ORIGINAL ARTICLE

Evaluation of the differential gene expression of the impact of epicatechin-3-gallate (EGCG) on antioxidant enzymes in mice at risk for cardiovascular disease

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ABSTRACT

The polyphenol epigallocatechin-3-gallate (EGCG), which is present in green tea, has been demonstrated to possess antioxidant qualities, possible advantages for cardiovascular health, and therapeutic effects in non-alcoholic fatty liver disease (NAFLD), a common ailment that impairs liver function. This work looked at the effects of EGCG on the differential gene expression levels of the superoxide dismutase 2(SOD2), Glutathione peroxidase-1 (Gpx1), Catalase (CAT), and Thioredoxin Reductase (TrxR1) genes coding for antioxidant defense enzymes to determine whether EGCG could enhance antioxidant defense capacity in mice at risk of cardiovascular disease caused by a high-fat diet and sedentary lifestyle. It was shown that liver tissue samples from male mice fed a high-fat diet (HFD) and two control diets (CD) with or without EGCG supplementation showed distinct expression patterns for the genes Sod2, Gpx1, Catalase, and other transcripts. For this investigation of differential gene expression levels, male mice with sedentary lifestyles and two different control groups that underwent physical activity with or without EGCG treatment were also utilized. GraphPad Prism version 6 was used to conduct Tukey-Kramer multiple comparison tests between the treatment and control groups. For the results, p 0.05 was chosen as the significant level. The study results show that EGCG increases the enzyme activities of Sod2, Gpx1, CAT, and TrxR1 which are related to antioxidant defense differential gene expression. The results of this study demonstrated that cycle threshold (CT) values before EGCG supplementation were greater than CT values after EGCG supplementation (p-value = 0.424 versus p-value = 0.2541 for diets and p-value = 0.5547 versus p-value = 0.5149 for physical exercise), suggesting that EGCG enhanced the differently expressed genes.

Keywords: Epigallocatechin-3-gallate, High Fat Diet, Sedentary lifestyle, cardiovascular disease, DNA methylation, differential gene expression.

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INTRODUCTION

One promising approach to creating medications that promote health is the use of natural components [1, 2]. The potential therapeutic effects of tea polyphenols in treating a variety of clinical disorders have been extensively studied [3, 4]. Tea polyphenols, the primary bioactive component of green tea leaves, account for 30% of the leaves' dry weight. Interestingly, the catechin with the highest concentration of EGCG and the health-promoting qualities of tea polyphenols are significantly correlated [5,6]. Several types of cancer, cardiovascular disease, and neurological illnesses are delayed or prevented by EGCG [7-8]. Other studies have shown that plant meals and many items made from them contain a variety of necessary and non-essential bioactive chemicals, in addition to the polyphenols present in green tea. These compounds have received approval for a wide range of biological actions that could help to prevent or promote several inflammatory and chronic illnesses. Preclinical research has frequently demonstrated that in human culture or animal models, exposure to some of the bioactive chemicals and some of their produced metabolites changes the levels of a wide variety of molecular targets, suggesting that this may be one of the processes.

The beneficial effects of dietary bioactive compounds are mediated by particular mechanisms of action [12]. In addition to protecting the cardiovascular and nervous systems, these compounds have been shown in numerous studies to have anti-proliferative [13], anti-inflammatory [14], and anti-obesity [15] properties. These actions have been linked to changes in the expression of genes associated with cancer development, cell cycle control, transcription, inflammation, xenobiotic metabolism, or redox processes. The precise mechanisms by which dietary bioactive compounds or their derivatives can affect the various biological targets remain mostly unclear. Direct molecular interactions that either stimulate or repress gene expression or epigenetic mechanisms have been proposed as potential mediating mechanisms for the effects of bioactive chemicals [18, 19].

A group of antioxidant enzymes found in cellular antioxidant defense systems prevent ROS formation or neutralize ROS to maintain equilibrium [20]. Important members of this group of enzymes include glutathione reductase (GSR), GPX1, CAT, and SOD [21, 22]. Cancer cells and cardiovascular disorders may benefit from altered expression of these enzymes [21, 22].

As a result, further investigation is constantly needed into the use and characteristics of the EGCG, which is covered in this paper. In this study, we examined the impact of epigallocatechin-3-gallate (EGCG) on differential expression change levels of SOD2, GPX1, CAT, and TRXR1 genes involved in antioxidant defense using RT-qPCR in a mouse model fed a high-fat diet and leading a sedentary lifestyle.

MATERIALS AND METHODS

Ethics Statement

The procedures and protocols utilized in this work were approved by the Uganda National Council of Science and Technology and Kampala International University's Animal Ethics Committee; NS 645 is the approval number for these committees. The way that the animals were treated adhered to both the European Council of the Animals' Guiding Principles in the Care and Use of Experimental Animals [23] and the US National Institutes of Health's Guide to the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996) [23].

