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SAMBUCUS EBULUS L. FRUIT AQUEOUS INFUSION MODULATES GCL AND GPX4 GENE EXPRESSION

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Abstract

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Glutamate-cysteine ligase and glutathione peroxidase 4 are enzymes involved in cellular antioxidant defense. Biologically active compounds, such as polyphenols, may modulate their gene expression. *Sambucus ebulus* L. is a plant, which fruit infusions exhibits high antioxidant activity, due to its high polyphenol content. We analyzed the effect of *Sambucus ebulus* fruit aqueous infusion on glutamate-cysteine ligase catalytic subunit and glutathione peroxidase 4 gene expression levels in the presence and in the absence of oxidizing agent tert-butyl hydroperoxide in a 3T3-L1 preadipocyte cell culture model. Gene expression was analyzed by Real-Time qPCR, and relative gene expression levels were calculated using $2^{-\Delta\Delta Ct}$ method. Viability of treated preadipocytes was evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromid reduction assay. In the range of 1.25% to 12.5% content of the infusion in the culture medium cell viability was not affected significantly. Glutamate-cysteine ligase mRNA levels were increased significantly ($p < 0.05$) after treatment. Pretreatment decreased tert-butyl hydroperoxide-induced gene expression of glutamate-cysteine ligase ($p < 0.05$) and of glutathione peroxidase 4 ($p < 0.05$). These data suggest that *Sambucus* fruit infusion exhibits strong antioxidant activity by modulating glutamate-cysteine ligase and glutathione peroxidase 4 gene expression. The study provides first scientific data about the antioxidant activity of *Sambucus ebulus* fruit infusion in a cell culture model.

Key words: antioxidant, glutamate-cysteine ligase, glutathione peroxidase 4, *Sambucus ebulus*, 3T3-L1cells
Abbreviations: GCLc – glutamate-cysteine ligase catalytic subunit; GPx4 – glutathione peroxidase 4; GSH – gluthatione; SE – *Sambucus ebulus*; FAI – fruit aqueous infusion; tButOOH – tert-butyl hydroperoxide; Real-Time qPCR – Real Time quantitative polymerase chain reaction; ROS – reactive oxygen species; cAMP – cyclic adenosine monophosphate; PKA – protein kinase A; ARE/EpRE – antioxidant response element/electrophile response element; CREB – cAMP response element binding protein

Introduction

GCL and GPx4 are enzymes involved in cell antioxidant defense. The cells respond to oxidative stress by increasing the expression of GCLc (Kobayashi et al., 2009). Thus, compounds modulating positively antioxidant enzymes gene expression may be considered potentially protective against oxidizing agents. Polyphenols are known to increase GSH levels by activating expression of GCLc (Myhrstad et al., 2002; Moskaug et al., 2005). Another enzyme, which activity is related to GSH levels, is GPx4, and its expression is modulated by polyphenols (Oliva et al., 2011).

Fruit juices, jams, teas from fruits and other preparations from dry and fresh fruits are rich in polyphenol compounds contributing to their antioxidant properties. *Sambucus ebulus* L. is a plant which fruit and flower infusions and extracts exhibit high antioxidant activity, due to high polyphenol content (Tasinov et al., 2012; Hosseinimehr et al., 2007;

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Ebrahimzadeh et al., 2008). However, by the mechanism of boosted antioxidant defense is not clear. Therefore, we aimed to explore the effect of SE FAI on the expression of GCLc and GPx4 as a part of the cell antioxidant defense in 3T3-L1 preadipocytes in norm and in oxidatively challenged cells.

Materials and Methods

Plant material: *Sambucus ebulus* fruits collected from Northeastern Bulgaria in July-August, 2011 were dried at room temperature. SE FAI was prepared from 150 mg fruits finely ground and extracted three times with 3 ml of dH_2O for 3 min on vortex, at room temperature. After centrifuging (5 min, 3500 r.p.m.) the supernatants were collected and diluted to 15ml with PBS buffer (pH = 7.4). Cells were treated for 24 hours with: 100μM tButOOH and/or with different concentrations of SE FAI in the culture medium. After treatment, the cells were lysed and total RNA extracted.

Cell culture: 3T3-L1 preadipocyte cells (ATCC, Manassas, VA, USA) were cultured as previously described (Kiselova-Kaneva et al., 2012).

Cell viability: Viability of treated cells was evaluated using MTT reduction assay as previously described (Kiselova-Kaneva et al., 2012).

