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Research Article

Effect of Grape Seed Extract on the Oxidative and Proliferative Status of Porcine Intestinal Epithelial Cells

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Abstract

The aim of this study was to investigate the oxidative and proliferative effects of grape seed extract (GSE). Piglet intestinal epithelial cells (IPEC1) were selected as an unstressed cell model, or they were exposed to 400 μM H_2O_2 to establish a H_2O_2-stimulated cell model. The glutathione (GSH) and total antioxidant capacity in response to GSE addition were tested in the unstressed and H2O2-stimulated cell models. The relative mRNA levels of antioxidant or antioxidant enzymes and apoptosis-related genes were measured by Real-Time RT-PCR. In the unstressed status, the cell survival ratio and GSH increased with the addition of GSE at 1 and 10 μg/mL but diminished at 60 μg GSE/mL. The addition of 1 μg/mL GSE upregulated the mRNA expression levels of B-Cell Lymphoma protein-2 (Bcl-2), cysteine aspartases-3 (Caspase-3), cysteine aspartases-8 (Caspase-8) and glutathione peroxidase-1(GPx-1), while it downregulated that of Bcl2-associated X protein (Bax), copper-zinc superoxide dismutase, glutathione S-transferase (GST), thioredoxin, thioltransferase and thioredoxin reductase. As GSE reached 60 μg/mL, the tumor protein p53 (p53) and caspase-8 gene expressions were upregulated. In stressed status, 1 and 10 μg GSE/mL promoted the increase of GSH. H2O2-induced increases in Bax, p53, and Caspase-3 mRNA expressions were attenuated by the subsequent addition of 1 μg GSE/mL and promoted the gene expression of tumor necrosis factor-α, GPx-1 and thioltransferase (Ttas). Treatment with 60 μg GSE/ml resulted in a significant reduction in Bax, p53, manganese superoxide dismutase and GST mRNA expressions. These results indicate that GSE exhibits antioxidative and proliferative functions on unstressed IPEC1 cells at low and medium levels and oxidative and antiproliferative functions at high levels.

Keywords: *Grape seed extract, Antioxidation, Proliferation, Intestinal epithelial cells*

Üzüm Çekirdeği Ekstraktının Domuz Barsak Epitel Hücreleri Üzerine Oksidatif ve Proliferatif Etkileri

Öz

Bu çalışmanın amacı üzüm çekirdeği ekstraktının (ÜÇE) oksidatif ve proliferatif etkilerini araştırmaktır. Domuz barsak epitel hücreleri (IPEC1) strese maruz kalmamış veya 400 μM H_2O_2 uygulanarak H_2O_2 ile uyarılmış modeller olarak kullanıldı. ÜÇE ilavesinde strese maruz kalmayan ve H_2O_2 ile uyarılmış modellerde glutatyon (GSH) ve total antioksidan kapasite tespit edildi. Antioksidanlar veya antioksidan enzimler ile apoptozisle ilişkili genlerin orantısal mRNA seviyesi gerçek zamanlı RT-PCR ile ölçüldü. Strese maruz kalmayanlarda hücre hayatta kalma oranı ve GSH seviyesi 1 ve 10 μg/mL miktarlarında ÜÇE ilavesi ile artarken 60 μg/mL ile azalma gösterdi. 1 μg/mL ÜÇE ilavesi; B-hücre Lenfoma protein-2 (Bcl-2), sistein aspartaz-3 (Kaspaz-3), sistein aspartaz-8 (Kaspaz-8) ve glutatyon peroksidaz-1 (GPx-1) mRNA ekspresyonlarında upregulasyona neden olurken Bcl2-alakalı X protein (Bax), Bakır-çinko süperoksit dismutaz, glutatyon S-transferaz (GST), thioredoksin, thioltransferaz ve thioredoksin reduktaz mRNA ekspresyonlarında downregulasyona neden oldu. 60 μg/mL ÜÇE; tümör protein p53 (p53) ve kaspaz-8 gen ekspresyonunda upregulasyona yol açtı. Strese maruz kalınmada 1 ve 10 μg/mL miktarlarında ÜÇE; GSH miktarındaki artmayı destekledi. H2O2 ile oluşturulan Bax, p53 ve kaspaz-3 mRNA ekspresyonlarındaki artmalar 1 μg/mL ÜÇE ilavesi ile düşürülürken tümör nekroz faktör-α, GPx-1 ve thioltransferaz (Ttas) gen ekspresyonları arttı. 60 μg/mL ÜÇE uygulaması Bax, p53, manganez süperoksit dismutaz ve GST mRNA ekspresyonlarında anlamlı oranda azalmaya neden oldu. Bu sonuçlar; strese maruz kalmamış olan IPEC1 hücrelerinde düşük ve orta seviyede dozlarda ÜÇE uygulamasının antioksidatif ve proliferatif, yüksek dozda ise oksidatif ve antiproliferatif etkileri olduğunu göstermektedir.

