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Effect of Tea Catechins on Regulation of Antioxidant Enzyme Expression in H_2O_2-Induced Skeletal Muscle Cells of Goat in Vitro

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ABSTRACT: Skeletal muscle cells (SMCs) of goats were stress induced with 1 mM H_2O_2 in the absence or presence of 0.5, 5, and 50 μg/mL tea catechins (TCs) incubation. Cells were harvested at 48 h postincubation with TCs to investigate the effects of TCs on cell proliferation, cell membrane integrity, antioxidant enzyme activities, and antioxidant enzyme genes and protein expression levels. Results showed that H_2O_2 induction inhibited cell proliferation with or without TC incubation; moreover, the inhibition effect was enhanced in the presence of TCs ($P < 0.001$). H_2O_2-induced stress increased the lactate dehydrogenase (LDH) activity in the absence or presence of TC incubation, but concentrations of TCs, less than 5 μg/mL, showed protective functions against LDH leakage than in other H_2O_2-induced treatments. The catalase (CAT) activity increased when SMCs were stress induced with H_2O_2 in the absence or presence of TC incubation ($P < 0.001$). H_2O_2-induced stress decreased CuZn superoxide dismutase (CuZn-SOD) and glutathione peroxidase (GPx) activities, whereas this effect was prevented by incubation with TCs in a concentration-dependent manner. H_2O_2-induced stress with or without TC incubation had significant effects on mRNA and protein expression levels of CAT, CuZn-SOD, and GPx ($P < 0.001$). CAT and CuZn-SOD mRNA expression levels were increased by different concentrations of TC incubation, and this tendency was basically consistent with corresponding protein expression levels. The GPx mRNA expression level increased with a low concentration of TCs but decreased with concentrations greater than 5 μg/mL of TCs, whereas GPx protein expression in all TC-incubated groups was lower than in the control treatment. The current findings imply that TCs had an inhibitory effect on cell proliferation and enhanced damage to the cell membrane integrity, but TCs affected antioxidant status in SMCs by modulating antioxidant enzyme activities at mRNA and protein expression levels.

KEYWORDS: Tea catechins, cell proliferation, membrane integrity, antioxidant enzyme

INTRODUCTION

The oxidation of membrane phospholipids is associated with the loss of meat quality because of reduced shelf life as a result of rancid odor formation.[1,2] A growing body of evidence suggests that excessive reactive oxygen species (ROS), which impair cellular membranes, organelles, and DNA, are one of the most important causes of lipid oxidation.[3] Organisms have endogenous enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx), to protect cells against ROS-induced oxidation;[4] these enzymes may not be in physiologically proportionate amounts to counteract excessive pro-oxidants.[5] It has been well demonstrated that alternative practices of exogenous antioxidants supplies became feasible to improve the antioxidant activity of animal products.[6−8] Although synthetic antioxidants have been widely used in the meat industry, natural antioxidants have been proven to be better than synthetic ones due to lower toxicity and higher safety in animal products.[9,10]

Tea catechins (TCs), a predominant group of polyphenols present in green tea leaves (*Camellia sinensis* L.), comprise mainly four compounds, namely, (−)-epicatechin (EC), (−)-epicatechin gallate (ECG), (−)-epigallocatechin (EGC), and (−)-epigallocatechin gallate (EGCG). Our previous studies have shown that dietary TC supplementation inhibited lipid oxidation in *longissimus dorsi* muscle (LD), improved meat color stability, and increased the ratio of polyunsaturated fatty acid to monounsaturated fatty acid in LD of goats.[11,12] However, the underlying mechanisms are not yet well understood. It will be beneficial if the antioxidant capacity of TC in modulating lipid oxidation in meat could be probed at the gene and protein expression levels.