Animal groups, preparation, anesthesia, euthanasia, and liver tissue samples

A total of sixty-three-month-old male Swiss albino mice were purchased from the animal facility of the College of Veterinary Medicine, Animal Resources and Biosecurity (COVAB) at Makerere University. The mice were housed in standard cages with a 12-hour light/dark cycle and standard humidity and temperature levels (27 2°C and 55 5%, respectively). Twelve groups of five mice each were created out of the animals. The first six groups received physical exercise therapy as described by Banzubaze et al., 2022 [11, 24], while the

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remaining six groups received diet treatment as described by Banzubaze et al., 2022 [11, 24]. EGCG was given as a daily dosage of 30 mg/kg/day after being dissolved in drinking water. The mice were put to sleep and put to death by the earlier reports of Gail et al. (2013) [25], ILAR (2014) [26], and Grandjean (2014) [27], which were referenced by Banzubaze et al., 2022 [11, 24]. Samples of liver tissue were obtained for cDNA synthesis, RT-qPCR, and RNA extraction.

RNA extraction and quantitative reverse transcription–polymerase chain reaction (qRT–PCR)

RNA extraction

RNA was extracted following the GeneJET RNA Purification Kit (Thermo Scientific, catalog number #K0731). In summary, 10 mg of liver tissue samples that had been previously preserved in liquid nitrogen (-80°C) were meticulously pulverized with a mortar and pestle. During the grinding process, 1 ml of RNA was added to preserve the integrity of the RNA. Following crushing, the materials were put right away in a 1.5 ml microcentrifuge tube together with 300µl of lysis buffer that had been enhanced with DTT or -mercaptoethanol. After vortexing the tube for ten seconds, it was allowed to sit at ambient temperature for three minutes. These samples were vortexed, heated at 20°C for 10 minutes, and then treated with 600l of diluted proteinase K (10µl of the contained proteinase K diluted in 590 µL of TE buffer). After five minutes of spinning at 13,000 g, the samples and supernatant were placed into a sterile microcentrifuge tube. For every sample, 450 µl of ethanol (96–100%) was added and mixed using a pipette. Subsequently, the lysate was transferred to the column, put in a collecting tube, and centrifuged for a minute at 13,000 g. After adding 700 µl of Wash Buffer 1 (which included ethanol as a supplement), the column was centrifuged for one minute at 13,000 g, the stream was disposed of, and the column was returned.

This was repeated using 600 µL and 250 µL of ethanol-infused Wash Buffer 2. The column was then transferred to a sterile 1.5 mL microcentrifuge tube after the collection tube holding the run-off solution was disposed of. After adding 100µl of nuclease-free water, the mixture was centrifuged at 13,000 g for one minute to extract the RNA. After that, the isolated RNA was kept at -20 °C until it was needed.

RNA integrity

The purity and quantity of the extracted total RNA were assessed using a spectrophotometric technique using a Nanodrop-2000C (Thermos Scientific, USA). The integrity of the RNA was evaluated using whole RNA electrophoresis on an agarose gel (Figure 1). The GoScriptTM Reverse Transcription System (Promega, USA) was used to reverse transcribe total RNA in a PCR thermal cycler (Applied Biosystems, USA) by the manufacturer's instructions. The entire RNA that wasn't used for RT-PCR was frozen at 80 °C in case more analysis was needed.

cDNA synthesis

The Protocol for LunaScript® RT SuperMix Kit from New England Biolabs (catalog number E3010) was used to convert total RNA to cDNA by the manufacturer's instructions. The polymerase chain reaction with reverse transcription (RT-PCR; New England Biolabs) Following the manufacturer's instructions, cDNA was synthesized from 100 ng of RNA using the LunaScript® RT SuperMix Kit First-Strand Synthesis System. The cDNA synthesis reaction was prepared using the following steps: 1µg of the whole RNA sample was mixed with 4 µl of LunaScript RT SuperMix (5X). To give the combination a total volume of 20µl, nuclease-free water was added. As with the last mixture, the no-RT control reaction was likewise prepared. Then, 4µl of LunaScript RT SuperMix (5X) was combined with 20µl of nuclease-free water to create the no template controls reactions. As stated in Table 1, thermal gradient polymerase chain reactions (PCRs) were carried out to determine the ideal primer annealing temperatures for each primer set.

Quantification of cDNA and Ensuring Quality

One microliter of the first-strand reaction was used to measure the distribution and amount of cDNA molecules using the Agilent 2100 bioanalyzer with RNA 6000 LabChip kit. By running the experiment on triplicates of cDNA from four independent reverse transcription procedures using the same animal's RNA,

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the reproducibility of this assay was evaluated. A serial dilution of the sample in triplicates was quantified to confirm the measurement's linearity.