Anahtar sözcükler: *Üzüm çekirdeği ekstraktı, Antioksidasyon, Proliferasyon, Bağırsak epitel hücreleri*

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INTRODUCTION

Grape seed extract (GSE) comprises diverse types of bioactive phenolic substances, such as anthocyanin, flavanol and resveratrol, and can effectively scavenge the reactive oxidative species (ROS) of mammalian cells [1]. Moreover, relevant evidence suggests that a series of enzymes are altered at the gene and/or protein levels in response to GSE in certain cells; this alteration brings about a wider interest in how GSE removes excessive ROS in organic systems [1].

In practical applications, GSE has been used extensively to alleviate damage from harmful chemical substances and fatty foods, particularly in terms of antioxidation and antiapoptosis. The exact mechanisms of the damaging effects of a wide range of chemical substances, such as cisplatin, cadmium, doxorubicin and cyclosporine A, as well as high-fat diets, are not fully understood; however, the formation of free radicals, which lead to oxidative stress, is primarily responsible, and the generation of ROS, depletion of glutathione (GSH), inhibition of antioxidant enzyme activity and lipid peroxidation are common signs of these damaging processes [2,3]. The generation of massive ROS is a strong signal of the initiation and stimulation of cell apoptosis, further inducing serious damage, whereas the experimental co-administration of GSE successfully counteracts the apoptotic effect, with a significant decrease in the apoptotic index and improvement in antioxidant capacity [4,5].

Grape seed extract, however, shows oxidative and apoptotic effects in some cases. GSE has been widely utilized to deter the growth of diverse types of cancers, such as breast cancer [6] and liver cancer [7], primarily by facilitating apoptosis in these cancer cells. Although the mechanisms by which GSE causes apoptosis are still controversial, increasing evidence points to the excessive generation of ROS that is induced by GSE. Some studies have found that GSE-mediated apoptosis is remarkably reversed by antioxidants.

Therefore, the objective of this study was to investigate the effect of different concentrations of GSE on the *in vitro* oxidative and proliferative status of porcine intestinal epithelial cells under unstressed and H_2O_2-stressed conditions.

MATERIAL and METHODS

Materials

Commercially available, dried and powdered GSE obtained from Tarac Technologies (Nuriootpa, South Australia) contained 5.01% (+)-catechin, 4.78% (2)-epicatechin, 2.35% (2)-epigallo-catechin, 14.1% dimericproanthocyanidin, 11.60% trimericproanthocyanidins, 7.69% tetramericproanthocyanidins and 40.0% polymeric proanthocyanidins.

Cell Culture and Treatment

Piglet intestinal epithelial cells were kindly provided by Dr. Bi-e Tan from the Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha, China. Cells were grown in DMEM/F-12 (1:1) medium supplemented with 5% fetal calf serum, 5 µg/mL insulin, 5 µg/mL transferrin, 5 ng/mL selenium, 5 ng/mL epidermal growth factor and 1% penicillin-streptomycin under standard culture conditions. The growth medium was changed every other day until nearly 80% confluence was reached. Subsequently, the cells were harvested by treatment with 0.25% trypsin and 0.53 mM EDTA. Without a specific indication, IPECs were used between passages 42 and 48 and starved of FBS for 24 h before any experimental treatment. For all studies, GSE was dissolved in dimethyl sulfoxide (DMSO) as a 200 mg/mL stock solution and diluted as desired directly in the medium.