Various properties of skeletal muscle cells (SMCs), including high metabolic activity and high concentration of myoglobin, render it particularly susceptible to injury by free radicals. Indeed, cellular injuries from ROS have been implicated in lipid peroxidation in cell membranes, which reduces meat quality.[13,3] Dietary antioxidants can be delivered to the muscle to counteract the action of ROS together with the native antioxidant defense system.[14] Lamosová et al. reported that Rooibos tea extract inhibited the growth of chick embryonic primary SMCs due to potent ROS-scavenging activity.[15] Murase et al. suggested that mice fed TCs combined with exercise increased fatty acid β-oxidation in skeletal muscle.[16] On the basis of the above evidence, TCs can exert antioxidant activities to regulate antioxidant status in SMCs. If this is the case, the underlying reasons

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Table 1. Sequences of Primers (Forward, For; Reverse, Rev), Primer Sequences Size, and PCR Products Size of Real-Time Quantitative RT-PCR

target genes	primers (5′→ 3′)	primer size (bp)	product size (bp)
CuZn-SOD, for	TGCAGGCCCTCACTTTAATC	20	207
CuZn-SOD, rev	CTGCCCAAGTCATCTGGTTT	20	
GPx, for	ACATTGAAACCCTGCTGTCC	20	216
GPx, rev	TCATGAGGAGCTGTGGTCTG	20	
CAT, for	TGGGACCCAACTATCTCCAG	20	178
CAT, rev	AAGTGGGTCCTGTGTTCCAG	20	
β-actin, for	CCAACCGTGAGAAGATGACC	20	201
β-actin, rev	CGCTCCGTGAGAATCTTCAT	20	

of meat quality improvement by dietary TC supplementation in goats may be attributed to the alteration of antioxidant status in SMCs in the presence of TC delivery to the muscle. Therefore, further studies are needed to validate this inference.

The objectives of this study were to determine effects of TC incubation on cell proliferation, cell membrane integrity, antioxidant enzyme activities, and antioxidant enzyme mRNA and protein expression in H_2O_2 stress -induced SMCs of goats.

MATERIALS AND METHODS

Chemicals. All chemicals and cell culture reagents were obtained from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated. Primary antibodies were purchased from Abcam Co. (San Francisco, CA). Secondary antibodies and all Western blot reagents were ordered from KenGen Co. (Nanjing, China).

The TCs (purity of 80.11%) were isolated from green tea leaves (*C. sinensis L.*) by high pressure liquid chromatography (HPLC) (model Waters 600, Waters Corp., Milford, MA) according to the procedure of Nonaka et al.[17] The extracted TCs contained (+)-catechin and (−)-catechin (DL-C) (1.61%), EC (5.77%), EGC (0.47%), ECG (13.03%), (−)-gallocatechin gallate (GCG) (1.56%), and EGCG (57.67%).

Isolation and Culture of Primary SMC of Goat. All experimental procedures complied with the animal care and use guidelines of the Animal Care Committee, Institute of Subtropical Agriculture, the Chinese Academy of Sciences (Changsha, China). One male goat at the age of 8 months was sacrificed, and 10 g of LD was quickly removed from the left side of the carcass to prepare SMC culture as described by Lynge et al.[18] The SMC was cultured according to the method reported by Lamosová et al.[15] Briefly, the isolated cells were seeded at a density of 2.0×10^5 cells/mL on two 96-well plates and 2×10^6 cells/mL in 90 25 cm^2 flasks in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (Sigma Aldrich), 100 U of penicillin-streptomycin/mL, and 5 μg/mL gentamicin. The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2/95% O_2.

After 24 h of preculture, cells were treated with 0.5, 5, and 50 μg/mL TC, respectively, for 45 h and subsequently were stress-induced with 1 mM H_2O_2 in the absence or presence of TC followed by additional 3 h incubation. Each treatment was replicated six times. Cells were harvested at 48 h postincubation with TC. Media in two 96-well plates were used to analyze cell proliferation and cell membrane integrity, respectively. Cells in 30 flasks were disrupted in media and centrifuged, and then, the supernatant was used to analyze antioxidant enzyme activities. Cells in other flasks were collected by centrifugation to analyze antioxidant enzyme mRNA and protein expression levels.