Absolute a quantitative RT - PCR- using StepOnePlus™ system (Applied Biosystems, USA)

Using quantitative polymerase chain reaction (qPCR) testing on a Step One Plus TM system (Applied Biosystems, USA) with Lun Universal qPCR Master Mix (New England Biolabs, catalog number 3003S), the Sod2 gene, GPx1 gene, Cat gene, and Trx1 gene were examined in tissue liver samplesThe reaction mixture was made using the procedure indicated in Table 2. The oligonucleotide primers used for qRT-PCR are displayed in Table 3 [28], per Bustin et al. 2009 [28].

Each cDNA sample was diluted until the final concentration of 5ng/ml was reached. 20 ml of 2 X Fast SYBRH Green Master Mix (Applied Biosystem), 10 µl of cDNA, 200 nM forward and reverse primers, 10 ng of cDNA, UP (Ultra-Pure), UDG, ROXTM dye Passive Reference, dNTPs, and adjusted buffer components made up the final reaction volume for real-time PCR.

The StepOne Plus Real-Time PCR System from Applied Biosystems was used to perform qRT-PCR utilizing a temperature profile that included 20 seconds of Taq DNA.

Each cDNA sample was diluted until 5ng/ml was the final concentration. The final reaction volume for real-time PCR consisted of 20 ml of 2 X Fast SYBRH Green Master Mix (Applied Biosystem), 10 µl of cDNA, 200 nM forward and reverse primers, 10 ng of cDNA, UP (Ultra-Pure), UDG, ROXTM dye Passive Reference, dNTPs, and adjusted buffer components. qRT-PCR was carried out using the StepOne Plus Real-Time PCR System from Applied Biosystems, utilizing a temperature profile consisting of 20 seconds for Taq DNA. After 40 cycles of primer annealing for 30 s at 60 °C, primer denaturation for 15 s at 95 °C, and primer annealing for 30 s at 60 °C, the polymerase was activated for 60 s at 95 °C. To help with product identification, the melting curves of the real-time PCR products were analyzed from 65°C to 95°C. Each was measured three times.

In addition, standard curves were always used in one-step real-time RT-PCR to assess the relative copy number of the target gene. In quantitative real-time PCR (qRT-PCR), based on Ct values, a housekeeping gene called -Actin was used as an internal control. Target gene copy number/-actin ratios were used to calculate the relative values of target gene expression in the SOD2, GPX1, CAT, and TRXR1 groups relative to the control group. The relative amounts of RNA expression for each target gene were used to provide this data.

Statistical Analysis

Graph Pad Prism Software version 6 was used to analyze variance (ANOVA). The results, which were presented as mean SD, were assessed using a one-way ANOVA. Kruse multiple comparison tests were used to compare means when suitable. For each cDNA sample used in the real-time PCR study, the threshold cycle (CT) was determined using the Step One software version 2.3. According to the gene expression significance test, a p-value of 0.05 was deemed statistically significant. The 2-Ct approach, where Ct = CT (Target) CT (-Actin), was used to quantify changes in gene expression [29, 30]. Turkey's multiple comparisons test was used to compare the means of the treatment and control groups at a 95% confidence level (LFD).

RESULTS

3.1. Effect of EGCG on differential expression levels of antioxidant enzymes after diet treatment

For all diets supplemented with or without EGCG, the CT values were less than 29, indicating a significant level of differential gene expression or strong positive reactions pointing to a significant amount of target nucleic acid in the sample. The Cat and Sod2 genes had less differential expression than TrxR1 and Gpx1 (Figure 3). With a p-value (0.0004) less than 0.05, the

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results from diet-subjected mice showed a statistically significant relationship between diets supplemented with or without EGCG on variations in antioxidant enzyme expression levels of Sod2, Gpx1, Cat, and TrxR1 in mice (Table 4. A).

When compared to other genes, differential Gpx1 gene expression was significantly up-regulated, while differential Cat gene expression was dramatically down-regulated. The LFD showed up-regulation of genes whereas the HFD showed down-regulation of genes when comparing the same diet without EGCG to the same diet supplemented with EGCG, as well as the differential gene expression of the investigated genes. The mice fed the HFD increased the hepatic gene expression level of the investigated genes by 2.8 times when compared to animals fed the HFD with and without EGCG (Table 5).

Because target nucleic acid levels and sample amounts are inversely correlated, a sample's target nucleic acid content increases with decreasing Ct level. Table 6 and Fig. 4 demonstrate that the CT values in the meals supplemented with EGCG were lower than the CT values in the diets without EGCG, suggesting that EGCG increased the gene expression of the genes encoding the enzymes related to antioxidant defense.

The study's results demonstrate statistically significant differences between LFD vs LFD + EGCG and HFD vs HFD + EGCG by taking into account the effect of EGCG on the overall gene differential expression levels of genes involved in endogenous antioxidant defense (Sod2, Gpx1, Cat, and TrxR1) and dietary processing (LFD, ND, and HFD) in mice. The high values of the means of the CT-values were more observed for LFD which means that in LFD there was a low level of differential gene expression (Figure 4).