To characterize the effect of GSE on IPEC under unstressed and stressed conditions, two cell models were prepared as follows: one group of cells was separately cultured in the medium with 1, 10 and 60 µg GSE/mL for 24 h as the unstressed cell model, and the other group of cells was subjected to oxidative stress insult through incubation with 400 µM H_2O_2 for 1 h before being treated with GSE, as with the former group. Notably, after being incubated for 1 h with 400 µM H_2O_2, the stressed cells were washed twice with PBS to remove the H_2O_2 to avoid a reaction between the H_2O_2 and the GSE. Untreated cells that served as the control were also run in parallel and subjected to the same changes in the medium but were not supplemented with either GSE or H_2O_2.

Cell Survival Ratio Test

After treatment, the cells were washed twice with PBS to eliminate interference in the subsequent assay and were seeded in a 96-well plate (1×10^4 cells/well). The cell survival ratio was assessed by using a CCK-8 commercial kit (Dojindo, China) according to the manufacturer's instructions.

Total Antioxidant Capacity and GSH Measurement

The total antioxidant capacity (TAOC) was measured using the azino-diethyl-benzthiazoline sulfate (ABTS) method. In this assay, the incubation of ABTS with H_2O_2 and a peroxidase (metmyoglobin) resulted in the production of the blue-green radical cation $ABTS^+$, whereas the antioxidants in the tested samples suppressed the production of this color, which was proportional to their concentrations [8]. The system was standardized using Trolox, a water-soluble vitamin E analog. The results were expressed as mmol Trolox equivalents/protein concentration of plasma supernatants of cells lysed [9]. The measurement of GSH was based on a Glutathione Quantification Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

RNA Isolation and Real-Time RT-PCR

These studies were performed as previously described [10] *(Table 1)*.

Statistical Analysis

Data are presented as the mean ± SEM for analysis. Statistical differences were analyzed by one-way ANOVA or Mann-Whitney U tests, as appropriate. $P<0.05$ was considered to indicate statistically significant differences [11].

Table 1. *Oligonucleotide primer sequences for the real-time PCR reactions*

Target Gene	Orientation	Primer (5′-3′)
Catalase	Forward	CAGCTTTAGTGCTCCCGAAC
	Reverse	AGATGACCCGCAATGTTCTC
Mn-SOD	Forward	TGCAGGCCCTCACTTTAATC
	Reverse	CTGCCCAAGTCATCTGGTTT
CuZn-SOD	Forward	CAGGTCCTCACTTCAATCC
	Reverse	CCAAACGACTTCCASCAT
GPx1	Forward	TGGGGAGATCCTGAATTG
	Reverse	GATAAACTTGGGGTCGGT
GPx2	Forward	GACATCAAGCGCCTCCTC
	Reverse	AGACCAGAAAGGCAAGGTTC
GPx4	Forward	GATTCTGGCCTTCCCTTGC
	Reverse	TCCCCTTGGGCTGGACTTT
GST	Forward	TTTTTGCCAACCCAGAAGAC
	Reverse	GGGGTGTCAAATACGCAATC
Trx	Forward	GCTGCCAAGATGGTGAAGCAGATT
	Reverse	GCAACATCCTGACAGTCATCCACA
TR	Forward	GCTTTGGAGTGCGCTGGATTTCTT
	Reverse	CGTGAAAGCCCACAACACGTTCAT
Ttas	Forward	CCTGTCAGCATGGCTCAAGCATTT
	Reverse	ATCCACCAGGAAGCGCTGTCATTA
Bax	Forward	CTACTTTGCCAGTAAACTGG
	Reverse	TCCCAAAGTAGGAGAGGA
Bcl-2	Forward	GGAGCTGGTGGTTGACTTTC
	Reverse	CTAGGTGGTCATTCAGGTAAG
TNF-α	Forward	CGTTGTAGCCAATGTCA
	Reverse	TAGGAGACGGCGATGC
p53	Forward	GTCACGAACTGGCTGGATG
	Reverse	GAAGGGACAAAGGACGACAG
Caspase-3	Forward	TCTAACTGGCAAACCCAAACTT
	Reverse	AGTCCCACTGTCCGTCTCAAT
Caspase-8	Forward	TCCCAGGATTTGCCTC
	Reverse	AAGCCAGGTCATCACTGTC
β-Actin	Forward	CTGCGGCATCCACGAAACT
	Reverse	AGGGCCGTGATCTCCTTCTG

RESULTS

Cell survival ratios are presented in *Fig. 1*. Cells were actively growing and had the greatest proportions of viable cells under the unstressed status with the addition of 1 and 10 μg GSE/mL. However, instead of continuing this growing trend, the addition of 60 μg GSE/mL notably inhibited ($P<0.01$) the cell survival ratio compared to the control. The treatment with H_2O_2 resulted in marked decreases ($P<0.01$) in cell survival ratios in comparison with the control, and the low cell survival ratio rebounded after the addition of 1 and 10 μg GSE/mL, even though this ratio decreased with the addition of 60 μg/mL GSE.

