Centaurium erythraea extract reduces redox imbalance and improves insulin expression and secretion in pancreatic β-cells exposed to oxidative and nitrosative stress


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Abstract: Oxidative stress is one of the major mechanisms that underlies the damage of pancreatic β-cells and defects in insulin secretion in diabetes. As herbal preparations can alleviate oxidative stress through their redox-active secondary metabolites, in this study we investigated the cytoprotective effects of Centaurium erythraea extract (CEe) against H₂O₂- and SNP-induced oxidative/nitrosative stress in Rin-5F β-cells. The antioxidant activity of CEe and its effect on cell survival and insulin expression/secretion were evaluated. The CEe increased cell viability and ameliorated the disturbance of redox homeostasis in H₂O₂- and SNP-treated cells by decreasing DNA damage, lipid peroxidation and protein S-glutathionylation. The CEe restored GSH homeostasis in H₂O₂-treated β-cells and attenuated the SNP-induced disturbance of the GSH/GSSG ratio. The H₂O₂- and SNP-induced disruption of CAT, GPx, GR, MnSOD and CuZnSOD activities was adjusted by the CEe towards control values, as well as mRNA and protein levels of GPx, MnSOD and CAT. The CEe increased insulin expression/secretion particularly in H₂O₂-treated β-cells, which was in accordance with the more pronounced antioxidant effect of the CEe observed in H₂O₂-treated β-cells as compared to SNP-treated cells. These findings support the beneficial effect of the CEe in preventing or slowing down β-cell damage and dysfunction caused by oxidative/nitrosative stress during diabetes development.

Keywords: oxidative stress; nitrosative stress; β-cells; Centaurium erythraea; antioxidant; cytoprotective

Abbreviations and acronyms: CEe – Centaurium erythraea extract; SNP – sodium nitroprusside; GSH – glutathione; GSSG – glutathione disulfide; GSSP – S-glutathionylated proteins; CAT – catalase; GPx – glutathione peroxidase; GR – glutathione reductase; MnSOD – manganese superoxide dismutase; CuZnSOD – copper-zinc superoxide dismutase

INTRODUCTION

Diabetes is a complex metabolic disorder with insulin producing pancreatic β-cell loss and dysfunction at its core. Studies indicate that oxidative stress-activated redox-signaling pathways damage β-cells, cause defects in insulin expression and secretion, leading to eventual cell death [1]. In type 1 diabetes, β-cell death is driven by pro-inflammatory cytokines and free radical species such as the superoxide anion (O₂⁻) and nitric oxide radical (NO⁻) released from immune cells infiltrating pancreatic islets [2]. Elevated glucose concentrations in type 2 diabetes lead to β-cell dysfunction through increased glucose metabolism associated with mitochondrial production of reactive oxygen species (ROS) [2]. In the physiological state, ROS such as O₂⁻, the hydroxyl radical (·OH) and hydrogen peroxide (H₂O₂), and reactive nitrogen species (RNS), including NO⁻ and the peroxynitrite anion (ONOO⁻), are present in low concentrations that play important roles in the regulation of various cellular processes [3]. Thus, H₂O₂ acts as a signaling molecule in glucose-stimulated insulin secretion (GSIS) in β-cells [4] while...
NO* is a physiological regulator of insulin secretion at lower concentrations [5]. High concentrations of ROS and RNS, resulting either from their overproduction or from the disruption of antioxidant protection, produce oxidative stress associated with damage and dysfunction of proteins, lipids and DNA [6]. To maintain redox homeostasis, the cell must ensure proper functioning of the endogenous antioxidant system. MnSOD and CuZnSOD catalyze the dismutation of \( \text{O}_2^- \) to \( \text{H}_2\text{O}_2 \), which is neutralized to \( \text{H}_2\text{O} \) by CAT or by GPx that utilizes GSH as a reducing factor [7-9]. During catalytic activity of GPx, GSH is oxidized to GSSG, which is reduced to GSH by GR [10]. As a non-enzymatic antioxidant, GSH plays an important role in cellular redox buffering [11].