By comparing the gene expression levels for HFD and HFD + EGCG, the high CT-values were higher for HFD, which means that HFD decreases differential gene expression which negatively affects the endogenous antioxidant defense of the enzymes involved in antioxidant defense. The lowest average CT values are observed for HFD+EGCG. This implies that EGCG enhances differential gene expression and therefore increases endogenous antioxidant defense.

3.2. Effect of EGCG on differential expression levels of antioxidant enzymes after exercise treatment exercise

When the p-value (p-value = 0.0037) is less than 0.05, our results show a significant difference in the differential gene expression of the Sod2, Gpx1, Cat, and TrxR1 genes in mice given physical exercise supplemented with or without EGCG (Table 4. B). According to the results, the Cat and Sod2 genes had a lower differential expression than TrxR1 and Gpx1. Compared to other genes, differential Gpx1 gene expression was highly up-regulated while differential Cat gene expression was significantly down-regulated (Figure 5 and Table 7). Comparing the type of exercise with EGCG with the same type of exercise supplemented with EGCG and the differential gene expression of the studied genes, chronic exercise, and voluntary exercise showed gene down-regulation while the sedentary lifestyle presented up-regulation. Meanwhile, sedentary lifestyle mice had increased the hepatic gene expression level of the studied genes by 2.368 times when comparing a sedentary lifestyle without EGCG and a sedentary lifestyle with EGCG. Similarly, mice subjected to voluntary exercise had increased hepatic expression levels of the genes studied by 1.910 times when comparing voluntary exercise without EGCG and voluntary exercise supplemented with EGCG (Table 8). In addition, mice subjected to voluntary exercise without EGCG had increased hepatic expression levels of the studied genes by 2.762 times when compared with chronic exercise supplemented with EGCG. For all types of physical exercise (voluntary physical exercise, chronic physical exercise, and sedentary lifestyle) supplemented or not by EGCG, the CT values were lower than 29 which always reflects a high differential gene expression. However, with the types of physical exercise supplemented with EGCG, the CT values were lower than the CT values of the types of physical exercise without supplementation with EGCG, which means that EGCG increased the differential gene expression. Regarding the effect of EGCG on the global gene differential expression of Sod2, Gpx1, Cat, and TrxR1 genes and physical exercises (CE, VE, and SL) in mice, there were statistically significant differences for SL vs SL+EGCG (Figure 6). The high values of CT-values are observed for SL without EGCG which means that SL decreases

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the level of differential expression and consequently decreases the endogenous antioxidant defense of the enzymes involved.

DISCUSSION

This study looked at how the green tea extract epigallocatechin-3-gallate (EGCG) affected the expression of genes related to the antioxidant response in mice given a high-fat diet (HFD) and mice kept in a sedentary position. These genes included superoxide dismutase 2, glutathione peroxidase 1, catalase, and thioredoxin reductase 1. Elevated levels of free radicals are a hallmark of chronic inflammatory illnesses like atherosclerosis, which damages the liver and induces oxidative stress. A high-fat diet (HFD) and sedentary lifestyle (SL) influence the development and course of these conditions as well (Siti et al. 2015) [31].

The purpose of this work is to examine the feasibility of using green tea polyphenols as a natural antioxidant in meals to regulate the differential gene expression of Sod2, Gpx1, Cat, and TrxR1 in the liver of mice with HFD and SL. Numerous research investigations have shown how flavonoids can treat conditions including insulin resistance [32]. Other investigations have shown that oxidative stress, inflammation, and several molecular signaling pathways are EGCG's likely mechanisms of action [33].

In a previous investigation, we found that supplementing mice on HFD and SL with EGCG derived from green tea polyphenols resulted in liver antioxidant qualities [24] as well as a lowered lipid profile and blood glucose levels [11]. The study's findings indicate that the liver genes Sod2, GPx1, CAT, and TrxR1, which are associated with antioxidant defense, regulate the EGCG's differential expression. Polyphenols increased the expression of genes linked to antioxidants, according to research Yang *et al*. published in Chinese Yellow Chicken in 2019 [34]. Tao et al. (2014) have shown that oral cancer cells treated with EGCG experience mitochondrial oxidative stress and that the expression of antioxidant response systems associated with mitochondria is decreased [35]. The same researchers found that the EGCG-treated mice had lower expression levels of the mitochondrial-related antioxidant enzymes SOD2 and GPX1. Other investigations have produced results that are comparable to ours.

An investigation conducted in 2019 by Ni et al. [36] discovered that the liver's antioxidant defense mechanism is significantly engaged to shield laying hens from oxidative damage brought on by heat stress and to sustain body antioxidant levels. Total RNA, SOD, and GPX1 liver expression levels were higher in the tea polyphenol supplement group, according to the Ni et al., 2019 study [36].

Zhang R et al. reported in 2020 [37] those patients with idiopathic pulmonary arterial hypertension, both male and female, showed higher levels of all three SOD isoforms than healthy individuals. The relationship between SOD activity and vascular disease is critical for the creation of a novel diagnostic biomarker and treatment approach, according to these studies. According to the study conducted by Hwang et al. in 2021, CAT is regulated by pathways connected to OxS and several transcription factors [38].