Cell anti/oxidant status was evaluated by TAOC assay. As shown in *Fig. 2,* the TAOC numerically increased after cells were incubated with 1 or 10 μg GSE/mL under the unstressed status, but this increase did not reach statistical significance ($P>0.05$). In contrast, the TAOC level was found to decrease to less ($P<0.05$) than half that of the control after the 60 μg GSE/mL treatment. Exposure to H_2O_2 was associated with a nonsignificant decline in the TAOC. The subsequent addition of GSE, regardless of the doses applied in our study, also did not show a significant difference ($P>0.05$) in TAOC, although a marginal elevation was shown at 10 μg GSE/mL compared to the control.

In the unstressed cells, GSH increased with GSE doses from 0 to 10 μg/mL, as shown in *Fig. 3,* but was followed by a pronounced depletion ($P<0.01$) of GSH, which was approximately 30% of that in the control cells. The level of GSH in the H_2O_2-treated IPEC1 was also less than the control, but this difference was unremarkable. Following treatment with 1 and 10 μg GSE/mL, the intracellular GSH level was notably enhanced ($P<0.05$) compared with the H_2O_2-treated IPEC1, but little or no effect on GSH level was observed when GSE was added at 60 μg/mL.

The alterations in the gene expression related to anti-oxidation and apoptosis in response to GSE addition in

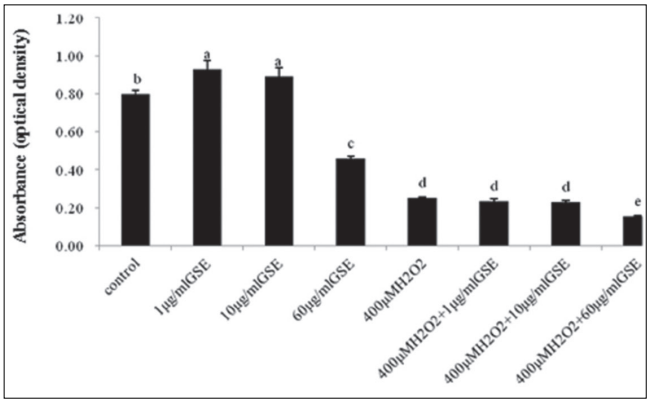

Fig 1. Alteration of the cell survival ratio after IPEC1 cells were incubated with different grape seed extract (GSE) concentrations for 24 h with or without H_2O_2 pretreatment. Bars represented mean ± SEM (n = 6). Bars not sharing a common letter differ *(P*<0.05)

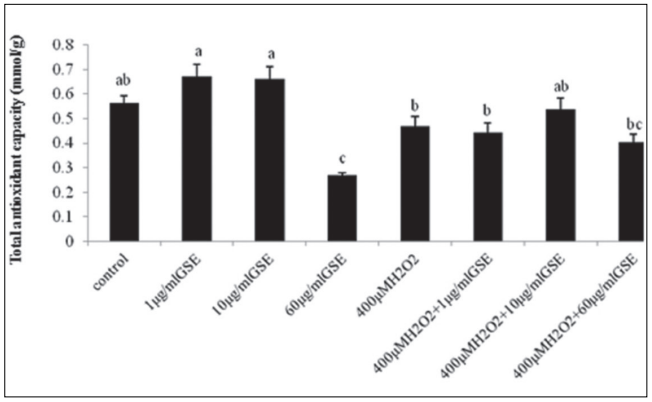

Fig 2. Alteration of total antioxidant capacity (TAOC) after IPEC1 cells were incubated with different grape seed extract (GSE) concentrations for 24 h with or without H_2O_2 pretreatment. Bars represented mean ± SEM (n = 3). Bars not sharing a common letter differ *(P*<0.05)