Because of the relatively low expression and activity of antioxidant enzymes and redox buffers in comparison to other cell types, β-cells are more susceptible to oxidative stress [6,12]. As redox-homeostasis in β-cells can easily shift to a state of harmful oxidative stress, exogenously assisted lowering of excess ROS/RNS levels and/or the improvement of endogenous antioxidant defenses can contribute to the preservation of structural and functional properties of β-cells in diabetes.

A growing body of evidence indicates that herbal preparations can alleviate oxidative stress through additive and synergistic effects of their redox active polyphenolic compounds. *Centaurium erythraea* Rafn (CE), also known as a common centaury, is a plant from the *Gentianaceae* family, which is used in Serbian traditional medicine for treating different diseases, including diabetes [13]. We previously presented the phytochemical characterization of the extract prepared from CE (CEe), with secoiridoid glycosides and phenolics (including xanthones and flavonoids) as its major components [14]. We demonstrated a significant in vitro antioxidant potential of CEe, including strong \( \text{H}_2\text{O}_2^- \) and NO-scavenging activities, as well as the ability to improve the structural and functional properties of pancreatic islets in streptozotocin (STZ)-induced diabetic rats [14, 15].

The aim of the present study was to examine the antioxidant effects of CEe in β-cells treated with \( \text{H}_2\text{O}_2 \) and with SNP, a prooxidant with a different mechanism of action: while hydrogen peroxide directly leads to oxidative damage and is a source of highly reactive \( \cdot\text{OH} \) [16], SNP is a complex compound that in aqueous solution releases NO* and is most commonly used to investigate the cytotoxicity induced by nitrosative stress [17,18]. The antioxidant action of the CEe was estimated by analyzing DNA damage, lipid peroxidation, protein S-glutathionylation (GSSP), the GSH/GSSG ratio, as well as the expression and activities of CAT, GPx, GR, MnSOD and CuZnSOD antioxidant enzymes. The protective effect of the CEe was also evaluated by analyzing β-cell survival as well as insulin expression and secretion.

**MATERIALS AND METHODS**

**CE methanol extract**

Procedures for the collection of plant material, preparation and phytochemical characterization of the methanol CEe used in the present study were described previously [14].

**Cell culture**

Rin-5F (ATCC-CRL-2058) cells were seeded in sterile plates and cultivated at 37°C under 5% CO\(_2\) in a humidified atmosphere (95%) in RPMI 1640 medium (Biological Industries, Beit HaEmek, Israel) supplemented with L-glutamine (2 mM) (Biological Industries, Beit HaEmek, Israel), antibiotics penicillin (100 U/mL) and streptomycin (100 μg/mL) (Biological Industries, Beit HaEmek, Israel) and fetal bovine serum (FBS) (10%) (Bio West, Nuaille, France), exchanged every 72 h. Cells were subjected to all treatments after reaching 60-70% confluency. \( \text{H}_2\text{O}_2 \), SNP and CEe were dissolved in RPMI medium. Concentrations of CEe were chosen after performing a cytotoxicity test that showed that extract concentrations ranging from 0.05 to 0.25mg/mL were not cytotoxic to Rin-5F cells [15]. For the determination of IC\(_{50}\) doses of prooxidant agents, the cells were treated with a series of concentrations of \( \text{H}_2\text{O}_2 \) (10, 20, 40, 50, 75, 100, 125, 150 and 200 µM) for 3 h, and with SNP (0.1, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75 and 2 mM) for 24 h. In all subsequent experiments the cells were treated with \( \text{H}_2\text{O}_2 \) (75 µM, for 3 h), SNP (1.25 mM for 24 h) and the CEe (0.25 mg/mL either for 3 h or for 24 h). For the deter-
mination of secreted insulin, the medium in which the cells were grown and treated was harvested and centrifuged at 500 x g for 10 min; 10 µL of the obtained supernatant was used to determine the concentration of released insulin by enzyme immunoassay (Rat/Mouse Insulin ELISA Kit, EMD Millipore, MO, USA).

**MTT viability assay**

The cell viability test is based on the measurement of the mitochondrial activity detected by the formazan formation in living cells after reduction of the tetrazolium ring of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma). Rin-5F cells cultured in 96-well plates were treated with the indicated concentrations of H₂O₂ (for 3 h), SNP (for 24 h) and CEe (for 3 h and 24 h as indicated); immediately after the treatments, 200 µL of MTT (0.5 mg/mL RPMI medium) was added to each well, followed by incubation for 2 h in the dark. The insoluble formazan was dissolved in dimethyl sulfoxide (DMSO) and quantified by measuring the absorbance at 570 nm. Cell viability was expressed in comparison to control cells assumed to be 100%.