Gene expression: GCLc and GPx4 genes expression was analyzed using Real-Time qPCR as previously described (Kiselova-Kaneva et al., 2012). Relative gene expression levels were calculated using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). The sequences of the primers (Alpha DNA, Canada, and Sigma-Aldrich, Germany) for each gene are indicated in Table 1.

Statistical analysis: Data are presented as mean±SEM. All measurements were performed in triplicate. Graph Pad Prism 5.0 software was used for statistical analysis (*t*-test), $p < 0.05$ was considered as significant.

Results and Discussion

Prior to gene expression analyses the cytotoxicity of SE FAI on 3T3-L1 cells was evaluated. The infusion in concentrations in the range of 1.25% to 12.5% content in medium did not affect cell viability significantly (Figure 1), so the concentrations of 2.5%, 5% and 10% of SE FAI were used for the treatments in all subsequent experiments.

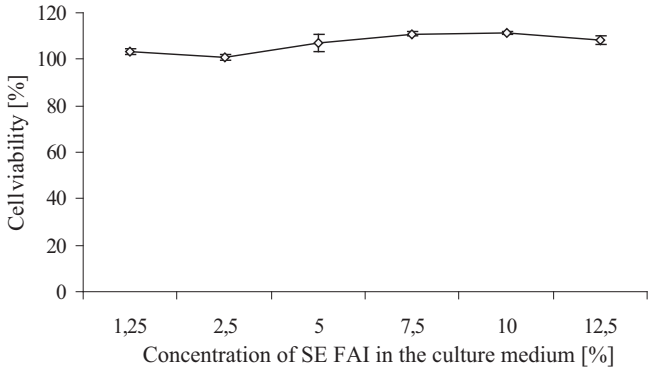

Fig. 1. Viability of 3T3-L1 cells treated with different concentrations of SE FAI. Data are presented as mean±SEM

Real-Time qPCR analyses demonstrated that GCLc mRNA levels were increased after treatment with SE FAI (Figure 2) and the increase was statistically significant

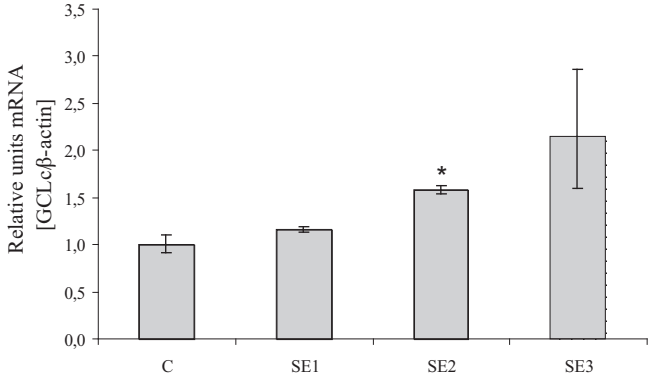

Fig. 2. Changes of GCLc gene expression in 3T3-L1 preadipocytes treated with different concentrations of SE FAI. Data are presented as mean±SEM. *p < 0.05 (compared to control group)
Legend: **C – control group (untreated cells); SE1 – 2.5%, SE2 – 5%, SE3 – 10% SE FAI in the culture medium**

Table 1

Oligonucleotide sequences for primer sets used in Real-Time qPCR

Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)
GCLc	AATGGAGGCGATGTTCTTGAG	CAGAGGGTCGGATGGTTGG
GPx4	CCCCACTGCGCTCATGA	GGCACACCGGAGACCAAA
β-Actin	CAAGAAGGAAGGCTGGAAAAG	ACGGCCAGGTCATCACTATTG

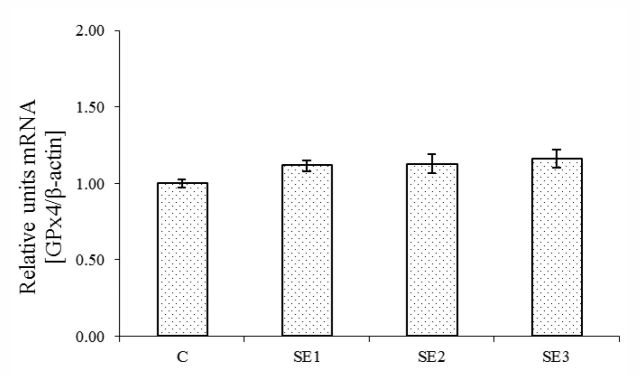

Fig. 3. Changes of GPx4 gene expression in 3T3-L1 preadipocytes treated with different concentrations of SE FAI. Data are presented as mean±SEM.
***Legend:* C – control group (untreated cells); SE1 – 2.5%, SE2 – 5%, SE3 – 10% SE FAI in the culture medium**