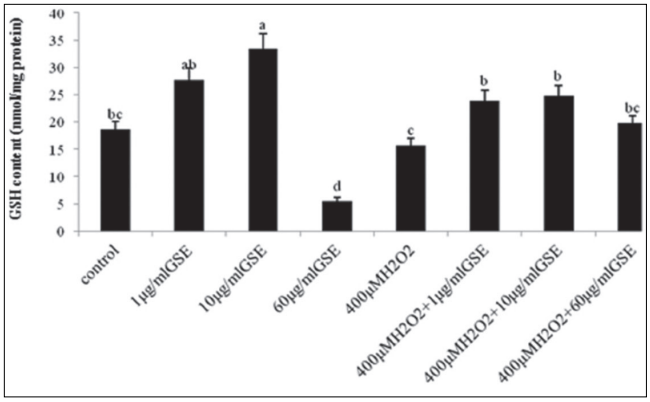

Fig 3. Alteration of glutathione (GSH) after IPEC1 cells were incubated with different grape seed extract (GSE) concentrations for 24 h with or without H_2O_2 pretreatment. Bars represented mean ± SEM (n = 3). Bars not sharing a common letter differ *(P*<0.05)

Table 2. *Effects of grape seed extract (GSE) (*μg GSE/mL*) on the mRNA expression of antioxidant or antioxidant enzymes in IPEC1 pretreated with or without H_2O_2*

Item	Control	Unstressed IEPC1			400 μM H_2O_2-stressed IPEC1				SEM	*P* Value
		1	10	60	H_2O_2-stressed	1	10	60		
Catalase	1.01^{ab}	0.83^{bc}	1.25^{a}	0.70^{bcd}	0.46^{cd}	0.34^{d}	0.58^{cd}	0.50^{cd}	0.11	<0.005
GuZn-SOD	1.01^{a}	0.37^{bc}	0.57^{b}	0.21^{c}	0.15^{c}	0.26^{c}	0.45^{bc}	0.21^{c}	0.07	<0.001
Mn-SOD	1.02^{b}	0.87^{b}	0.84^{b}	0.40^{c}	1.44^{a}	1.69^{a}	0.92^{b}	0.43^{c}	0.37	<0.005
GPx-1	1.04^{b}	1.60^{a}	1.33^{ab}	0.32^{c}	0.59^{c}	1.60^{a}	0.89^{bc}	0.48^{c}	0.20	<0.005
GPx-2	1.07^{b}	0.70^{b}	0.97^{b}	0.86^{b}	1.11^{b}	1.58^{ab}	2.02^{a}	1.30^{ab}	0.26	<0.005
GPx-4	1.00^{a}	1.20^{a}	0.91^{a}	0.37^{b}	0.35^{b}	0.44^{b}	0.93^{a}	0.37^{b}	0.14	<0.005
GST	1.01^{a}	0.36^{c}	0.68^{ab}	0.47^{c}	0.66^{ab}	0.34^{c}	0.41^{c}	0.47^{c}	0.11	<0.005
Trx	1.01^{a}	0.42^{b}	0.20^{c}	0.40^{b}	0.13^{c}	0.23^{bc}	0.10^{c}	0.23^{bc}	0.05	<0.001
Ttas	1.03^{a}	0.36 ^{bc}	0.26^{c}	0.15^{c}	0.16^{c}	0.63^{b}	0.13^{c}	0.18^{c}	0.07	<0.001
TR	1.00^{b}	0.73^{c}	0.51^{cd}	0.24^{d}	1.22^{b}	1.10^{b}	0.40^{d}	1.68^{a}	0.10	<0.005

$^{a-d}$ Means within a row with different superscripts differ (P<0.05)

Table 3. *Effects of grape seed extract (GSE) (*μg GSE/mL*) on the mRNA expression of apoptosis-related genes in IPEC1 with or without H_2O_2 pretreatment*