**Comet assay**

DNA damage was estimated using the Comet assay performed as previously described [15]. The tail moment, which takes into account both the relative amount of DNA in the tail and the migration of the DNA, was used as an indicator of the displacement of the genetic material between the nucleus (‘comet head’) and the resulting ‘comet tail’. Quantification of images was performed by TriTekCometScore Freeware version 1.5.

**Lipid peroxidation assay**

Cells grown in 6-well plates were resuspended in 200 µL of phosphate-buffered saline (PBS); 10 µL of the cell suspension was used for the Comet assay, while the rest was centrifuged at 300 x g for 10 min. The cell pellet was dissolved in 1.15% KCl solution, sonicated (5 s/30 kHz/+4°C three times), and the obtained homogenate was stored at -80°C until use. The level of lipid peroxidation was estimated by measuring the level of malondialdehyde (MDA) in the thiobarbituric acid-reactive substance (TBARS) assay [19]. The cell homogenate (0.1 mL) was mixed with 0.2 mL of 8.1% sodium dodecyl sulfate (SDS), 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of 0.8% thiobarbituric acid (TBA) and 0.7 mL of water and heated at 95°C for 60 min. After cooling to room temperature, 1 mL of water and 5 mL of n-butanol-pyridine (15:1, v/v) were added to samples that were mixed and centrifuged at 3000 x g for 10 min. The absorbance of the upper colored layer was measured at 532 nm. The concentration of MDA was calculated from a calibration curve prepared using MDA standards (concentration range of 25 to 500 nM/mL) and expressed as nM MDA/100 mg of proteins. Protein concentrations were determined according to Lowry et al. [20].

**Determination of GSH, GSSG and GSSP**

Measurement of GSH, GSSG and GSSP was performed as described [21]. Rin-5F cells were resuspended in PBS (500 µL) and centrifuged at 300 x g for 10 min. The cell pellet was resuspended in 2.5% sulfosalicylic acid, homogenized and centrifuged at 8000 x g for 5 min. The supernatant was neutralized by the addition of 5 µL of 4 M triethanolamine (TEA) per 100 µL of the supernatant and used to determine the GSH/GSSG ratio, while the acid-precipitated proteins were used for measurement of GSSP. For GSH analysis, samples supplemented with the reaction mixture (0.1 M sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 0.3 mM-5,5'-dithiobis-(2-nitrobenzoic acid), 0.4 mM NADPH and 1 U/mL glutathione reductase I (GR) were incubated for 30 min at room temperature. Absorbance was measured at 412 nm and the GSH content was calculated using a calibration curve. The concentration of GSH was expressed as µM GSH/mg of protein. GSSG was quantified after derivatization of GSH with 10 mM 2-vinylpyridine and assayed as described above. For GSSP determination, acid-precipitated proteins were washed twice with 1.5% trichloracetic acid (TCA), resuspended in 0.5 mL of basic solution (9:1, v/v, 0.1 mM phosphate buffer (pH 7.4) and 0.25 mM NaOH), and stirred for 30 min at room temperature. Proteins were precipitated with 60% TCA, centrifuged at 5000 x g for 5 min and GSH was determined in the supernatant as described above. Protein concentrations were determined according to Lowry et al. [20].
Antioxidant enzyme activity assays

Homogenates were prepared by resuspension of cells in sucrose buffer (0.25 M sucrose, 1 mM EDTA and 0.05 M Tris–HCl, pH 7.4); cell suspension was sonicated at 20 kHz/30 s on ice, centrifuged at 14000 × g/4°C for 1 min, and the supernatants were used for the determination of enzyme activities and protein concentrations. CAT activity was measured by the rate of \( {H}_2{O}_2 \) decomposition as described by Beutler [22]. Total SOD activity was determined according to the epinephrine method [23]. MnSOD activity was performed after pre-incubation with 8 mM KCN, and CuZnSOD activity was calculated from the difference between total SOD and MnSOD activities. The activity of GPx was determined following oxidation of NADPH as a substrate with tert-butyl hydroperoxide [24]. The activity of GR was measured as described by Glatzle et al. [25]. Enzyme activity was expressed as U activity/mg of protein.

Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) and Western blot analysis

Homogenate proteins (20 μg) were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Amersham Hybond P 0.45 PVDF, GE Healthcare Life Sciences, UK). After blocking with 5% non-fat dry milk (Blotto, non-fat dry milk, Santa Cruz Biotechnology, USA) in blotto base buffer (0.2% Tween 20, 20 mM Tris–HCl pH 7.6, 150 mM NaCl) for 1 h at room temperature, samples were subjected to analysis using the following antibodies: anti-CAT, anti-GR and anti-GPX 1 (all from Abcam, USA) and anti-MnSOD (FL-222), anti-CuZnSOD (C-17) and anti-GAPDH (FL-335) (all from Santa Cruz Biotechnology, USA). The membranes were probed with adequate HRP-conjugated secondary antibodies (all from Santa Cruz Biotechnology, USA). The fragments were amplified using the following primers (Invitrogen, USA): for the rat CAT gene: Fw 5’-GCGAATGGAGGCTGTAGC-3’ and Rev 5’-GAGTGACGTTGCTTCACTAGC-3’ (652 bp); for the rat GPx gene: Fw 5’-AGTTCCGAGCATTACGAGAAATGG-3’ and Rev 5’-TAAAGAGC-GGTTGAGGCCCTT-3’ (141 bp); for the rat GR gene: Fw 5’-CAGTCCGGTGGAAACC-3’ and Rev 5’-GATCCAGGCTGAAGGA-3’ (227 bp); for the rat MnSOD gene: Fw 5’-CAGATCAGCTGCACCA-3’ and Rev 5’-AGTCCAGGCTGAAGGA-3’ (133 bp); for the CuZnSOD gene: Fw 5’-GGCAGAGGCAACGCTC-3’ and Rev 5’-GGCAGAGGCAACGCTC-3’ (282 bp); for the rat insulin 1 (Ins1) gene: Fw 5’-ATGGCCCTG-3’ and Rev 5’-ATGGCCCTG-3’ (275 bp); for the rat GAPDH gene: Fw 5’-ATGGCCCTG-3’ and Rev 5’-ATGGCCCTG-3’ (496 bp).

RNA isolation and real-time quantitative PCR analysis (RT-qPCR)

Total RNA was isolated from Rin-5F cells using the GeneJET RNA Purification Kit (Thermo Fisher Scientific, USA) following the manufacturer’s instructions. Cells were seeded in 6-well plates and treated with \( {H}_2{O}_2 \) (75 μM) for 3 h or with SNP (1.25 mM) for 24 h in parallel with the Ceo (0.25 mg/mL). Synthesis of cDNA was performed using total RNA (1 μg) treated with DNase I and reverse transcribed with oligo(dT) primers using the RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, USA). The levels of mRNA were quantified by Maxima SYBR Green/ROX qPCR Master Mix (2X) (Thermo Fisher Scientific, USA) and QuantStudio 3 Real-Time PCR system (Applied Biosystems, Carlsbad, CA, USA). The cycles for RT-qPCR included an initial denaturation step (95°C/10 min), followed by 40 cycles of a two-step PCR program at 95°C for 15 s and at 60°C for 1 min. In all RT-qPCR reactions negative controls without the template were used. The expression levels of the target genes were calculated in relation to the averaged expression of rat GAPDH gene. RT-qPCR reactions were carried out in triplicate. The fragments were amplified using the following primers (Invitrogen, USA): for the rat CAT gene: Fw 5’-GCGAATGGAGGCTGTAGC-3’ and Rev 5’-GAGTGACGTTGCTTCACTAGC-3’ (652 bp); for the rat GPx gene: Fw 5’-AGTTCCGAGCATTACGAGAAATGG-3’ and Rev 5’-TAAAGAGCGGTTGAGGCCCTT-3’ (141 bp); for the rat GR gene: Fw 5’-CAGTCCGGTGGAAACC-3’ and Rev 5’-GATCCAGGCTGAAGGA-3’ (133 bp); for the CuZnSOD gene: Fw 5’-GGCAGAGGCAACGCTC-3’ and Rev 5’-GGCAGAGGCAACGCTC-3’ (282 bp); for the rat insulin 1 (Ins1) gene: Fw 5’-ATGGCCCTG-3’ and Rev 5’-ATGGCCCTG-3’ (275 bp); for the rat GAPDH gene: Fw 5’-ATGGCCCTG-3’ and Rev 5’-ATGGCCCTG-3’ (496 bp).

