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INFLUENCE OF SELECTED POLYPHENOLS ON ANTIOXIDANT ENZYMES ACTIVITY AND EXPRESSION OF BAX AND BCL-2 GENES IN CACO-2 CELLS EXPOSED ON OXIDATIVE STRESS

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Abstract

The main purpose was to study the influence of antioxidants on human colon adenocarcinoma cells Caco-2 exposed to H_2O_2-induced oxidative injury. Caco-2 cells were exposed twice to 400 or 800 µM of H_2O_2, with 6 hours of interval between the applications. To assess the impact of quercetin (Qw), chlorogenic acid (ChlA) and epigallocatechin (EGC), cells were pre-treated with the antioxidants solutions (100 or 200 µM) for 24 h prior to H_2O_2 treatment. The expression of mRNA for bax and bcl-2 genes and the activity of intracellular antioxidant enzymes (catalase /CAT/, glutathione peroxidase /GPx/) in Caco-2 cells were estimated 24 and 48 hours after exposition to the stress agent. We demonstrated that pretreatment with analyzed antioxidants can activate anti-apoptotic signal in cells exposed on oxidative injury and prevent their apoptosis. On the basis of the results we found that protective effect of antioxidants is independent on antioxidant enzymes activity.

Key words: *antioxidants, apoptosis, catalase, bax, bcl-2, Caco-2 cells, glutathione peroxidase, oxidative stress*

1. INTRODUCTION

Reactive oxygen species (ROS) decide about cell survival or death. This effect is completely dependent on ROS concentration and time of exposition. High amounts as well as long exposition to harmful action of those reactive molecules often exceed the defensive possibilities of cell resulting in sufficient cellular damage and finally its apoptosis (Duda-Chodak & Tarko 2007).

Apoptosis, called programmed cell death, is identified on the basis of biochemical and morphological changes; cell dying by apoptosis process differs from damaged cell dying by necrosis. Characteristic cell morphology of cells undergoing apoptosis include blebbing, changes to the cell membrane such as loss of membrane asymmetry and attachment, cell shrinkage, nuclear fragmentation, and chromatin condensation. Visible in apoptotic cells is chromosomal fragmentation due to the controlled digestion of DNA by DNAses. Formation of apoptotic bodies is also characteristic for apoptosis. The end result of apoptosis is cell death without inflammation of the surrounding tissue (Nagata 1997, Robertson et al. 2000, Elmore 2007).

Apoptosis is a complex process controlled on several levels. Cellular signal and enzymatic apparatus is necessary for initiation of the apoptotic cell death. Main membrane receptors initiating apoptosis cascade are special receptor proteins from Fas/APO-1 family that contain “death domain”. Activated receptors induce synthesis of proteolytic enzymes that degrade elementary proteins. One of the

important factors, that can induce apoptosis, is elevated concentration of intracellular calcium resulting in phospholipase C and caspase cascade activation. Caspases are executioners of apoptosis (Cohen 1997). Important role in apoptosis regulation play proteins from Bcl-2 family. They govern mitochondrial outer membrane permeabilization. It is thought that some Bcl-2 family proteins can induce (pro-apoptotic members) or inhibit (anti-apoptotic members) the release of cytochrome c into the cytosol which, once there, activates caspase-9 and caspase-3, leading to apoptosis. Among pro-apoptotic members of this proteins family Bax, BAD, and Bak should be mentioned, while Bcl-2 proper, Bcl-xL, and Bcl-w are anti-apoptotic (Robertson et al. 2000, Gewies 2003, Elmore 2007).

Free radicals and reactive oxygen species contribute in programmed cell death at different levels, modulating signal cascades that promote or inhibit anti-apoptotic transcriptions factors (NF-κB, AP-1). They can also induce p53 translocation to nucleus or activate $p21^{WAF1/CIP1}$ and GADD45 (Johnsosn et al. 1996). There are some researches suggesting that H_2O_2 can induce apoptosis through phosphorylation and ubiquitination Bcl-2 family proteins (Grajek 2007).

In the course of evolution the mammalian cells had been equipped in specialized intercellular antioxidant system. The enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and S-glutathione transferase (GT) are destined for scavenging and neutralizing ROS (Wijeratne et al. 2005). However, in many situations the internal antioxidant defensive system is insufficient and needs to be enhanced by external antioxidants. Those can be provided by diet rich in fruits and vegetables. A considerable body of evidence indicates that compounds such as polyphenols play a critical role in the prevention of oxidative damage to biomolecules and associated pathologies in humans, including heart diseases, tumors, cell aging and dying (Bergamini et al. 2004).

Polyphenols are secondary plant metabolites, which exhibit a wide range of biological effects because of their antioxidant properties. Polyphenols inhibit LDL oxidation (Frankel et al. 1993, Ivanov et al. 2001) and protect DNA from oxidative damage (Cook & Samman 1996, Grajek 2007). Moreover, polyphenols show antitoxin, antimicrobial (Friedman 2007), anticarcinogenic and antimutagenic effects (Bravo 1998). Chlorogenic acid, quercetin, epigallocatechin are the main representatives of different polyphenol classes present in the diet. Chlorogenic acid (ChlA) is one of the hydrocinnamic acids. The main source of chlorogenic acid is coffee, but is also present in apples, pears, and plums. Quercetin (Qw) is an aglycone of many flavonols common in berries, onion, cabbages and lettuces. Epigallocatechin (EGC) is one of the main tea catechin monomers and belongs to flavanols (Manach et al. 2004).

It is interesting that some polyphenols have been reported to be detrimental. Some of these effects may depend on the concentration of the polyphenol utilized since high doses of some phenolic compounds may be prooxidant and negatively affect cell growth and viability (Agullo et al. 1994, Raza & John 2005). Several studies have shown that some antioxidants, particularly in the presence of transition metal ions, exhibit prooxidant activity under certain conditions and may cause oxidative DNA damage (Sakihama et al. 2002, Azam et al. 2004, Labieniec & Gabryelak 2006). Some researches postulate that this prooxidant action of plant polyphenols may be an important mechanism of their anticancer properties (Yang et al. 1998, Azam et al. 2004, Lee et al. 2005, Bhat et al. 2007).

Caco-2 cells are cell line isolated from a primary colonic tumor in a 72-year-old Caucasian male and constitute one of the most utilized *in vitro* models of colon cancer (Rösmann et al. 2002, Lee et al. 2005). The aim of the study was to evaluate the influence of different concentrations of quercetin, chlorogenic acid, and epigallocatechin on antioxidant enzymes activity and expression of bax and bcl-2 genes in Caco-2 cells exposed on H_2O_2-induced oxidative injury.