3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide (MTT) Assay for Cell Proliferation. Cell proliferation after culturing for 48 h in the presence of TC and H_2O_2 was assayed using a Cell Growth Determination kit based upon the use of MTT (Sigma Aldrich). This assay is based on the cellular reduction of a MTT salt into formazan product by mitochondrial enzymes; therefore, the metabolic activity of proliferation cells was assessed. Data were calibrated by measuring the absorbance at 570 nm according to previous reports.[19,20]

Cell Membrane Integrity [Lactate Dehydrogenase (LDH) Release Assay]. Media in 96-well plate were collected and centrifuged from all tested wells after H_2O_2 induction in the absence or presence of 48 h-incubated TC to test LDH leakage into the growth media using an in vitro LDH activity assay kit (Jiancheng Biology Co., Nanjing, China). The unit of LDH activity was expressed as U/L.

Antioxidant Enzyme Activity Assays. The CAT activity was determined using the method reported by Aebi,[21] where residual H_2O_2 is determined as a yellow "peroxy titanium sulfate". One unit of CAT activity is defined as the amount of CAT required to decompose 1 mmol of H_2O_2 per min at pH 7.5 and 25 °C. The CuZn-superoxide dismutase activity was measured according to the method of McCord and Fridovich.[22] One unit of CuZn superoxide dismutase (CuZn-SOD) activity is defined as the amount of required SOD to inhibit the maximum rate of cytochrome C reduction by 50%. The GPx activity was measured spectrophotometrically according to the method of Levander et al.[23] Enzyme activities were expressed as units of enzyme activity per mg protein. The total protein content of cells was estimated by the method of Lowry et al.[24] To prevent the TC interference with Folin reagent, protein was precipitated using trichloroacetic acid and phosphortungstic acid according to the method of Lindeboom and Wanasundara[25] before Lowry assay.

RNA Isolation and cDNA Synthesis. Total RNA from SMC was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of isolated total RNA was quantified with a ND-1000 UV−vis spectrophotometer (NanoDrop Ltd., TX). Then, 1 μg of RNA was treated with RNase-Free DNase I (#EN0523, Fermentas Inc., Ontario, CA) to remove contaminating DNA. Thereafter, first-standard cDNA was synthesized using a RevertAidTM First Strand cDNA Synthesis Kit (K1622, Fermentas Inc., Ontario, CA). The cDNA products were separated by agarose gel electrophoresis (1.5% agarose), visualized with UV light after ethidium bromide staining, and then were stored at −20 °C for real-time quantitative RT-PCR (qRT-PCR).

Real-Time Quantitative RT-PCR. Oligonucleotide primers of CuZn-SOD, CAT, GPx, and β-actin gene were designed using Premier 5 software (Premier Co., Canada). The details of the oligonucleotide primer sequences, primer lengths, and predicted amplified product lengths are listed in Table 1. To quantify gene expression, synthesized cDNA was subjected to real-time qRT-PCR to measure all genes in a total reaction volume of 10 μL using a Power SYBR Green PCR Master Mix (2×) (PN44367659, Applied Biosystems Inc., Foster City, CA). Briefly, the real-time qRT-PCR reaction components were 5 μL of mix (1×), 1 μL of cDNA template (20 ng), 1 μL of forward primer (400 nM), 1 μL of reverse primer (400 nM), and 2 μL of DEPC-treated distilled water. Reactions were conducted on an ABI 7900 HT fast real-time qRT-PCR system (Applied Biosystems Inc.) that combined 384-well plate comparability with fully automated robotic loading using the following cycling conditions: 95 °C for 10 min holding, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. In addition, β-actin cDNA was amplified in parallel for all genes to provide an appropriate internal PCR control. For each experiment, a nontemplate reaction served as a negative control. The real-time qRT-PCR products of all the genes were evaluated in comparison with the real-time qRT-PCR product of β-actin. Changes in mRNA expression level determined from the real-time qRT-PCR were calculated according to the $2-\Delta\Delta CT$