Statistical analysis

Data were analyzed by GraphPad Prism v5.00 (GraphPad Software, San Diego, CA, USA). Statistical differ-
ences between groups in comparison to the corresponding control group were analyzed using one-way analysis of variance (one-way ANOVA), followed by Bonferroni’s Multiple Comparison Test. The data were expressed as the mean±SEM. (standard error of mean). The difference was considered statistically significant at $p<0.05$. The statistical significance ($p$) for all obtained results is shown in the tables and figures.

RESULTS

**CxEE increases cell-viability and insulin expression/secretion in $\text{H}_2\text{O}_2$- and SNP-treated $\beta$-cells**

Treatment of Rin-5F cells with a series of concentrations of either $\text{H}_2\text{O}_2$ or SNP revealed their IC$_{50}$ doses as follows, 75 $\mu$M for $\text{H}_2\text{O}_2$ and 1.25 mM for SNP (Fig. 1A), which were then used in all further analyses. Combined treatment of Rin-5F cells with the IC$_{50}$ dose of $\text{H}_2\text{O}_2$ and increasing concentrations of CxEE resulted in an increase in the percentage of viable cells in comparison to the $\text{H}_2\text{O}_2$ treatment (Fig. 1B). A slight increase (5%) was observed when 0.05 mg/mL of the extract was applied, while concentrations of the CxEE of 0.1 and 0.25 mg/mL increased the number of viable cells by 15% and 26%, respectively. Co-treatment of cells with IC$_{50}$ SNP and increasing concentrations of the CxEE revealed that the greatest improvement in cell viability (24%) was when 0.25 mg/mL of the CxEE was applied (Fig. 1B). Therefore, the concentration of 0.25 mg/mL of the CxEE was chosen for subsequent investigation in combination with IC$_{50}$ doses of $\text{H}_2\text{O}_2$ and SNP.

The effect of the CxEE on the functional properties of $\beta$-cells was examined by measuring the mRNA level of the insulin gene ($\text{Ins1}$), and by determining the level of insulin released into the growing...
Following H2O2 treatment, the relative level of insulin mRNA was reduced by 45% relative to the mRNA level observed in control cells. Simultaneous H2O2/CEe treatment increased the level of insulin mRNA relative to H2O2-treated cells by 46%. Accordingly, reduction of insulin secretion by 28% after H2O2 treatment returned to the control level after H2O2/CEe co-treatment. Both insulin expression and secretion were also impaired in SNP-treated Rin-5F cells as the level of insulin mRNA was reduced by 38% (Fig. 1C), while insulin release was decreased by 27% (Fig. 1D) when compared to the control. SNP/CEe co-treatment increased the level of insulin mRNA by 36% and increased the level of released insulin by 16% in comparison to SNP-treated cells. Treatment of control cells with the CEe had no influence on gene expression and the level of insulin secretion.

**CEe improves the β-cell response to H2O2- and SNP-mediated redox challenge**

To assess the impact of the CEe on the degree of oxidative damage in β-cells exposed to H2O2 and SNP treatments, oxidative stress parameters such as DNA damage, lipid peroxidation, GSSP formation, the GSH/GSSG ratio and antioxidant enzyme activities were analyzed. As can be seen in Fig. 2, in H2O2- and SNP-treated cells the tail moment was increased 6.6- and 7-fold, respectively, relative to the control cells. Both H2O2/CEe and SNP/CEe co-treatments reduced the quantity of DNA in the comet tail (by 2.9- and 2.5-fold, respectively). In comparison to the control, the levels of MDA and GSSP in H2O2-treated cells were increased (2.3- and 3.2-fold, respectively), while the GSH/GSSG ratio was reduced 39% (Table 1). In H2O2/CEe co-treated cells, the GSH/GSSG ratio was at the level of the control while the levels of MDA and GSSP were reduced (34% and 55%, respectively) when compared to H2O2-treated cells. Treatment of Rin-5F cells with H2O2 affected the activities of the antioxidant enzymes by inducing CAT, MnSOD and CuZnSOD (by 32%, 51% and 48%, respectively), and by lowering the activities of GPx and GR (by 30% and 23%, respectively). In H2O2/CEe co-treated cells, the activities of CAT and CuZnSOD were lowered (both for 17%), whereas the activities of GPx and GR were increased (40% and 20%, respectively) in comparison to the H2O2 treatment.