2. MATERIALS AND METHODS

2.1. Chemicals

Quercetin dihydrate, (-)epigallocatechin from green tea, chlorogenic acid, Eagle's Minimum Essential Medium (EMEM), Dulbecco's Modified Eagle's Medium (DMEM), Non-essential Amino Acids (NEAA), trypsin, Phosphate Buffered Saline (PBS), dimethyl sulfoxide (DMSO), Fetal Bovine Serum (FBS), L-glutamine, antibiotics and other chemicals for cell culture were obtained from Sigma-Aldrich (Poznań, Poland). TRIzol was provided by Invitrogen (Groninen, Holandia) and DNA-zol Reagent was from Gibco BRL (Paisley UK). Chemicals for DNA and mRNA analysis and RT-PCR were from Promega (Germany), and assays for antioxidant enzymes activity evaluation from Cayman Chemicals (Cayman Chemical Company, Ann Arbor, USA). Isopropanol (analytical grade) and other basic laboratory chemicals were purchased from POCh Company (Gliwice, Poland).

2.2. Cell culture

A human colon adenocarcinoma cell line Caco-2 (ATCC No. HTB-37) was purchased from LGC Promochem (UK). In the study, Caco-2 cells treated with hydrogen peroxide were used as an *in vitro* model of oxidative stress in intestinal cells. Frozen Caco-2 cells were storage in liquid nitrogen. Then, cells were resuscitated and proliferated in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% NEAA and 1% antibiotics. The cells were maintained for 48 h in controlled conditions (37°C, 98% of humidity, 5% CO_2, Nuaire, Plymouth, USA) up to reaching confluence. At confluence, i.e. when monolayer was formed, cells were washed in PBS (without Ca^{2+}/Mg^{2+}), harvested by treatment with 0.25% trypsin and centrifuged at 200×g for 15 min. Then the cells pellet was resuspended in fresh growth medium of reduced FBS content (5%) and transferred into 6-well plates. All experiments were performed with Caco-2 cells at least of 70% confluence ($1x10^9$ cells×cm^{-3} of medium).

2.3. Oxidative stress

Caco-2 cells were exposed twice to 400 or 800 µM of H_2O_2, with 6 hours of interval between the applications. To assess the influence of antioxidants (chlorogenic acid, epigallocatechin and quercetin), cells were pre-treated with the antioxidants solutions (100 or 200 µM in DMSO) for 24 h prior to H_2O_2 treatment. The control cells were treated with DMSO only. No stressed Caco-2 cells (with no addition of H_2O_2) were also used as control. Experiments were performed in triplicate and the results were shown as an arithmetic mean.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR) for detection of messenger RNA (mRNA) for bax and bcl-2

Total RNA was extracted from Caco-2 cells using guanidine isothiocyanate/phenol single-step method developed by Chomczynski and Sacchi (1987). Cells were incubated 24 or 48 hours after exposition to the stress agent. Then, the medium was removed by aspiration. Cells were lysed directly in the wells by adding 0.5 ml of TRIzol reagent, to each well and passing the cell lysate several times through a pipette tip. The homogenized samples were transferred into cap tubes and incubated for 5 minutes at room temperature (RT) to permit the complete dissociation of nucleoprotein complexes. Then, 0.1 ml of chloroform was added and the mixture was shaken vigorously by hand for 15 second and incubated for 3 minutes at RT. Samples were centrifuged (15 min, 16 000 × g, 4°C) and the aqueous phase was transferred to a fresh tube. Organic phase was saved for DNA and protein isolation. Precipitation of RNA was conducted by mixing the aqueous phase with 0.25 ml of isopropyl alcohol and incubation for 10 min at RT. Sample were then centrifuged (15 min, 16 000 × g, 4°C) and the supernatant was carefully removed. The RNA pellet was washed with 0.5 ml of 75% ethanol, vortexed, and centrifuged (5 min, 16 000 × g, 4°C). The RNA pellet was dried at ice for 5-10 min, and dissolved in 20 µl of

DEPC-water (RNase free) by passing a few times through a pipette tip and incubating for 10 minutes at 55°C. RNA was stored in -80°C until analysis.

Single stranded cDNA was generated from 2 µg of total RNA using Moloney murine leukemia virus reverse transcriptase (MMLV-RT) and oligo-(dT)-primers. Briefly, 2 µg of total RNA was uncoiled by heating (70°C, 5 min) and then reverse transcribed (42°C for 1h followed by 5 min at 90°C) into complementary DNA (cDNA) in a 50 µl reaction mixture that contained 50 U MMLV-RT, 0.3 µg oligo-(dT)-primer, 20 U RNase Block Ribonuclease Inhibitor, 5 µl of a 100-mM mixture of dNTP, and 5 µl 10x MMLV buffer.

The resultant cDNA (2 µl) was amplified in a 50-µl reaction volume containing 2 U Taq polymerase, dNTP (200 µM each), 1.5 mM MgCl2, 5 µl 10 × PCR buffer and specific primers used at final concentration of 1 mM. The mixture was overlaid with 25 µl of mineral oil to prevent evaporation. The polymerase chain reaction mixture was amplified in a thermal cycler Biometra T3. The nucleotide sequences of the primers and thermal profiles of particular PCR reaction are presented in the table 1.

Gene		Primer sequence 5' → 3'	Thermal profile of reaction	Product size (bp)
β-actin	sense	GGGTACATGGTGGTGCCG	94°C/1 min; (94°C/1 min, 54°C/1 min, 55,5°C/1 min, 72°C/2 min) x 29 cycles, 72°C/10 min, 4°C – pause	307
	antisense	AGCGGGAAATCGTGCGTG		
bax	sense	CGTCCCAACCACCCTGGTCT	94°C/4 min; (94°C/45 s, 55°C/45 s, 72°C/2 min) x 29 cycles, 72°C/7 min, 4°C – pause	195
	antisense	TGGCAGCTGACATGTTTTCTGAC		
bcl-2	sense	CCCAGCCTCCGTTATCCTGGA	94°C/2 min; (94°C/1 min, 60°C/1 min, 72°C/2 min) x 29 cycles, 72°C/7 min, 4°C – pause	235
	antisense	CAGATGCACCTGACGCCCTT		

Table 1. Primer sequences and thermal profile of PCR reaction

Products of PCR reaction were detected by electrophoresis in a 1.5% agarose gel containing ethidium bromide. Location of predicted products was confirmed by using a 100-bp ladder as a standard size marker. The gel was then photographed under UV transillumination and the intensity of PCR products was measured densitometrically in Gel-Pro Analyzer (Fotodyne Incorporated, USA). The signal for bax and bcl-2 mRNA was normalized against that of the β-actin mRNA from each sample and the results were expresses as bax or bcl-2 mRNA over the β-actin mRNA ratio.