It is well known that CAT protects against ROS, and some writers claim that decreasing CAT activity enhances the visual appearance of abdominal aortic dilatation [39]. Another internal antioxidant enzyme, GPx1, plays a significant role in reducing ROS by converting lipid peroxides and H_2O_2 to alcohol [40].

We discovered that experimental settings had an impact on the dynamic expression of antioxidant enzymes. Our research has been supported by other studies, like Fukai et al. (2011) who claimed that antioxidant enzyme expression is very dynamic and that variations in both expressions have been associated with atherosclerosis [41]. According to Brinkmann et al. (2013), antioxidant enzyme expression is very dynamic, and changes in both expressions have been connected to exercise [42]. According to Oelze et al. (2014), antioxidant enzyme expression is very dynamic, and variations in expression are crucial for maintaining cellular activities [43].

The expression of the antioxidant enzymes Trx1, Sod2, and Prdx3 responded to the

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environment in different ways, according to research done in 2016 by Nguyen et al. [44]. These results are consistent with earlier research showing increased expression of antioxidant enzymes during the start and development of oxidative stress-related diseases [45]. Exercise and diet appear to raise CT values, which in turn reduces the amount of the antioxidant response by blocking the upregulation of antioxidant enzymes.

However, EGCG supplementation turned this constant around. In WT and ATX mice, Gpx1 expression is elevated by exercise above basal levels, according to Nguyen et al.'s 2016 findings [44]. The 2003 study by Blankenberg et al. [46] showed that low Gpx1 activity is an independent risk factor for cardiovascular events.

Our research on the regulatory function of Sod2, Gpx1, Cat, and TrxR1 in the differential expression of genes encoding endogenous antioxidant defense-related enzymes is supported by our findings.

CONCLUSION

This study focuses on EGCG, a kind of catechin and polyphenol found in green tea. The purpose of this work is to investigate the potential of green tea polyphenols as a natural antioxidant dietary additive to regulate the differential expression of Sod2, Gpx1, Cat, and TrxR1 genes in the liver of mice. This work demonstrated the regulatory function of EGCG in the differential gene expression of enzymes encoding antioxidant defense in the liver, such as Sod2, GPx1, CAT, and TrxR1. Green tea catechins have the potential to be a useful treatment for liver and cardiovascular illnesses because they improve the differential gene expression of genes involved in antioxidant defense.

Limitation

The experimental procedures were the study's main drawback. Because of the high expense of the experimental procedure, the study did not compare the effects of EGCG on differential gene expression for different organs in mice. It would have been interesting to investigate the effect of EGCG on the differential gene expression for different tissues, such as cardiac muscle, femoral artery, skeletal muscle, and circulating leukocyte tissues, to ascertain whether the differential gene expression involved in antioxidant defense would vary depending on the tissues studied. Nonetheless, the investigation yielded noteworthy findings indicating that variables related to experimentation and surroundings impact the varying expression of genes associated with antioxidant defense.

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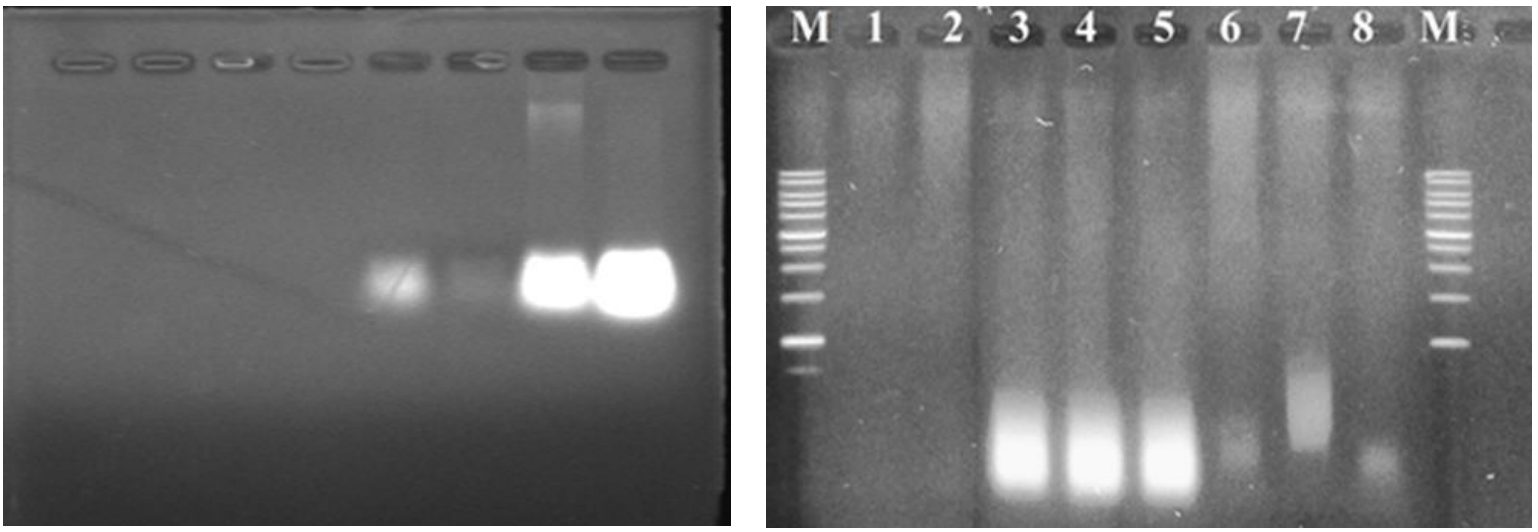