SNP treatment of β-cells induced lipid peroxidation (a 4.9-fold increase in MDA), increased the level of GSSP (3.6-fold) and decreased the GSH/GSSG ratio (21%) (Table 2). Compared to control cells, increases...
in MnSOD and CuZnSOD activities (2.1- and 1.9-fold, respectively) and decreases in CAT, GPx and GR activities (by 26%, 30% and 51%, respectively) were observed after SNP treatment (Table 2). In relation to the SNP treatment, co-treatment with SNP/CEe was characterized by lower levels of MDA and GSSP (by 27% and 21%, respectively), as well as by decreases in MnSOD and CuZnSOD activities (by 29% and 20%, respectively) and increases in CAT, GPx and GR activities (by 19%, 32% and 43%, respectively). Compared to the SNP treatment, the GSH/GSSG ratio was increased in SNP/CEe co-treated cells although the observed increase was not statistically significant.

**DISCUSSION**

Studies on the animal model of diabetes have revealed the antidiabetic and antioxidant effects of extracts pre-
pared from CE, supporting its traditional use in diabetes management and stimulating further investigation of its biological activities [14,15,26,27]. Medicinal plants are a valuable source for drug discovery, and a number of active components have been isolated for direct use as drugs, or as a lead compound or pharmacological agent [28,29]. Thus, galegine, prepared from the plant *Galega officinalis* L., which has been used in traditional medicine for the treatment of diabetes has provided a template for the synthesis of the oral hypoglycemic agent metformin and opened up interest in the synthesis of other biguanidine-type antidiabetic drugs [28]. In the present study we investigated the cytoprotective effects of the CEe on β-cells in H$_2$O$_2$- and SNP-induced oxidative and nitrosative stress. Our results revealed that the CEe ameliorated disturbed redox homeostasis in β-cells, contributing to a decrease in DNA, lipid and protein damage and to an increase in cell viability and insulin expression/secretion. Suppression of oxidative and nitrosative stress in β-cells points to the beneficial effect of the CEe in preventing or slowing down the processes of β-cell loss and dysfunction in diabetes.

The increase in DNA damage, lipid peroxidation and protein S-glutathionylation, the decrease in the GSH/GSSG ratio and alterations in the activities of antioxidant enzymes in β-cells observed after treatments with H$_2$O$_2$ and SNP are in accordance with their prooxidant action. H$_2$O$_2$ exerts its effect through the oxidation of DNA, lipids and protein thiol (-SH) groups [30]. Also, H$_2$O$_2$ is a source of ‘OH that causes damage to DNA, proteins and lipids [16,31]. H$_2$O$_2$ can be converted to hypochlorous acid (HOCl), which in reaction with the amino group (-NH$_2$), followed by free radical formation, leads to protein degradation, enzyme inactivation, nucleic acid denaturation and lipid peroxidation [32]. On the other hand, SNP reacts with -SH groups of proteins and releases NO$, which in reaction with other radicals such as O$_2$ forms extremely reactive ONOO$^{-}$ [33,34]. The protonated form of peroxynitrite (ONOOH) leads to depletion of -SH groups, fragmentation of DNA, lipid and protein oxidation [16,31]. The pre-