2.5. Evaluation of antioxidant enzymes activity

The activity of intracellular antioxidant enzymes (catalase /CAT/, glutathione peroxidase /GPx/) in Caco-2 cells were estimated 24 and 48 hours after exposition to the stress agent. At particulate time point, the medium was aspirated and cells were washed in PBS (by scraping and centrifuging). Cells pellet was then re-suspended in special lysis buffer provided by Cayman Chemicals. Samples were centrifuged (15.000 × g, 15 min) and the supernatant was used for determination of antioxidant

enzymes activity. The activity of intracellular enzymes in Caco-2 cells was evaluated by using the commercial kits (Cayman Chemical Company) and performed in conformity with producer protocols.

Cayman's catalase Assay Kit utilizes the peroxidase function of catalase for determination of enzymes activity. The method is based on the reaction of the enzyme with methanol in the presence of of an optimal concentration of H2O2. The formaldehyde produced is measured colorimetrically with Purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple color (determined at 540 nm, microplates spectrophotometer PowerWave XS, BIOTEK).

Glutathione peroxidase (GPx) catalyzes the reduction of hydroperoxides, including hydrogen peroxide, by reduced glutathione and functions to protect the cell from oxidative damage. Cayman's GPx Assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG), produces upon reduction of hydroperoxide by GPx, is recycled to its reduced state by GR and NADPH. The oxidation of NADPH to $NADP^+$ is accompanied by a decrease in absorbance at 340 nm. Under condition in which the GPx activity is rate limiting, the rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

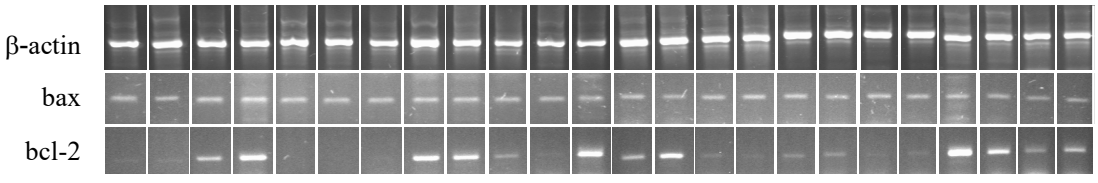
2.6. DNA laddering assay

The DNA was extracted from Caco-2 cells 24, 48, or 72 hours after exposition to the oxidative stress. Then, the medium was removed by aspiration. Cells were lysed directly in the wells by adding 0.5 ml of DNAzol reagent to each well and passing the cell lysate several times through a pipette tip. The samples were transferred into cap tubes and DNA precipitation was performed by adding 250 µl of 99.8% ethanol. Precipitated DNA was wash twice in 95% ethanol, evaporated and dissolved in 100 µl of water. Samples of DNA were stored at -80°C until analysis

The DNA laddering technique is used to visualize the endonuclease cleavage products of apoptosis. DNA samples were separated in 2% agarose gel in TBE buffer containing ethidium bromide addition. The presence of characteristic DNA ladder was assessed by Gel-Pro Analyzer.

3. RESULTS

The aim of the study was to assess if H_2O_2-induced oxidative stress can direct cells on the apoptosis pathway or gives them the survival signal. The expression level of bax and bcl-2 mRNA was determined in the experiments (Fig. 1).



h	24	48	24	48	24	48	24	48	24	48	24	48	24	24	48	48	24	24	48	48	24	24	48	48
1	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
2	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	-	-	-
3	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+
4	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+	-	+	-	+	-	+	-	+	-
5	-	-	-	-	-	-	-	-	-	-	+	+	-	+	-	+	-	+	-	+	-	+	-	+

Fig. 1. RT-PCR analysis of mRNA for β-actin, bax and bcl-2 in Caco-2 cells 24 and 48 h after exposition to different H_2O_2 concentrations; numbers of lines denote addition (+) or lack (–) of:

1 – 100 μM quercetin, 2 – 100 μM epigallocatechin, 3 – 100 μM chlorogenic acid, 4 – 400 μM H_2O_2, 5 – 800 μM H_2O_2

The initial level of bax mRNA was low in control cells. It indicates that even in non-stressed cell the pro-apoptotic genes are expressed. Hydrogen peroxide did not influenced the expression of bax mRNA. In contrary, the expression of mRNA for anti-apoptotic bcl-2 gene was relatively high and constant in control cells, but changed significantly by the action of H_2O_2. 24 h after exposition to H_2O_2 the violent decrease in bcl-2 mRNA level was observed. The expression after 48 h depended on the H_2O_2 concentration. When low doses of stressing agent (400 μM of H_2O_2) were used the expression still decreased, while in cells exposed to strong stress (800 μM) the level of bcl-2 mRNA increased twice when compared with control. It should be highlighted that the most important for cell is not the level of gene expression, but the proportion between them. The ratio of bcl-2 mRNA to bax mRNA decides which signal obtains the cell: "to survive" or "to die by apoptosis". When bcl-2/bax ratio is higher than 1 cell is switch on the survival pathway, but when the ratio is lower than 1 it gives the death signal and apoptosis pathway starts.

Our results showed that hydrogen peroxide at 800 μM concentration is a strong stress agent that can induce apoptosis on genetic level during first 24 h. However, it also activated the repair mechanisms in cells within next hours, so the executive phase of apoptosis has been blocked and the cells returned to the initial status. The lower concentration of H_2O_2 could not induce those processes.

The hydrogen peroxide at concentration of 800 μM can induce apoptosis in Caco-2 cell. Supposing that antioxidant can play protective role against oxidative stress induced by hydrogen peroxide, the exposition to H_2O_2 should not activate apoptosis in cells pretreated with antioxidants. Our results showed that chlorogenic acid used alone acted pro-apoptotically in Caco-2 cells, causing the decrease of bcl-2 mRNA expression and not affecting bax mRNA. However, pretreatment with chlorogenic acid before oxidative stress had the protective effect. The expression of both bcl-2 and bax genes was stimulated; the stronger induction was observed for anti-apoptotic bcl-2. Hence, upon an action of ChlA in cells that had been stressed with H_2O_2 the ratio of bcl-2 to bax changed and reached the status similar that this in non-stressed cells. This means that pretreatment with chlorogenic acid can attenuate the oxidative stress consequences (Fig 2).