Figure 1: Agarose gel electrophoresis of purity and quantity of the isolated total RNA

Table 1: Showing cDNA synthesis conditions for the incubate reactions in a thermocycler

CYCLE STEP	TEMPERATURE	TIME	No. Of CYCLES
Primer Annealing	25°C	2 minutes	1
cDNA Synthesis	55°C	10 minutes	
Heat Inactivation	95°C	1 minute	

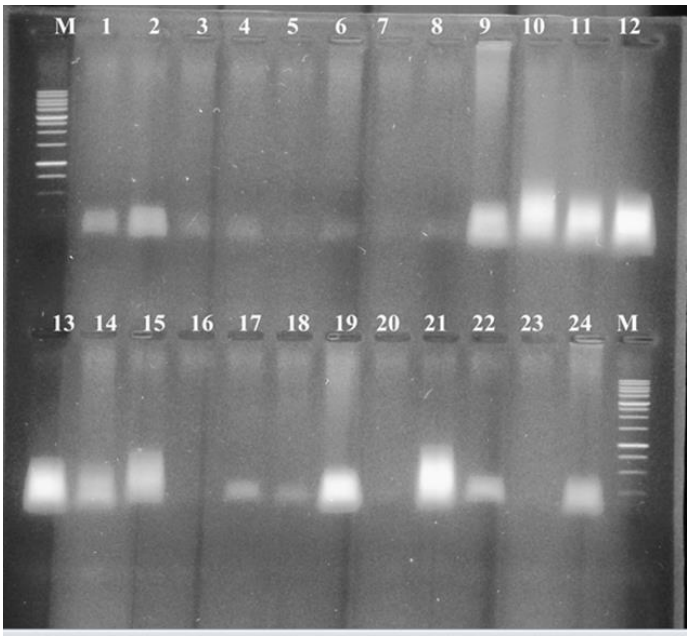

Figure 2: Agarose gel electrophoresis of purity and quantity of cDNA synthesis

Table 2: RT-PCR reaction mixture

Components	Reaction mixture (20 µL)
Master mix	10 µL
Forward primer	0.5 µL
Reverse primer	0.5 µL
DNA Template	2 µL
Nuclease free water	7 µL

Table 3: Primers sequences used for qRT-PCR

Gene	Forward Primer	Reverse Primer
Sod2	5′-CGACCTGCCCTACGACTACG-3’ (20)	5’-TGACCACCACCATTGAACTT-3’(20)
Gpx1	5’-CCACCGTGTATGGCCTTCTCC-3’ (20)	5’-GATCGTGGTGCCTCAGAGAG-3’(20)
Cat	5’-GCCTGGGACCCAATTATCTT-3’(20)	5’-GAATCTCCGCACTTCTCCAG-3’ (20)
TrxR1	5’-CGTGGTGGACTTCTCTGCTACC GTGGTG-3’ (27)	5’-GGTCGGCATGCATTTGACTTCACA GTC-3’ (27)
β-Actin	5´-GTCGGTGAAGGGGACTTACA-3´	5´-TTCATACAGCAGGCAAGCAC-3´

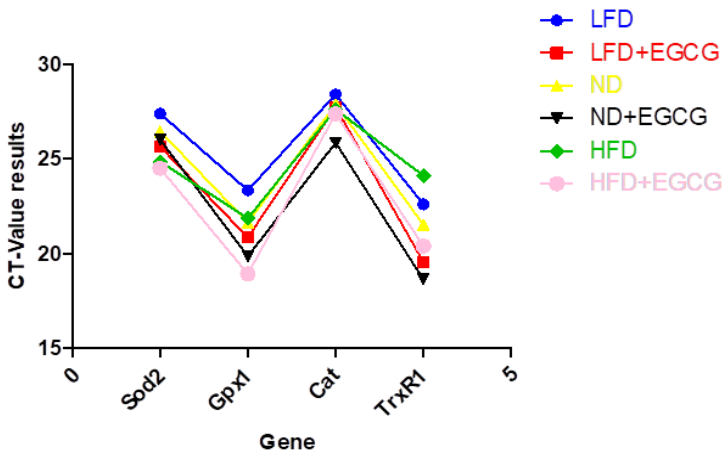

Figure 3: Effects of EGCG on mice eating diets enriched with or without EGCG on the various levels of gene expression of antioxidant enzymes.