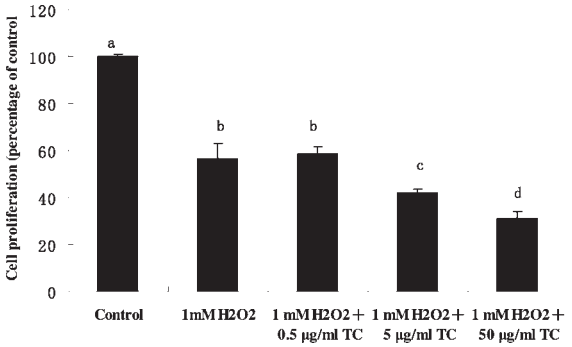

Figure 1. Effects of TC incubation on cell proliferation of H_2O_2-induced SMCs. Cell proliferation was determined by the MTT assay. Results were expressed as a percentage of control and listed as means ± SEs ($n = 6$). Mean values with different letters differ significantly ($P < 0.05$).

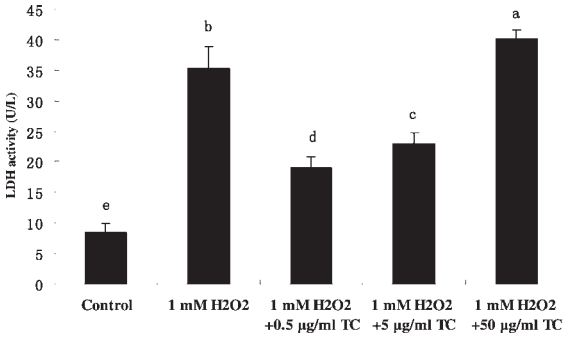

Figure 2. Effects of TC incubation on cell membrane integrity of H_2O_2-induced SMCs. Results were indicated by LDH activities in media and expressed as means ± SEs ($n = 6$). Mean values with different letters differ significantly ($P < 0.05$).

method,[26] where $-\Delta\Delta CT = -(\Delta CT$ experiment group $- \Delta CT$ control group) and $\Delta CT = CT$ samples $-$ CT β-actin.

Western Blot Analysis. Total proteins of SMC were extracted using a Total Protein Extraction Kit (KEP210, KeyGEN Biotech, Nanjing, China) and measured by the method of Lowry et al.[23] Equivalent quantities of protein were denatured at 100 °C for 10 min and separated by reducing 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gel. The isolated proteins were transferred into Polyvinylidene Fluoride membranes (Millipore, United States) with 0.45 μm apertures at a constant 200 mA for 70 min. Molecular weight standards of protein (Fermentas, United States) were run simultaneously. Nonspecific binding sites of the membranes were blocked overnight in 5% bovine serum albumin (BSA) and 0.1% Tween. Then, membranes were washed and subsequently incubated in the presence of CuZn-SOD, CAT, GPx, and β-actin primary antibodies (Abcam Com., ab62800, ab50434, ab36140, and ab15263, United Kingdom) at a concentration of 0.02, 3, 2, and 2 μg/mL, respectively, for 24 h at 4 °C. Thereafter, the protein affinity with primary antibodies was tested by corresponding horseradish peroxidase conjugated secondary antibodies for 2 h at 4 °C. The intensity of each protein was detected by chemiluminescent reaction performed with a Super ECL Detection Kit (KeyGEN Biotech, Nanjing, China). Densitometric signals were obtained after exposure to an X-ray film. The band intensities were quantified by Quantity One software (Bio-Rad) and normalized versus β-actin as an internal control for total protein loading. The relative expression levels of three antioxidant enzymes were expressed as the ratio of band intensities of enzymes to β-actin.

Statistical Analysis. Data were analyzed by GLM followed by Duncan's multiple range tests of SAS.[27] Statistical significance was declared at $P \leq 0.05$.

RESULTS

Cell proliferations were inhibited when SMCs were stress induced by 1 mM H_2O_2 in the absence or presence of 0.5, 5, and 50 μg/mL TC incubation (Figure 1). Moreover, TC enhanced the inhibitory effect of H_2O_2 induction in a concentration-dependent manner, and cell proliferations of SMCs decreased with increasing concentrations of TC ($P < 0.001$).