![Fig. 3. The CEe modulates the expression of the antioxidant enzymes in H$_2$O$_2$-treated Rin-5F cells. A – The levels of CAT, GPx, GR, MnSOD and CuZnSOD mRNAs were analyzed using quantitative RT-PCR. Preparation of RNA and complementary DNA (cDNA) was performed after treatment of Rin-5F cells with H$_2$O$_2$ (75 µM) for 3 h with or without CEe (0.25 mg/mL). The levels of mRNA for antioxidant enzymes were calculated against GAPDH mRNA and the plotted values are the means±SEM from three separate experiments performed in triplicate. B – CAT, GPx, GR, MnSOD and CuZnSOD protein levels were determined by immunoblot analysis of cell homogenates isolated from Rin-5F treated with H$_2$O$_2$ (75 µM) for 3 h with or without CEe (0.25 mg/mL). Blots were quantified using TotalLab (Phoretix) electrophoresis software. The relative protein levels of antioxidant enzymes were calculated against GAPDH protein level and are presented on the graphs as the means±SEM relative to the control arbitrarily taken as 100%. CAT – catalase; GPx – glutathione peroxidase; GR – glutathione reductase; MnSOD – manganese superoxide dismutase; CuZnSOD – copper-zinc superoxide dismutase; GAPDH – glyceraldehyde 3-phosphate dehydrogenase. C – control cells; C/CEe – control cells treated with CEe (0.25 mg/mL) for 3 h; H$_2$O$_2$ – cells treated with H$_2$O$_2$ (75 µM) for 3 h; H$_2$O$_2$/CEe – cells co-treated with H$_2$O$_2$ (75 µM) and CEe (0.25 mg/mL) for 3 h. ** p<0.01, *** p<0.001 as compared to C; + p<0.05, ++ p<0.01, +++ p<0.001 when H$_2$O$_2$/CEe is compared to H$_2$O$_2$.](image-url)
presented results indicate that both agents caused about the same level of DNA damage in β-cells, while SNP had a greater effect on the induction of lipid peroxidation and protein S-glutathionylation than H₂O₂, which in turn had a more pronounced influence on the decrease of the GSH/GSSG ratio. Such differences in the extent of changes of oxidative stress parameters could be ascribed to different mechanisms of H₂O₂ and SNP prooxidant actions.

The CEe alleviated H₂O₂- and SNP-induced DNA damage in β-cells that is crucial for their survival and functioning, especially with regard to the fact that β-cells possess a weak repair machinery for oxidative DNA damage [35]. An additional contribution of the CEe to β-cell protection was achieved by the reduction of lipid peroxidation that causes oxidative damage of membrane proteins, inactivation of receptors, enzymes and transport proteins and consequently cell death [16,36]. It can be assumed that by lowering lipid peroxidation, CEe also reduced the production of various stable aldehydes known to act as secondary messengers at sites distant from the site of their formation, leading to DNA and protein damage [36]. By increasing the GSH/GSSG ratio, which contributes to the redox potential of the cell and to redox homeostasis [11], CEe further suppressed the H₂O₂- and SNP-induced redox imbalance in β-cells. Oxidative stress leads to oxidation of GSH to GSSG and to S-glutathionylation (GSSP) through the formation of mixed disulfide bonds between GSH or GSSG and redox-sensitive protein -SH groups [37]. Nitrosative stress, through the reaction of NO’ with GSH, produces high levels of S-nitrosoglutathione (GSNO) that contribute to protein S-glutathionylation as well [38]. Although S-glutathionylation was assumed to be a by-product of oxidative or nitrosative stress, under physiological conditions this modification is involved in redox regulation and signaling, while during oxidative stress it leads to functional alterations of proteins, including modulation of enzyme activities [37,39]. By lowering the level of protein S-glutathionylation in H₂O₂- and SNP-treated β-cells, CEe could lessen the negative effects of this modification on the structural
and functional properties of enzymes and regulatory proteins. CEe was more efficient in lowering lipid peroxidation and S-glutathionylation and in raising the GSH/GSSG ratio in H$_2$O$_2$-treated β-cells in comparison to the SNP treatment, suggesting its higher antioxidant potential in ROS-induced oxidative stress.

