downregulation of six genes at various time points without upregulating any of the genes (Fig. 1). More specifically, *sod1* was downregulated by 71% at 24 h, *hmox1* was less abundant by 65% at 3 h, *nrf2* mRNA was 71% less compared to the control at 12 h, *gsr* by 82 and 62% at 12 and 24 h, respectively, *gpox1* by 80% at 3 h and *gsta2* by 67 and 91% at 3 and 24 h, respectively. No statistically significant changes were observed for *cat*, *txn*, *nqo1* and *gclc*.

In the flow cytometric analysis of the previous study, both the roasted and the green coffee extracts caused a similar increase in GSH levels (i.e., 70 and 66%, respectively) at the concentrations used in the qPCR. However, the latter analysis showed that only the roasted extract upregulated the expression of Nrf2 target genes and especially those that partake in GSH metabolism (i.e., *gclc*, *gpox1* and *gsta2*). The increased abundance of these mRNAs, if translated into increased protein levels, may justify the increased GSH levels. In addition, the upregulation of *nqo1*, which encodes for an important antioxidant enzyme that is involved in quinone detoxification, has been displayed in previous studies with coffee (32). Therefore, it can be hypothesised that the roasted extract causes the derepression of Nrf2. This can be attributed to coffee polyphenols such as the 5-caffeoylquinic acid that has been shown to stimulate *Nrf2* gene expression previously, through oxidation of KEAP1 cysteine residues (e.g., Cys151) that result in the release of Nrf2, which subsequently enters the nucleus and induces gene expression (36). These results are in agreement with those of another study in which the same extract was administered in the same myoblast cell line at an equal concentration and was subjected to microarray analysis to examine alterations in gene expression levels (35).

By contrast, the green coffee extract resulted in reduced gene expression for some antioxidant enzyme-coding genes. This

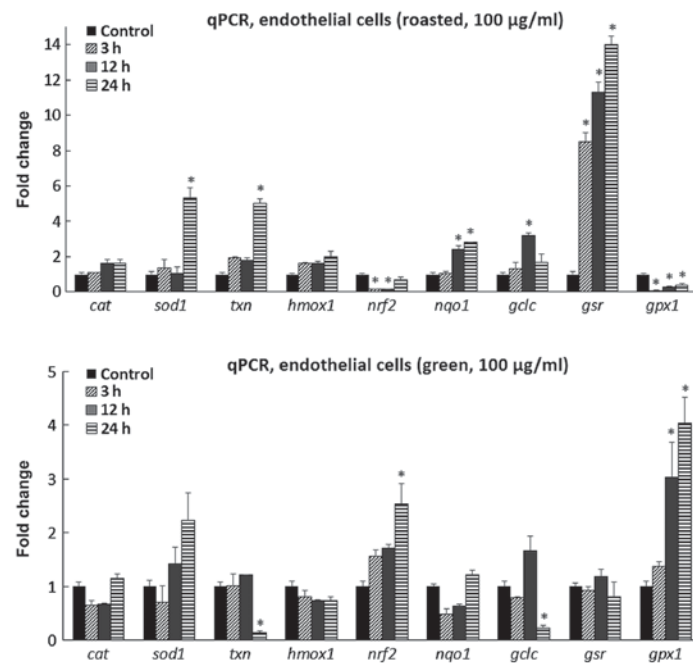


Figure 2. Effect of the tested coffee extracts on *Nrf2* target-gene expression in EA.hy926 cells. qPCR results are depicted, following administration of coffee extracts on endothelial cells for 3, 12 or 24 h. Gene expression has been normalised using *gapdh* expression and the fold-change in gene expression in comparison to the control cells (black bars) is displayed. The results are expressed as mean \pm SD and the asterisk (*) symbolises statistical significance at the $P < 0.05$ level. Nrf2, nuclear factor erythroid 2-related factor 2.

finding, to the best of our knowledge, has not been previously reported in the literature. Despite the fact that the green extract at this concentration (i.e., 2.5 $\mu\text{g/ml}$) resulted in increased GSH levels, this finding cannot be attributed to increased mRNA abundance of GSH metabolism-related genes. However, it is known that polyphenols exert antioxidant activity through a direct free radical scavenging capacity (37). Therefore, it is possible that this scavenging activity of the green coffee extract polyphenols, ‘saves’ endogenous GSH levels leading to the observed increase. These results, in combination with the high cytotoxicity of the green extract towards myoblasts (at just 20 $\mu\text{g/ml}$) indicate that the commercial green coffee extracts should be revisited for potential controversial effects.