Item[1]	Control	Unstressed IEPC1			400 μM H_2O_2-stressed IPEC1				SEM	*P* Value
		1	10	60	H_2O_2-stressed	1	10	60		
Bax	1.01^{b}	0.59^{c}	0.23^{d}	0.58^{c}	2.65^{a}	0.79^{bc}	0.11^{d}	0.17^{d}	0.24	<0.001
Bcl-2	1.03^{c}	2.73^{a}	1.88^{b}	0.70^{c}	1.42^{bc}	2.38^{ab}	2.10^{ab}	1.34^{bc}	0.43	<0.005
p53	1.12^{c}	6.97^{c}	11.53^{bc}	22.84^{b}	35.53^{a}	6.30^{c}	7.16^{c}	13.29^{bc}	4.01	<0.001
TNF-α	1.08^{b}	1.89^{ab}	1.48^{b}	1.03^{b}	1.71^{b}	3.07^{a}	2.76^{a}	1.59^{b}	0.65	<0.005
Caspase-3	1.10^{c}	4.01^{a}	3.44^{ab}	1.64^{bc}	5.01^{a}	2.46^{b}	2.89^{b}	4.34^{a}	0.66	<0.005
Caspase-8	1.08^{d}	4.19^{b}	2.24^{cd}	6.53^{a}	3.14^{bc}	2.92^{bc}	2.81^{bc}	1.63^{cd}	0.47	<0.005

$^{a-d}$ Means within a row with different superscripts differ (P< 0.05)

IPEC1 are listed in *Table 2* and *Table 3*. In the unstressed status, the addition of a low level of GSE (1 μg/mL) facilitated (P<0.05) the mRNA expression levels of glutathione peroxidase-1 (GPx-1), B-Cell Lymphoma protein-2 (Bcl-2), cysteine aspartases-3 (caspase-3) and cysteine aspartases-8 (caspase-8) genes, while suppressing (P<0.05) the mRNA expression levels of copper-zinc superoxide dismutase (CuZn-SOD), glutathione S-transferase (GST), thioredoxin (Trx), thioltransferase (Ttas), and thioredoxin reductase (TR), Bcl2-associated X protein (Bax) genes. A relatively high level of GSE at 10 μg/mL also increased Bcl-2 and caspase-3 but not caspase-8 and GPx-1 expression,

whereas it decreased the expression of the Bax, CuZn-SOD, Trx, Ttas and TR genes. As the GSE dose reached 60 μg/mL, a markedly positive effect ($P<0.05$) on the mRNA expression levels of p53 and caspase-8 genes was observed, but the Bax gene, as well as the antioxidant genes CuZn-SOD, manganese superoxide dismutase (Mn-SOD), GPx-1, GPx-4, GST, Trx, Ttas, and TR, were inhibited ($P<0.05$).

In H_2O_2-stressed cells, increases in the mRNA expression levels of p53 and caspase-3 were dramatically attenuated by the subsequent addition of 1 μg GSE /mL, but this addition of 1 μg GSE/mL also increased the mRNA expression level of tumor necrosis factor α (TNF-α). Meanwhile, 1 μg GSE/mL promoted ($P<0.05$) the GPx-1 and TtasmRNA expression that was suppressed by stress with H_2O_2 but reduced ($P<0.05$) the mRNA expression level of GST. The addition of 10 μg GSE/mL also alleviated the Bax, p53, caspase-3 and GST expression but enhanced TNF-α mRNA expression. Differences between the addition of 1 μg and 10 μg GSE/mL were noted with the addition of 10 μg GSE/mL, which increased GPx-2 and GPx-4 mRNA expression instead of increasing GPx-1 and TtasmRNA expression, and decreased Mn-SOD mRNA expression. Incubation with 60 μg GSE/mL caused a significant reduction in the mRNA expression levels of the Bax, p53, Mn-SOD and GST, in addition to a significant increase in the mRNA expression level of TR.

DISCUSSION

In this study, the contribution of GSE to the TAOC at low and medium levels (1 and 10 μg/mL, respectively) was moderate in unstressed or stressed IPEC1, but GSH was increased at 10 μg GSE/mL in unstressed cells, as well as at 1 and 10 μg/mL in the stressed cells. These results may represent the first direct evidence that a certain level of GSE can elevate the GSH contents in intestinal epithelial cells, although a few related studies have reported that dietary supplementation with GSE promotes an increase in GSH in the rat liver, the hippocampus and human serum [12,13]. Evidence indicates that some compositions of GSE (such as Quercetin, Epicatechin and Epicatechingallate) have the ability to stimulate GRed and γ-GCS activity, which act as the rate-limiting and key enzymes, respectively, in two major processes of GSH synthesis [14], suggesting that GSE accelerated the synthesis of GSH.