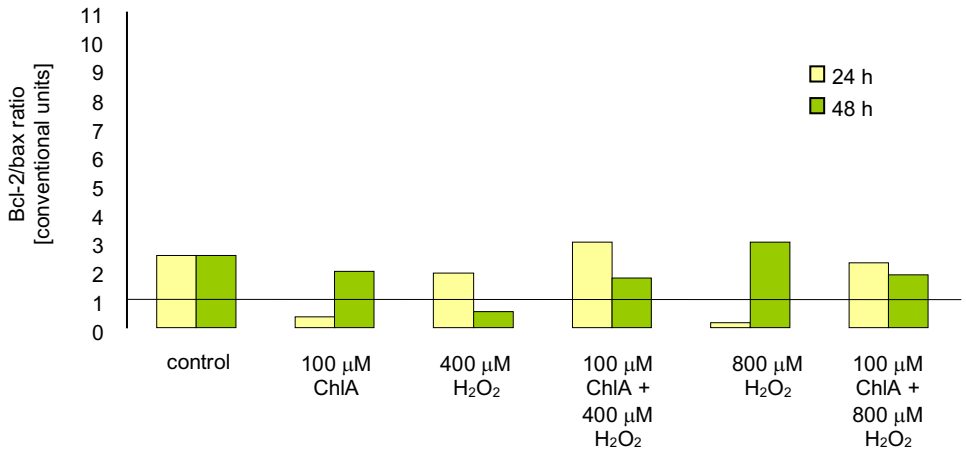


Fig. 2. Bcl-2:bax ratio in Caco-2 cells 24 and 48 hours after H_2O_2-induced oxidative stress with and without pretreatment with chlorogenic acid (ChlA)

The bax mRNA expression was relatively constant within 24 h after H_2O_2 addition, and there were no differences between cells treated and non-treated with quercetin. However, after 48 h, quercetin significantly reduced bax level in cells stressed with hydrogen peroxide. In cells incubated with quercetin alone, without the subsequent oxidative stress, the bax mRNA level grew by 50% at 24 h and more than twice after 48 h (Fig. 3).

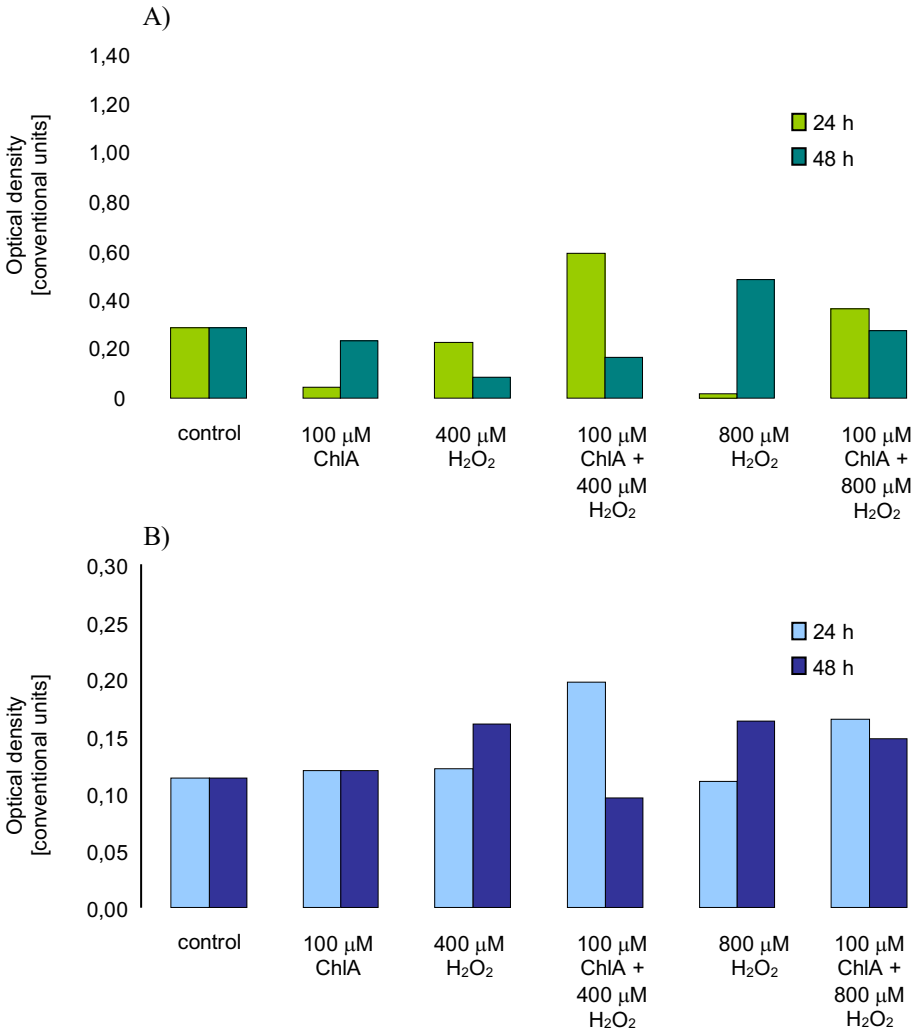


Fig. 3. The level of bcl-2 (A) and bax (B) mRNA in Caco-2 cells stressed by exposition to different H_2O_2 concentrations (400 and 800 µM) and treated or non-treated with chlorogenic acid (ChlA)

The influence of quercetin on the level of bcl-2 mRNA was similar like the effect of chlorogenic acid. However, the differences in values obtained were significantly higher. Both quercetin alone and H_2O_2-induced oxidative stress alone send signal for apoptosis. But, when oxidative stress in Caco-2 cells was preceded by incubation with 100 µM quercetin the bcl-2 gene expression increased. For the cells stressed with 400 µM hydrogen peroxide the bcl-2/bax ratio was about 10 and cells obtained strong

anti-apoptotical signal. When hydrogen peroxide was used at concentration of 800 μM the ratio returned to the initial level (Fig. 4 and 5).

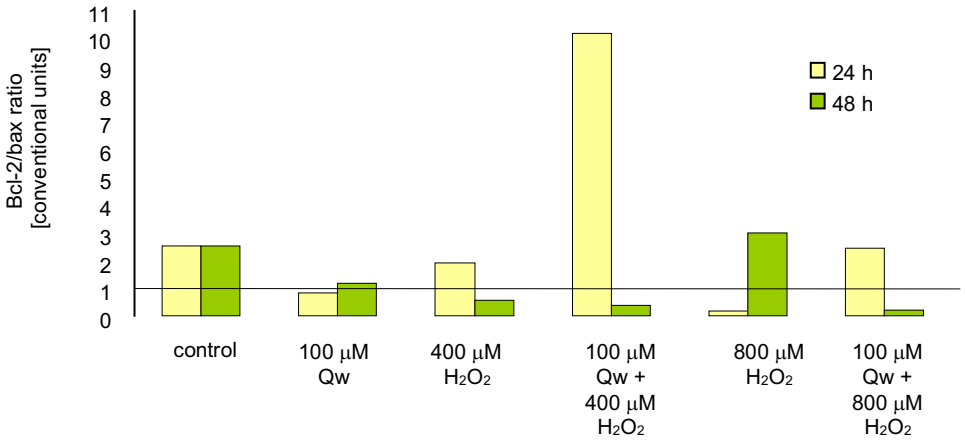


Fig. 4. Bcl-2:bax ratio in Caco-2 cells 24 and 48 hours after H_2O_2-induced oxidative stress with and without pretreatment with quercetin (Qw)