The LDH activities in all H_2O_2-induced groups increased ($P < 0.001$) regardless of the absence or presence of TC (Figure 2). However, 0.5 and 5 μg/mL TC incubation decreased the damage of H_2O_2 stimulation, while a concentration of 50 μg/mL TC enhanced the damage of H_2O_2 to cell membrane integrity.

The CAT activity increased when SMCs were stimulated with H_2O_2 in the absence or presence of TC incubation ($P < 0.001$) (Table 2); moreover, high concentrations greater than 5 μg/mL of TC incubation increased enzyme activities more than a low concentration of the TC group. Stress induction with H_2O_2 decreased CuZn-SOD and GPx activities. As a whole, the effect of TC incubation on enzyme activities showed a concentration-dependent manner. Concentrations of 0.5 and 5 μg/mL TC increased CuZn-SOD activities but decreased GPx activity in SMCs, whereas 50 μg/mL TC incubation showed a contrary pattern.

CAT mRNA expression levels were elevated 7-fold by H_2O_2 induction without TC and 1–6-fold in the presence of TC ($P < 0.001$) (Table 2). The mRNA expression levels of CuZn-SOD decreased by H_2O_2 induction and increased 4–6-fold by TC incubation in the presence of H_2O_2 ($P < 0.001$). The mRNA expression level of GPx decreased by H_2O_2 induction, but 0.5 μg/mL TC incubation enhanced its transcript level ($P < 0.001$).

CAT protein expression levels increased by H_2O_2 incubation with or without TC ($P < 0.001$), and the highest level was found in 50 μg/mL TC and H_2O_2 incubation group (Figure 3). CuZn-SOD protein expression levels decreased by H_2O_2 induction, but concentrations between 0.5 and less than 5 μg/mL of TC increased protein levels 2–3-fold. The GPx protein expression levels increased by H_2O_2 induction without TC preincubation, and TC incubation with H_2O_2 induction decreased GPx protein expression levels ($P < 0.001$).

DISCUSSION

Skeletal muscle can be readily exposed to oxidative stress caused by an imbalance between pro-oxidants and anti-oxidants.[28] Removal of toxic oxygen metabolites is the putative function of antioxidant enzymes. The regulation of these enzymes is important to improve antioxidant status of animal products. We used primary SMCs to study the effect of TC to improve antioxidant status of meat and prevent oxidative stress because primary SMC preserves most of its native functions and is useful in studying muscle at the cellular level.[29] Because literature is scarce on the measurement of normal physiological concentrations of polyphenols in muscles of goats, the criteria of TC concentration used in this study for cellular assay were referenced by our preliminary study and a previously related report.[30,31]

ROS induce a number of molecular alterations in cellular components, which lead to changes in cell proliferation.[32] One

Table 2. Effects of TC Incubation on Antioxidant Enzyme Activities and mRNA Expression Levels in H_2O_2-Induced SMCs[a]

	treatment						
item	control	1 mM H_2O_2	1 mM H_2O_2 + 0.5 μg/mL TC	1 mM H_2O_2 + 5 μg/mL TC	1 mM H_2O_2 + 50 μg/mL TC	SEM[b]	*P* value
			antioxidant enzyme activity (U/mg prot)				
CAT	5.15 c	6.99 b	6.65 b	8.46 a	8.05 a	0.16	<0.001
CuZn-SOD	19.1 b	15.9 c	24.3 a	25.6 a	19.0 b	0.44	<0.001
GPx	3.24 b	1.90 c,d	1.75 d	2.21 c	4.66 a	0.15	<0.001
			mRNA expression of antioxidant enzymes[c]				
CAT	1.00 e	7.07 a	1.39 d	4.26 c	6.15 b	0.11	<0.001
CuZn-SOD	1.00 c	0.88 c	4.05 b	6.58 a	6.50 a	0.19	<0.001
GPx	1.00 b	0.60 c	1.42 a	0.47 c,d	0.40 d	0.04	<0.001