Glutathione exerts great influence on the regulation of the apoptosis/survival balance of cells exposed to damaging stimuli. Cells that undergo apoptosis actively extrude GSH, abruptly creating a redox imbalance that favors the formation of ROS and protein disulfides [15], and antioxidants do not hamper apoptotic GSH efflux but instead protect GSH-depleted cells from apoptosis [16], indicating the importance of GSH serving as antioxidant. Further investigation has revealed more details on the GSH's role in maintaining critical protein sulfhydryls and donating hydrogens that are necessary for DNA repair, synthesis and expression [17]. GSH is a cofactor for anti-oxidative enzymes such as GPx and GST [17] and regulates relative transcriptional factors, such as nuclear factor kappa B and NF-E2-related factor 2, which take over cell survival/apoptosis [18]. Therefore, GSE's promotion of GSH presumably could explain why GSE exposure at the medium level promoted cell proliferation in unstressed cells.

As the addition of GSE reached 60 μg/mL, a decrease in the cell survival ratio was accompanied by a sharp decline in the TAOC and GSH, which was observed in the unstressed IPEC1 cells. Oxidative stress is well known to be the consequence of an imbalance between the formation of ROS and TAOC [19]. The low-level TAOC suggested that the antioxidant barrier was unable to fend off the amount of ROS present, and cells were prone to accumulate a mass of ROS. Moreover, the GSH-depleted cells could passively allow oxidative stress to take place, contributing to the formation of protein (S-) glutathionylation, which is thought to be an early molecular event in apoptosis induction [20]. Additionally, GSH depletion induces increased mitochondrial ROS exposure and cytochrome c release, representing another mechanism behind cells' commitment to apoptosis [21]. Therefore, a combination of a reduced cell survival ratio and the increase in ROS observed herein reinforced the previous suggestion that GSE exerts the ability to inhibit cell proliferative in IPEC1 cells by a ROS-mediated mechanism. Meanwhile, it is reasonable to believe that GSE at a high dose switches the cell from proliferation to antiproliferation, thereby partly accounting for the transformation of the role of the GSE from antioxidant to oxidant.

Interestingly, unstressed IPEC1 cells treated with 60 μg GSE/mL, compared to IPEC1 cells directly exposed to 400 μM H_2O_2 only, showed less TAOC and GSH but a higher cell survival ratio. One rational explanation is that the ROS generated by the excess GSE was different from that generated in the H_2O_2-stressed cells, and comparatively, H_2O_2 was presumably much more potent for inducing apoptosis, thereby resulting in a lower cell survival ratio. H_2O_2 is recognized as a strong oxidant and mediator of apoptosis, and millimolar concentrations of H_2O_2 can directly induce apoptosis [22]. The types of ROS generated by GSE and how they are generated remain elusive.

In our previous work, GSE was shown to have the capability to regulate a series of antioxidant enzymes that impacted the anti/oxidative status of lamb muscle cells in a chemical manner [1]. Similarly, the direct exposure of IPEC1 cells to 1 μg GSE/mL led to the downregulated mRNA expression of genes such as CuZn-SOD, GST, Trx, Ttas, and TR, with only GPx-1 being upregulated. With the addition of GSE at 10 μg/mL, the CuZn-SOD, Trx, Ttas and TR genes remained suppressed, and even the upregulation of GPx-1 was aborted. CuZn-SOD, GST, Trx,

Ttas and TR are critical antioxidant enzymes or proteins in organic systems that control the reduced intracellular redox environment. CuZn-SOD and GST are responsible for converting free radical superoxide to H_2O_2 and providing protection against electrophiles and products of oxidative stress, respectively [14,23]. The thioredoxin system comprises Trx and TR plus NADPH and functions in thiol-dependent thiol-disulfide exchange reactions, namely, the reduction of protein disulfides [24]. Ttas represents a pivotal partner with the Trx system in redox regulation, especially for ribonucleotide reduction [25]. Thus, the inhibition of these antioxidant enzymes and proteins seemingly run counter to our former conclusion that GSE at low and medium levels exerted primarily an antioxidant effect. It may still be premature to discuss how GSE regulated these genes, mainly due to the conflict between the sophisticated transcriptional mechanism of these genes and the relatively few studies in this field. Glutathione (GST) is regulated by at least 100 structurally diverse xenobiotics and chemicals. The ROS is the regulator of GST, and GST shows an adaptive upregulation response to ROS [26], which presumably explains, in part, why GST was suppressed by low levels of GSE that was supposed to create an antioxidant environment.