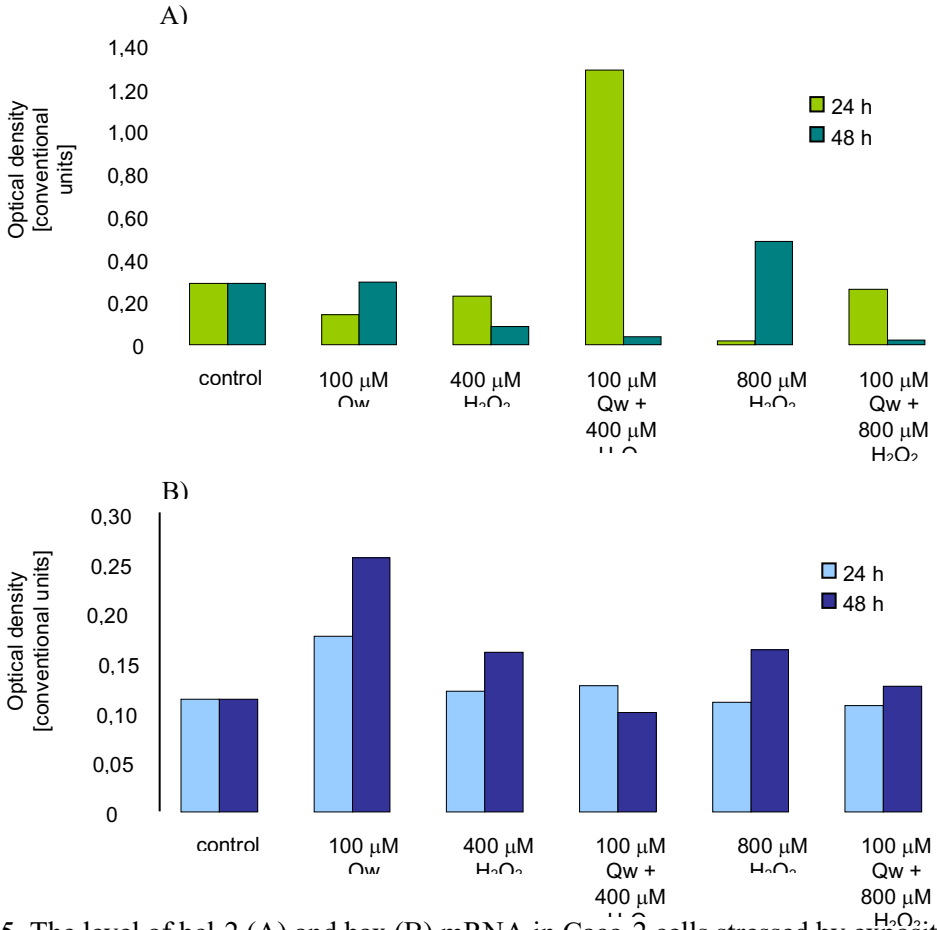


Fig. 5. The level of bcl-2 (A) and bax (B) mRNA in Caco-2 cells stressed by exposition to different H_2O_2 concentrations (400 and 800 μM) and treated or non-treated with quercetin (Qw)

On the basis of our results we demonstrated that epigallocatechin has little or no protective effect on cells exposed to oxidative stress. Pretreatment of Caco-2 cells with 100 μM of epigallocatechin 24 hours before adding the stress agent caused only slight decrease in bcl-2 with simultaneous increase in bax level, when compared with non-treated cells. Hence, both epigallocatechin alone and combination of EGC with H_2O_2 caused changes in gene expression that promoted cell death (Fig. 6 and 7).

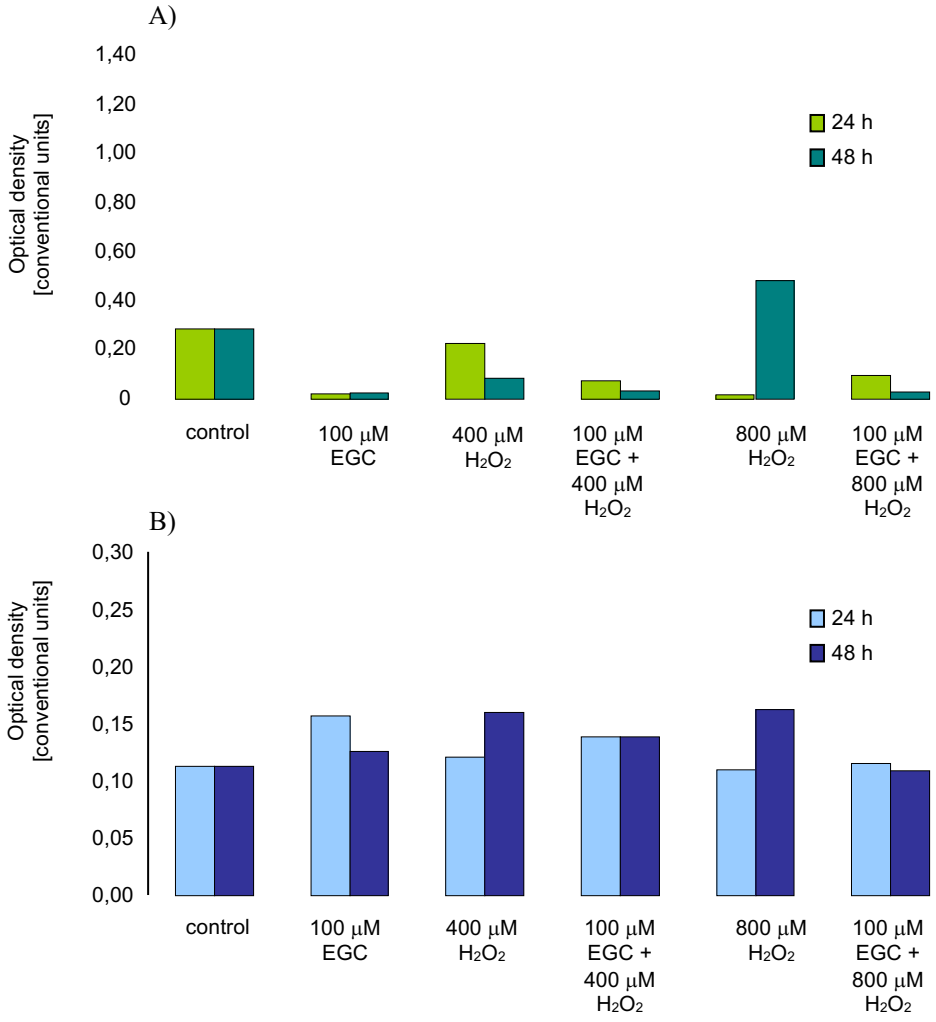


Fig. 6. The level of bcl-2 (A) and bax (B) mRNA in Caco-2 cells stressed by exposition to different H_2O_2 concentrations (400 and 800 μM) and treated or non-treated with epigallocatechin (EGC)

When the bcl-2 to bax ratio is considered, it can be seen that EGC impact depended on the H_2O_2 concentration (Fig. 7). However, in both experiments performed it was insufficient to prevent apoptosis signal. In the situation of weak oxidative stress (400 μM of H_2O_2), the EGC acted as additional stress agent causing further decrease of bcl-2/bax ratio from value 1.9 (stressed cells) to 0.5.