[a] Mean values with different letters in the same column differ significantly ($P < 0.05$). [b] SEM, standard error of mean. [c] mRNA expression levels were calculated according to the $2-\Delta\Delta CT$ method.[26]

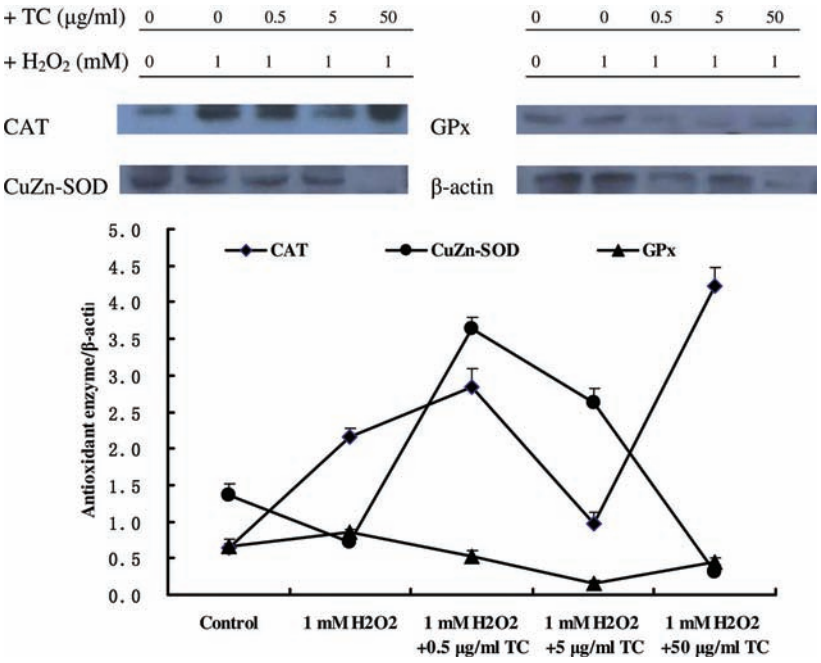

Figure 3. Effects of TC incubation on relative expression levels of antioxidant enzymes in H_2O_2-induced SMCs ($n = 6$). The five bands in each Western blot analysis picture were control, 1 mM H_2O_2, 1 mM H_2O_2 + 0.5 μg/mL TC, 1 mM H_2O_2 + 5 μg/mL TC, and 1 mM H_2O_2 + 50 μg/mL TC from left to right.

possible way of preventing ROS-mediated cellular injuries is to augment the endogenous oxidative defenses by consumption of natural antioxidants, such as TC.[33] Some studies revealed that TC reversed the inhibitory effect of ROS or other oxidants on cell proliferation.[34,35] However, our present results are not consistent with those studies. The current results showed that a high concentration of TC, greater than 5 μg/mL, decreased cell proliferation more than that of cells exposed to H_2O_2. This discrepancy may be due to different cell culture models and different concentrations of TC used in these studies. Overdose of TC incubation may be toxic to cells, leading to cell injury, and this inference could be validated by the cell membrane integrity seen the present study. In fact, Khafif et al. reported that TC had an inhibitory effect on cell proliferation by blocking cell cycle progression.[36]

Leakage of the membrane-bound enzyme LDH is a useful indicator to assess the cytotoxicity of chemicals.[37] It is well-known that DNA strand breaks occur when cells are exposed to H_2O_2.[38] In the present study, higher LDH activity in H_2O_2-induced group suggested that SMC suffered remarkable cell membrane damage. Although concentrations of 0.5 and 5 μg/mL TC protected SMC from injury, a higher concentration of 50 μg/mL caused even more serious damage to SMC. A similar study reported that fruit polyphenolics suppressed oxidative stress-induced SMC damage in vitro, with the protective capacity displayed in a dose-dependent manner.[39]