As the dose of GSE reached 60 μg/mL, several antioxidant genes, such as CuZn-SOD, Mn-SOD, GPx-1, GPx-4, GST, Trx, Ttas and TR, were inhibited, which, combined with the abovementioned evidence (remarkably decreased TAOC and GSH), suggested that the cells were subjected to strong oxidative stress. These results also suggested that GSE at high concentrations induced the generation of ROS, not only through the depletion of GSH but also through the downregulation of a number of crucial genes of antioxidant enzymes and proteins, further restraining the antioxidant function of these enzymes and proteins and the elimination of ROS.

In the stressed cells, 1 μg/mL GSE also reduced GST mRNA expression but promoted GPx-1 and Ttas mRNA expression that were suppressed by H_2O_2. In the group receiving 10 μg/mL GSE, instead of GPx-1 being upregulated, two isoforms of GP-x (GPx-2 and GPx-4) were dramatically upregulated. Enhanced mRNA expression of GPx-2 at the medium level of GSE in stressed cells was demonstrated in our previous study of the effects of GSE on lamb primary muscle cells as well [1]. Unfortunately, the mRNA expression of catalase in our work was not influenced by GSE in the stressed cells, and the treatment with 400 μmol H_2O_2 was speculatively beyond the handling capacity of the GPxs. Therefore, this may explain why the cell survival ratio barely recovered with GSE at 1 and 10 μg GSE/mL doses in stressed cells, even though the mRNA expression level of GPx was upregulated.

Apoptosis negatively affects the cell survival ratio, and several genes that modulate apoptosis varied differently in response to the GSE in our work. In unstressed cells, the addition of 1 μg and 10 μg GSE/mL promoted the mRNA expression level of Bcl-2 and mitigated that of Bax in the IPEC1 cells, thus leading to the downregulation of Bax/Bcl-2 ratio. The Bcl-2 and Bax belong to the Bcl-2 protein family, which is important in mitochondrial-mediated apoptotic events having either a proapoptotic or antiapoptotic function. In prior studies, Bax has been found to play an important role in GSE-induced apoptosis, which is blocked by the knockdown of Bax through Bax siRNA transfection to 4T1 cells [27]. A previous study verified that the ratio of Bax to Bcl-2 proteins is the determining factor in the transmission of the apoptotic signal [28]. The present study demonstrated that the proliferative effects of low and moderate levels of GSE on unstressed IPEC1 cells may be mediated by modulating the Bax/Bcl-2 ratio.

The most noteworthy variation at 60 μg GSE/mL in the unstressed IPEC1 cells was the exponential increase in the mRNA expression of p53. p53 uses various cellular inputs to regulate apoptosis and proliferation [29]. The ROS-mediated DNA damage and ROS-activated signaling pathways, such as the MAPK-mediated pathways, are able to activate p53 [30]. In our work, cellular exposure to H_2O_2 showed remarkable upregulation of the mRNA expression of p53. The results indicated that the addition of 60 μg GSE/mL inhibited the proliferation of the unstressed IPEC1 cells by increasing the p53.

In stressed cells, low and medium levels of GSE also exhibited antiapoptosis potential with the downregulation of the mRNA expression levels of Bax, p53, and caspase-3, but an increase in the TNF gene expression also suggested the apoptosis potential of GSE. The apoptosis mediated by TNF occurs through the extrinsic apoptosis pathway, unlike the intrinsic apoptosis pathway, which relies primarily on the Bcl-2 protein family and involves the acceptance of extracellular ligand-binding stimulation to initiate apoptosis [31]. The present results indicated that GSE regulates apoptosis-related genes differently in different circumstances.

In summary, the GSE primarily exerted an antioxidant and proliferative effect on unstressed IPEC1 cells at low and medium levels and an oxidative and antiproliferative effect at the high levels. Studies are needed to further verify the effect of GSE *in vivo*, possibly expanding the use of GSE supplementation to animal studies.

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