However, in the strong oxidative stress situation (800 μM), pretreatment with 100 μM EGC had stimulated cells (after 24 h) and attenuated pro-apoptotic influence of hydrogen peroxide.

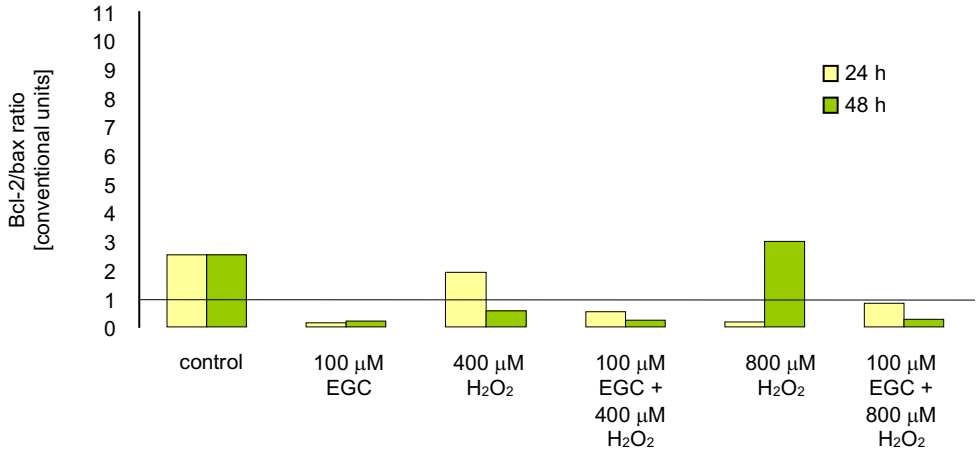


Fig. 7. Bcl-2:bax ratio in Caco-2 cells 24 and 48 hours after H_2O_2-induced oxidative stress with and without pretreatment with epigallocatechin (EGC)

In the study no executive phase of apoptosis was shown. Neither 400 μM nor 800 μM of H_2O_2 caused DNA ladder formation after gel electrophoresis (Fig. 8). However, since DNA fragmentation occurs in the later phase of apoptosis, the absence of a DNA ladder does not eliminate the potential that cells are undergoing early apoptosis.

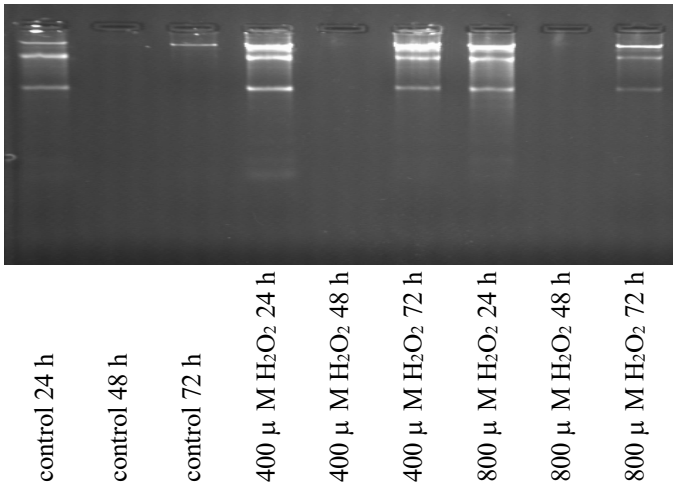


Fig. 8. The electrophoretical separation of DNA isolated from Caco-2 cells exposed to 400 μM or 800 μM of H_2O_2

The analyses were performed to check the way of cell protection by antioxidants against oxidative stress. One of the hypotheses was that protective effect consists in increased level and activity of

intracellular antioxidant enzymes. Another possibility was that antioxidants cooperate with those enzymes in fighting free radicals and reactive oxygen species (ROS). It was also taken into account that antioxidants do not influence the enzymes but directly scavenge ROS. The obtained results showed that differences in glutathione peroxidase activity are not statistically significant (Fig. 9-11). However, some tendency was demonstrated. During first 24 hours the activity of enzymes is usually lower in stressed cells than in control, non stressed cells. On the contrary, after 48 hours the slight increase of enzyme activity could be observed in stressed cells, both treated and non-treated with antioxidants, when compared with control cells.

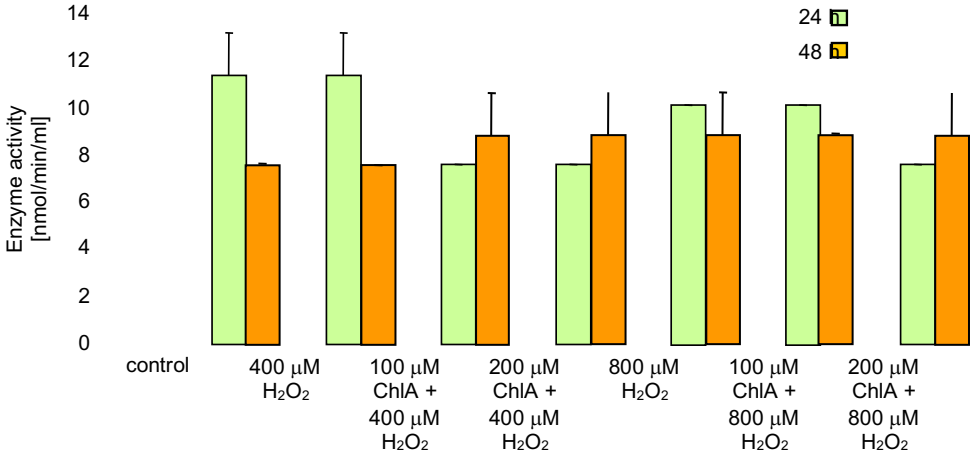


Fig. 9. Changes in activity of glutathione peroxidase 24 h and 48 h after oxidative stress induced by 400 µM or 800 µM of hydrogen peroxide with or without pretreatment with different concentrations of chlorogenic acid (ChlA)