In the endothelial cells, the roasted extract caused the increased abundance of *sod1* mRNA 5.3-fold at 24 h (Fig. 2). In addition, *txn* was also upregulated 5.0-fold at 24 h, while *nqo1* mRNA levels were increased by 2.4- and 2.82-fold at 12 and 24 h, respectively. The expression of the *gclc* gene was increased by 3.2-fold at 12 h, while *gsr* abundance was increased by 8.5-, 11.3- and 14.0-fold at 3, 12 and 24 h, respectively. On the other hand, *nrf2* mRNA levels were decreased by 84% at 12 h and 85% at 24 h compared with the control, while *gpx1* was downregulated by 92% at 3 h, 75% at 12 h and 64% at 24 h. No significant alterations were observed for *cat* and *hmox1*, although a rising trend was evident, particularly, at 24 h. The roasted extract exhibited a distinct profile of affecting the tested genes in the myoblasts versus the endothelial cells. As for the green extract, it caused a 2.5-fold increase in *nrf2* mRNA levels at 24 h and a 3.0- and 4.0-fold increase for *gpx1* at 12 and 24 h, respectively (Fig. 2). By contrast, lower mRNA levels were observed for *txn* by 86% at 24 h and *gclc* by 77% at 24 h. No significant changes were evident for the other examined genes.

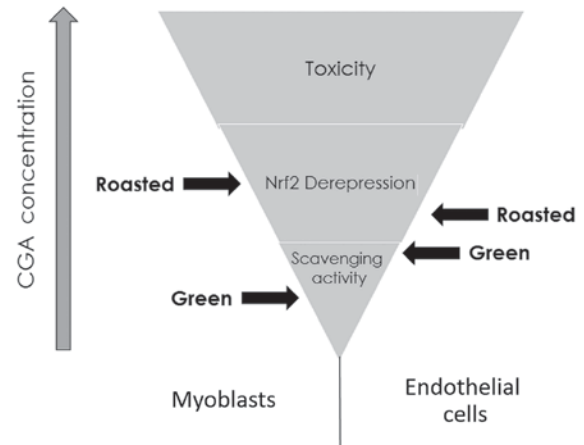


Figure 3. Suggested dose-dependent mechanism of action for the CGAs present in coffee. The potential mechanism of action of the tested coffee extracts is displayed. Their effect on the myoblasts and endothelial cells is dependent on CGA concentration. Low doses (as is the case for the green extract) exert a direct free radical scavenging activity sparing endogenous GSH levels, while at higher doses (as is the case for the roasted extract) Nrf2 derepression occurs, leading to the expression of its target genes. At higher doses, toxicity ensues, possibly due to the pro-oxidant effect of polyphenols. CGA, chlorogenic acids; GSH, glutathione; Nrf2, nuclear factor erythroid 2-related factor 2.

Taking into consideration the qPCR data along with the dose-dependent, hormetic effect of polyphenols (i.e., direct scavenging/antioxidant at low concentrations - pro-oxidant activity at higher doses, potentially leading to Nrf2 derepression - and toxic past a concentration threshold) (38), a potential mechanism of action for the aforementioned green and roasted extracts can be deduced for both cell lines (Fig. 3). According to the data obtained from the current and

previous studies, it can be assumed that moderate consumption (e.g., 2–4 cups per day) of lightly roasted coffee (as higher amounts may exhibit tissue-specific cytotoxicity) could elicit potentially beneficial effects through the expression of *Nrf2* target genes. In order to validate these *in vitro* results, an *in vivo* study is required so that the effects of bioavailability and metabolism on the potential bioactive compounds will also be taken into consideration.

To conclude, the two coffee extracts differentially affected gene expression in the two tested cell lines. On the one hand, the roasted extract (at 400 $\mu\text{g/ml}$) caused the upregulation of four genes (and especially those involved in GSH metabolism) and downregulation in one, while the green extract (at 2.5 $\mu\text{g/ml}$) resulted in the downregulation of six genes in the myoblasts. On the other hand, with respect to the endothelial cells, both extracts were administered at the same concentration (i.e., 100 $\mu\text{g/ml}$) as in that particular concentration maximum GSH levels were exhibited by flow cytometry. Again, the roasted extract had a more profound effect, as five genes were upregulated, including *gclc* and *gsr* that may explain the increased GSH levels and two were downregulated, including *gpx1* that could potentially lead to reduced GSH depletion as it utilises this tripeptide as a substrate. The green coffee extract caused an upregulation of only two genes whereas, it downregulated another two. These results highlight the complexity of coffee's molecular mechanism of action, which could partly be explained by the intricate regulation of the antioxidant mechanisms and the interplay between them. Specifically, when some antioxidant mechanisms are activated, some others remain inactive as a compensation adaptive cell response (39).

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

AP and ASV wrote the manuscript, AP and AEAT conducted the assays. DS collaborated with DK in reviewing the experimental design and the discussion. DK supervised the study while all authors reviewed the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

D.A. Spandidos is the Editor-in-Chief for the journal, but had no personal involvement in the reviewing process, or any influence in terms of adjudicating on the final decision, for this article.

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