Table 4: P-value of effect EGCG on differential gene expression levels of antioxidant enzymes using Step One real-time RT-PCR in mice

A. P-value of the effect of EGCG on differential gene expression levels of antioxidant enzymes after diet treatment		
Diet treatment	**P-value**	**Level of significant (s or ns)**
Diet without EGCG	0,4241	ns
Diet with EGCG	0,2541	ns
Diets with or without EGCG	0, 0004	s
B. P-value of the effect of EGCG on differential gene expression levels of antioxidant enzymes after exercise treatment		
Exercise treatment	P-value	Level of significant (s or ns)
Exercise without EGCG	0.5547	ns
Exercise with EGCG	0, 5149	ns
Exercise with or without EGCG	0.0037	s

Table 5: Tukey's Multiple Comparison Test of the effect of diets on differential gene expression levels of antioxidant enzymes using Step one real-time RT-PCR in mice subjected to a diet supplemented with or without EGCG.

Tukey's Multiple Comparison Test	**Mean Diff.**	**q**	**Significant? P < 0.05?**	**Summary**	**95% CI of diff**
CT LFD vs CT LFD+EGCG	1,989	4,602	Yes	*	0.003063 to 3.975
CT LFD vs CT ND	1,059	2,449	No	ns	-0.9274 to 3.044
CT LFD vs CT ND+ EGCG	2,848	6,590	Yes	**	0.8621 to 4.834
CT LFD vs CT HFD	0,3255	0,7531	No	ns	-1.660 to 2.311
CT LFD vs CT HFD +EGCG	3,134	7,251	Yes	**	1.148 to 5.120
CT LFD+EGCG vs CT ND	-0,9305	2,153	No	ns	-2.916 to 1.055
CT LFD+EGCG vs CT ND+ EGCG	0,8590	1,988	No	ns	-1.127 to 2.845
CT LFD+EGCG vs CT HFD	-1,664	3,849	No	ns	-3.649 to 0.3224
CT LFD+EGCG vs CT HFD +EGCG	1,145	2,649	No	ns	-0.8412 to 3.131
CT ND vs CT ND+ EGCG	1,789	4,140	No	ns	-0.1964 to 3.775
CT ND vs CT HFD	-0,7330	1,696	No	ns	-2.719 to 1.253
CT ND vs CT HFD +EGCG	2,075	4,802	Yes	*	0.08931 to 4.061
CT ND+ EGCG vs CT HFD	-2,522	5,836	Yes	**	-4.508 to -

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					0.5366
CT ND+ EGCG vs CT HFD +EGCG	0,2857	0,6612	No	ns	-1.700 to 2.272
CT HFD vs CT HFD +EGCG	2,808	6,498	Yes	**	0.8223 to 4.794

Table 6: Results of the CT-Value analysis of the effect of EGCG on the different gene expression levels of antioxidant enzymes in mice consuming a diet supplemented with or without EGCG.

CC	CT LFD	CT LFD+ EGCG	CT ND	CT ND+ EGCG	CT HFD	CT HFD+EGCG
Sod2	27,396	25,661	26,457	26,019	26,856	24,504
Gpx1	23,349	20,853	21,646	19,850	21,867	18,936
Cat	28,411	27,738	27,893	25,855	27,618	25,382
TrxR1	22,609	19,557	21,535	18,649	24,122	20,408

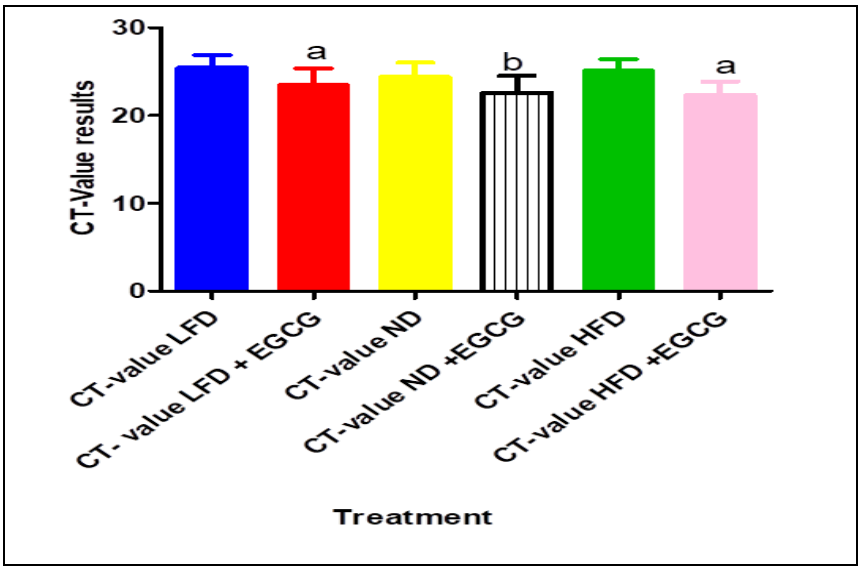

Figure 4: Results of the CT-Value analysis of the effect of EGCG on different levels of gene expression of antioxidant enzymes in mice fed diets containing or lacking EGCG. Bars with letter **a** was statistically significant from the control group and bar with letter **b** was not statistically significant.