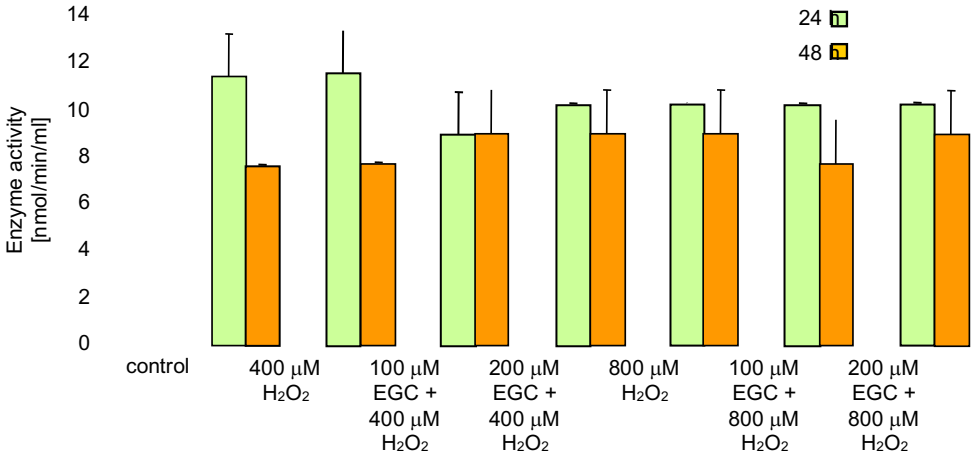


Fig. 10. Changes in activity of glutathione peroxidase 24 h (A) and 48 h (B) after oxidative stress induced by 400 µM or 800 µM of hydrogen peroxide with or without pretreatment with different concentrations of epigallocatechin (EGC)

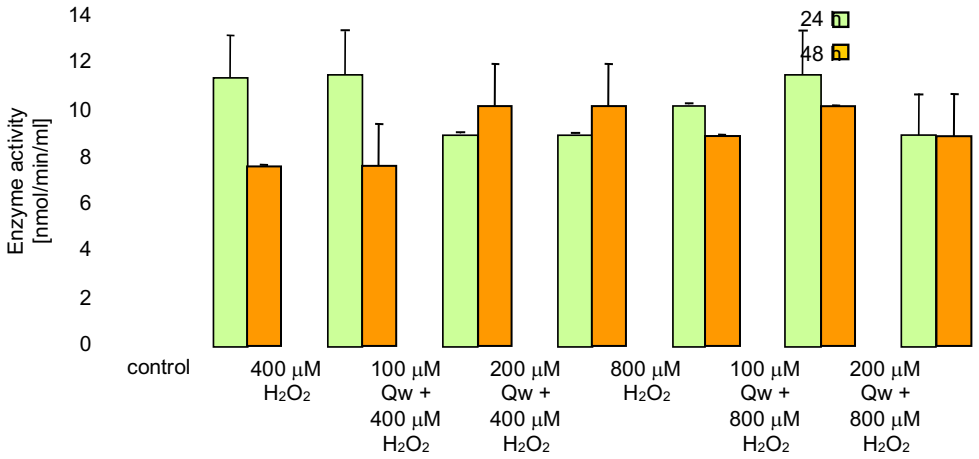


Fig. 11. Changes in activity of glutathione peroxidase 24 h (A) and 48 h (B) after oxidative stress induced by 400 μM or 800 μM of hydrogen peroxide with or without pretreatment with different concentrations of quercetin (Qw)

The analysis of catalase activity demonstrated more significant changes. It seems that each of used antioxidants influence the CAT activity in different way. Both weak and strong H_2O_2-induced stress resulted in lower CAT activity when compared with non-stressed cells. In cells exposed to higher H_2O_2 concentration (800 μM) the catalase activity was higher than in cells exposed to 400 μM. Moreover, in those cells addition of chlorogenic acid before oxidative stress caused increase of enzyme activity above the level noticed in non-treated stressed cells, after 24 hours. Nevertheless, chlorogenic acid neither in 100 μM nor 200 μM concentration could recover the enzyme activity to the control level after 24 h (Fig. 12).

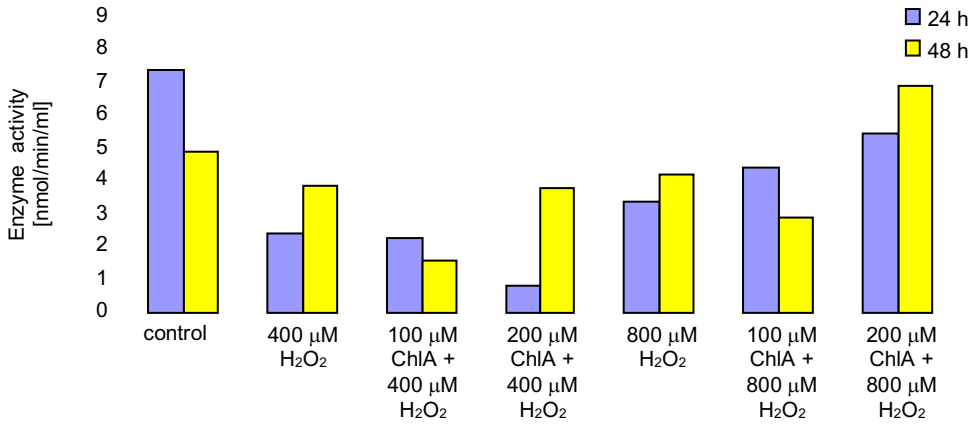


Fig. 12. Catalase activity in cells exposed to different doses of H_2O_2 and pretreated or non-treated with different concentration of chlorogenic acid (ChlA) before stress

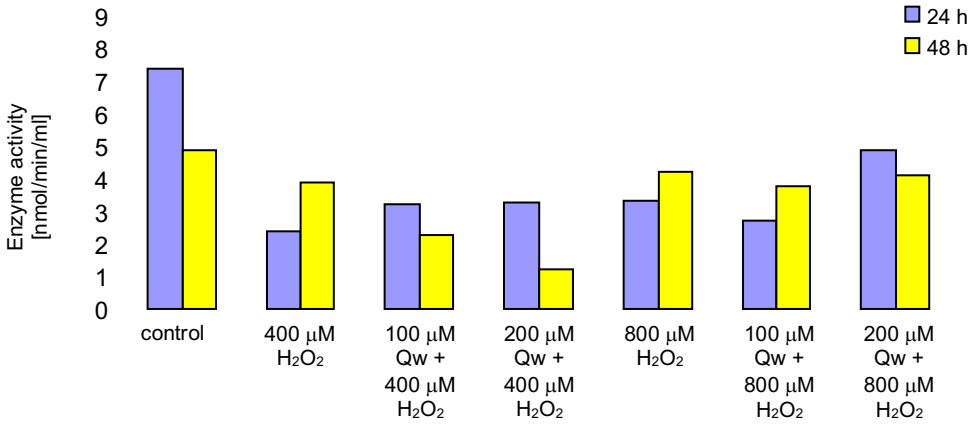


Fig. 13. Catalase activity in cells exposed to different doses of H_2O_2 and pretreated or non-treated with different concentration of quercetin (Qw) before stress

Similar situation was observed when quercetin was used as antioxidant; none of used concentrations could return the control level of catalase activity (Fig. 13). Different effect could be observed when epigallocatechin was used to protect cells (Fig. 14). Epigallocatechin used at concentrations of 100 μM and 200 μM caused growth of catalase activity both in weak and strong oxidative stress situation after 24 h, but only in cells exposed to 800 μM after 48 hours. It should be emphasized that only this antioxidant enables recovery of initial catalase activity 48 h after exposition to 800 μM of H_2O_2.