Suppression of the detrimental effects of H$_2$O$_2$ and SNP by CEe could be because of the H$_2$O$_2$- and NO-scavenging activities [14]. The CEe used in this study was previously phytochemically characterized and its free radical scavenging activity was attributed to major phenolic compounds quantified, such as quercetin, isoquercitrin, naringenin, astragalin, luteolin, apigenin, caffeic, p-coumaric, ferulic and sinapic acids and xanthones [14]. The mechanism involved in the scavenging activity of polyphenolic compounds includes the availability of their hydrogen atom and the possibility of stabilization of •OH and NO• through hydrogen donation or expansion of electron delocalization [40]. Accordingly, sinapic acid was reported to possess an appreciable NO• scavenging activity [41], while quercetin, apigenin and luteolin display DNA protective capacity against H$_2$O$_2$-generated free radicals [42]. Modulation of the activities of antioxidant enzymes in H$_2$O$_2$- and SNP-treated β-cells towards control values could also be due to the potential of CEe to neutralize free radicals [14]. By this mechanism of action CEe could reduce the need for further increases in enzyme activities, i.e. activation of CAT after the H$_2$O$_2$ treatment and activation of SOD enzymes after both treatments. On the other hand, CEe-mediated scavenging of free radicals could reduce their inhibitory effect on the enzyme activities of CAT, GPx and GR in SNP-treated β-cells. Namely, it has been shown that NO• reduces GPx activity and CAT activity by blocking the catalytic center of the enzyme [43,44]. Such inhibitory effects of free radicals in SNP-treated β-cells could drive the decrease in CAT, GPx and GR activities despite the detected increases in their gene and protein expression. Similarly, in H$_2$O$_2$-induced oxidative stress, GPx and GR activities were reduced although their protein levels were increased, suggesting ROS-mediated inhibition of their catalytic activity.

The observed increase in mRNA and protein levels of CAT, GPx and GR in SNP-induced nitrosative stress points to their regulation at the transcriptional level. In H$_2$O$_2$-treated β-cells, upregulation of GPx mRNA accompanied by the increase in GPx protein level suggests it is regulated at the transcriptional level, while the increase in GR protein level that is accompanied by a control level of GR mRNA, points to the greater stability of its mRNA or the involvement of post-translational regulation of protein stability. The rise in mRNA and protein levels of MnSOD after the H$_2$O$_2$ and SNP treatments suggests that the enzyme is regulated at the transcriptional level. By lowering the mRNA and protein levels of GPx and MnSOD after the H$_2$O$_2$ treatment, as well as of CAT after the SNP treatment, CEe affected their transcriptional regulation. Also, the decrease in the protein level of GR in H$_2$O$_2$-treated β-cells by CEe revealed its potential effect on post-translational regulatory mechanisms. These findings suggest that the antioxidant activity of CEe can influence the stability and/or the activity of redox-sensitive regulatory proteins involved in the control of gene and protein expression of antioxidant enzymes [15].

Elevated levels of H$_2$O$_2$ and NO• have detrimental effects on β-cell proliferation and insulin expression and secretion [45-47]. The underlying mechanisms are complex and are based on the post-translational modifications of proteins involved in the regulation of insulin gene expression, such as pancreatic and duodenal homeobox 1 (PDX1) and V-Maf avian musculoaponeurotic fibrosarcoma oncogene homolog A (MafA) [48]. We cannot exclude the possibility that CEe improved insulin expression and secretion in H$_2$O$_2$- and SNP-treated β-cells not just by increasing their viability, but also through the protection of regulatory proteins from oxidative damage. This assumption is further supported by the previously reported positive effect of CEe on PDX1 and MafA activities, as well as on the increase in insulin expression and secretion in STZ-treated β-cells [15]. It should be mentioned that CEe was more effective in enhancing the mRNA level and insulin secretion in H$_2$O$_2$-treated β-cells when compared to the SNP treatment.

**Conclusion**

The methanol extract prepared from *Centaurium erythraea* (CEe) attenuates the H$_2$O$_2$- and SNP-induced redox imbalance in pancreatic β-cells, thereby contrib-
ut ing to increased cell-viability and insulin expression and secretion. Application of the CEE improved the GSH/GSSG ratio and reduced DNA damage, lipid peroxidation and protein S-glutathionylation and modulated the activities of the antioxidant enzymes towards control values. Attenuation of the H2O2- and SNP-mediated detrimental effects in β-cells could rely on the H2O2 and NO scavenging properties of the CEE. The antioxidant effect of the CEE was more pronounced in H2O2-treated β-cells, indicating its higher efficiency against ROS-mediated oxidative stress, which was followed by a more effective induction of insulin expression/secretion. These findings disclose the beneficial effect of the CEE in preventing or slowing down the processes of β-cell damage and dysfunction. They support the traditional use of this plant in diabetes management.

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