In the present study, CAT activity increased and CuZn-SOD and GPx activities decreased when SMC were exposed to H_2O_2. All antioxidant enzyme activities increased in a concentration dependent manner when SMC were incubated with TC. Thus, it

is apparent that the responses of each enzyme to H_2O_2 or TC differ. The mechanisms of regulation of antioxidant enzyme activity and mRNA expression levels by ROS or TC have been well established.[40−42] The regulation of gene expression by ROS is related to nuclear factor-κB and AP-1,[43] while tea polyphenols have been suggested to activate redox-sensitive transcription factors to induce gene expression of antioxidant enzymes.[44,45] Therefore, different target molecules may be the main reason of different expression patterns of CAT, CuZn-SOD, and GPx. The elevated CuZn-SOD and CAT may be beneficial in scavenging super oxide anion radicals and H_2O_2, respectively.

Changes in CAT and CuZn-SOD mRNA expression or enzyme activities in SMC exposed to H_2O_2 and TC basically paralleled the changes in the corresponding protein levels; however, we did not found this correlation in GPx. Apparently, translational and post-translational regulation is important in determining these changes. It has been well demonstrated that antioxidant enzyme activities and gene expression will be affected when cells are exposed to oxidants or antioxidants. Moreover, CAT activity was found to be a major determinant of cellular resistance to H_2O_2 toxicity in different H_2O_2 resistant cell lines.[46] Röhrdanz and Kahl reported that CAT mRNA expression levels and enzyme activities decreased during rat hepatocyte culture, but exposure of hepatocytes to H_2O_2 prevented this decrease, and the CuZn-SOD mRNA expression level was not affected by H_2O_2 treatment.[47] Chan et al. revealed that CuZn-SOD activity increased consistently with the changes of mRNA levels in rat brain astrocytes.[48] Another study also demonstrated that incubation with soybean polyphenols increased SOD and CAT activities and their mRNA expression levels in porcine SMC.[49] This is partially consistent with the present results. Our results suggest that TC increased CAT and CuZn-SOD mRNA and protein expression levels and enzyme activities when SMC were exposed to H_2O_2, and this may be the potential mechanism of improved oxidative stability in meat by dietary TC supplementation.

Although the antioxidant enzymes work together in the defense against ROS, the mechanisms responsible for the regulation of the gene and protein expression can differ.[50,51] In the present study, no uniform change tendency was found among GPx mRNA levels, protein expression levels, and enzyme activities. The biochemical function of GPx is to reduce lipid hydroperoxides to their corresponding alcohols and to reduce free H_2O_2. Moreover, GPx is a glutathione-dependent enzyme, and glutathione can sometimes control GPx activity[52] because higher TC concentration in cells will enhance glutathione depletion.[45] Moreover, a change in mRNA level may not necessarily reflect that of corresponding proteins, especially in muscle where there is suppressed protein synthesis and increased protein degradation.[53] Decreased glutathione levels may cause abnormal GPx mRNA and protein expression levels in SMC in the presence of TC.

In conclusion, TC inclusion had an inhibitory effect on cell proliferation and damaged cell membrane integrity. However, TC incubation at concentrations less than 5 μg/mL increased CAT and CuZn-SOD mRNA and protein expression levels and enzyme activities, and this may be a possible mechanism to explain the effect of TC on improved oxidative stability in goat meat.

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ABBREVIATIONS USED

TCs, tea catechins; SMCs, skeletal muscle cells; LDH, lactate dehydrogenase; CAT, catalase; CuZn-SOD, CuZn superoxide dismutase; GPx, glutathione peroxidase; ROS, reactive oxygen species; EC, (−)-epicatechin; ECG, (−)-epicatechin gallate; EGC, (−)-epigallocatechin; GCG, (−)-gallocatechin gallate; EGCG, (−)-epigallocatechin gallate; LD, *longissimus dorsi* muscle; HPLC, high pressure liquid chromatography; DMEM, Dulbecco's modified Eagle's medium; MTT, 3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide; RTq-PCR, real-time quantitative RT-PCR

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