when SE FAI was applied in 5% concentration in the medium ($p < 0.05$). GPx 4 gene expression was not affected significantly by the addition of the infusion (Figure 3). We assume that SE affects the antioxidant defense by modulating the expression of key enzymes involved in GSH metabolism, the effect most likely mediated by polyphenol compounds in the infusion (Tasinov et al., 2012; Myhrstad et al., 2002; Moskaug et al., 2005). The involvement of cAMP-PKA-dependent signaling pathway is implicated in the activity of anthocyanins, demonstrated to induce CREB-regulated up regulation of GCLc transcription (Zhu et al., 2012), thus increasing GSH synthesis and protecting the cells against ROS. Another signaling pathway involving ARE/EpRE-mediated regulation of GCLc is suggested to be in the basis of the mechanism of action of flavonoids (Moskaug et al., 2005).

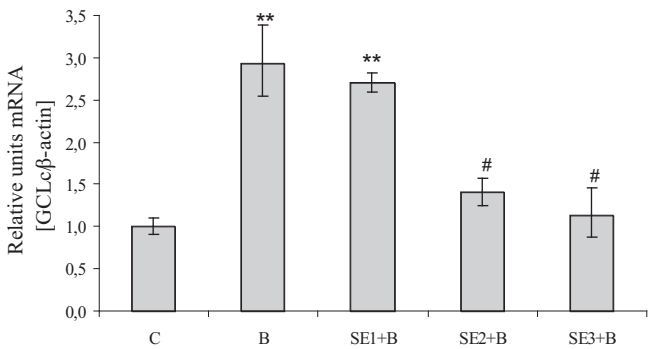

Fig. 4. Changes of GCLc gene expression in 3T3-L1 preadipocytes pretreated with different concentrations of SE FAI and treated with tButOOH.
Data are presented as mean±SEM.
****p < 0.01 (compared to control group),**
#p < 0.05 (compared to tButOOH group)
***Legend:* C – control group (untreated cells); B – 100μM tButOOH; SE1 – 2.5%, SE2 – 5%, SE3 – 10% SE FAI in the culture medium**

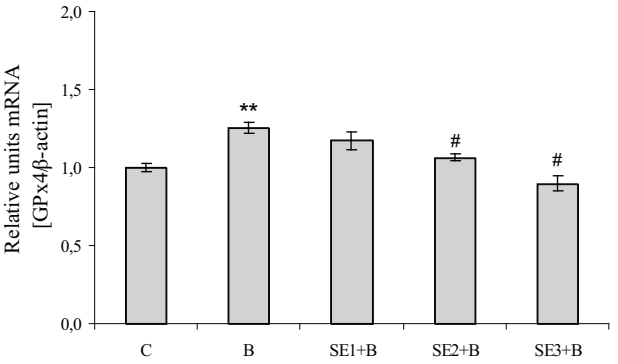

Fig. 5. Changes of GPx4 gene expression in 3T3-L1 preadipocytes pretreated with different concentrations of SE FAI and treated with tButOOH.
Data are presented as mean±SEM.
****p < 0.01 (compared to control group),**
#p < 0.05 (compared to tButOOH group)
***Legend:* C – control group (untreated cells); B – 100μM tButOOH; SE1 – 2.5%, SE2 – 5%, SE3 – 10% SE FAI in the culture medium**

Pretreatment with the infusion when applied in 5% and 10% concentrations decreased significantly tButOOH-induced gene expression of GCLc ($p < 0.05$) (Figure 4) and that of GPx4 ($p < 0.05$) (Figure 5). The effect may be contributed on the one hand to the direct radical trapping activity of the plant polyphenols acting as reductants and forming products with much lower activity. Alternatively, polyphenols may regulate cellular redox state through regulation of different signaling pathways activating cellular antioxidant defense systems.

In conclusion, we may assume that SE fruits infusion exhibit their antioxidant activity by modulating GCLc and GPx4 gene expression in a cell culture model. The fruits could be considered a good natural source of antioxidants in physiological conditions involving oxidative stress.

Acknowledgments

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