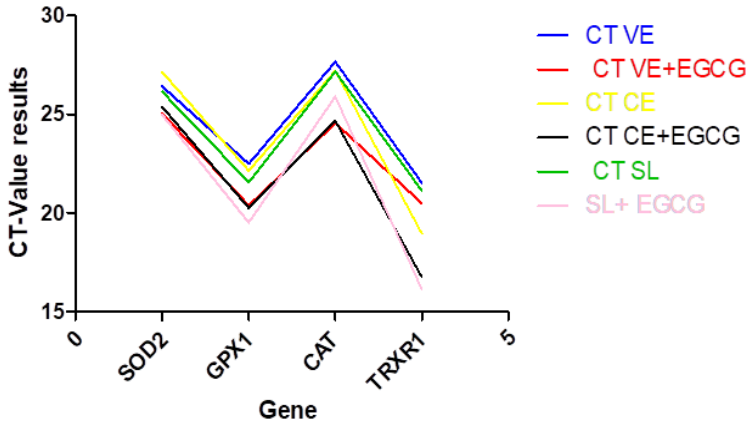

Table 8: Tukey's Multiple Comparison Test of the effect of exercise on differential gene expression levels of antioxidant enzymes using Step one real time RT-PCR in mice subjected to diet supplemented with or without EGCG.

Tukey's Multiple Comparison Test	Mean Diff.	q	Significant ? P < 0.05?	Summary	95% CI of diff
CT VE vs CT VE+EGCG	1,910	3,722	No	ns	-0.4481 to 4.268
CT VE vs CT CE	0,6643	1,295	No	ns	-1.694 to 3.022
CT VE vs CT CE+EGCG	2,762	5,383	Yes	*	0.4042 to 5.120
CT VE vs CT SL	0,5238	1,021	No	ns	-1.834 to 2.882
CT VE vs SL+ EGCG	2,892	5,635	Yes	*	0.5337 to 5.249
CT VE+EGCG vs CT CE	-1,245	2,427	No	ns	-3.603 to 1.112
CT VE+EGCG vs CT CE+EGCG	0,8523	1,661	No	ns	-1.506 to 3.210
CT VE+EGCG vs CT SL	-1,386	2,701	No	ns	-3.744 to 0.9718
CT VE+EGCG vs SL+ EGCG	0,9818	1,913	No	ns	-1.376 to 3.340
CT CE vs CT CE+EGCG	2,098	4,088	No	ns	-0.2601 to 4.456
CT CE vs CT SL	-0,1405	0,2738	No	ns	-2.498 to 2.217
CT CE vs SL+ EGCG	2,227	4,341	No	ns	-0.1306 to 4.585
CT CE+EGCG vs CT SL	-2,238	4,362	No	ns	-4.596 to 0.1196
CT CE+EGCG vs SL+ EGCG	0,1295	0,2524	No	ns	-2.228 to 2.487
CT SL vs SL+ EGCG	2,368	4,614	Yes	*	0.009941 to 4.726

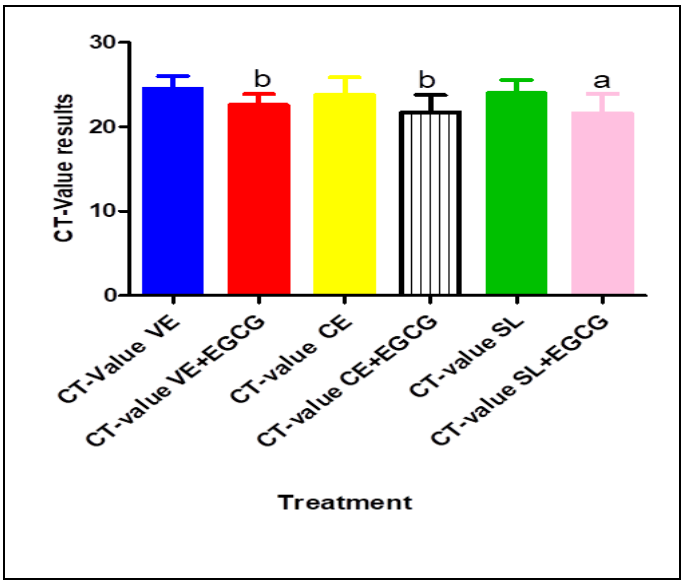

Figure 6: CT-Value results of the effect of EGCG on differential gene expression levels of antioxidant enzymes in mice subjected to exercise supplemented with or without EGCG. Bars with letter **A** was statistically significant from the control group and the bar with letter **b** was not statistically significant.