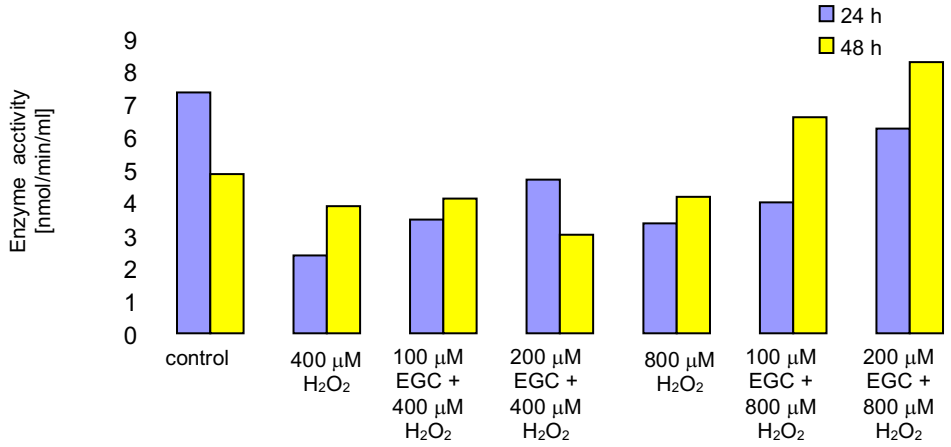


Fig. 14. Catalase activity in cells exposed to different doses of H_2O_2 and pretreated or non-treated with different concentration of epigalocatechin (EGC) before stress

4. DISCUSSION

Cells proliferation is an important process that enables regeneration and turnover of epithelial cells in gut, but uncontrolled proliferation might be dangerous and lead to tumours initiation. In our previous study (Duda-Chodak et al. 2009) we showed that chlorogenic acid and quercetin had activated Caco-2 cells proliferation in time- and concentration-dependent manner, and chlorogenic acid at low doses (25-50 μM) was more potent stimulator than quercetin. In contrary, epigallocatechin exhibit inhibitory influence on Caco-2 cells proliferation, particularly significant at concentration ≥200 μM. This EGC activity was probably caused by prooxidant action of epigallocatechin. It was already demonstrated that some antioxidants such as gallic acid and catechins could induce H_2O_2 generation in culture medium. In addition, the green tea components (e.g. epigallocatechin) can promote H_2O_2 production and generation of quinones and semiquinones, which can damage the cell. Hydrogen peroxide subsequently causes DNA oxidation and exerts strong antiproliferative effect, which can be abolished by the treatment with catalase (Lapidot et al. 2002, Chai et al. 2003, Furukawa et al. 2003). On the other side, H_2O_2 generated in small amounts as well as other ROS can activate cells, because those molecules act as secondary messengers (Rhee et al. 2003). Therefore ROS can induce defense mechanisms, for example synthesis of heat shock proteins or antioxidant enzymes. Thanks to it cells can react much faster and more efficient when another stronger stress agent will act on them. This effect, called preconditioning, is common in nature. It was found that ROS-generated injury in ischemia-reperfusion is less widespread if cells or tissue underwent before the short ischemic episode (Grajek 2007).

In our study we had shown that in Caco-2 cells double exposed to 400 μM or 800 μM of hydrogen peroxide the oxidative stress was induced. Those results comply with other researches. In the study of Wijeratne et al. (2005) the cell incubation for 30 min with H_2O_2 at concentrations <250 μM did not influence on cells viability; only higher doses were harmful.

One of the aims of this work was to check if oxidative stress induced by high concentration of H_2O_2 (800 μM) can promote apoptosis in cells or activates survival signal. Our results indicate that in the experiment conditions the apoptotic signal was regulated on the cell level by the changes in bcl-2 gene expression. The important role of bcl-2 gene in apoptosis regulation was confirmed by Ichimiya et al. (1998). They had shown that bcl-2 is anti-apoptotic and protects cells against oxidative stress-induced death. The mechanism of this protection is not known, but it is supposed that the Ca^{2+} ions are involved. The increased concentration of calcium ions in cytosol induces Ca-dependent endonucleases that cut DNA. Kidney cells transfected with bcl-2 gene were more resistant to H_2O_2 than control non-transfected cells (Ichimiya et al. 1998).

In our study Caco-2 cells were characterized by relatively high level of bcl-2, which can testify about their good condition. In cells stressed by 800 μM of hydrogen peroxide the level of bcl-2 rapidly fell, nearly to zero after 24 h. Then, slowly increased which can suggest the repair mechanism had started. The differences in bax level between stressed and control cells were not significant. When the ratio of bcl-2 to bax mRNA level is considered it can be seen that in cells stressed with 800 μM of H_2O_2 strong apoptotic signal was induced. However, our results show no DNA ladder in cells stressed with H_2O_2 at concentration of 400 and 800 μM. It means that the repair mechanism in cells succeeded and the executive phase of apoptosis was stopped.

DNA ladder is the fragmented DNA separated in electric field, so we can illustrate with this technique the last stage of apoptosis. Collins et al. (2001) used the comet assay for DNA damage evaluation. In the study, DNA in lymphocytes taken from volunteers was damaged already by 200 μM of hydrogen peroxide, and the long-lasting kiwi fruit consumption before the experiment provided the protection. The other study, performed on Caco-2 and HepG2 cell line, had shown that 30 min exposition to 10–

100 μM of H_2O_2 caused DNA fragmentation, while pretreatment with 200 μM of quercetin, myricetin or rutin protected DNA against H_2O_2-induced damage (Aherne & O'Brien 1999).

We did not demonstrate any significant changes in activity of glutathione peroxidase (GPx), one of the intracellular antioxidant enzymes. During cell culture, this enzyme is utilized by cells for neutralizing ROS formed in aerobic conditions. The decrease of enzyme activity observed in cells exposed to 800 μM of H_2O_2 after 24 h, followed by the growth in next hours could be caused by the exhaustion of enzyme possibilities and the *de novo* synthesis induced by oxidative stress.

Activity of catalase (CAT) decreased significantly 24 and 48 hours after exposition to both 400 μM and 800 μM of hydrogen peroxide. The discrepancy could be observed with results of Wijeratne et al. (2005). Although they had shown that glutathione peroxidase activity was almost constant, the catalase activity was dependent on H_2O_2 concentration. They demonstrated that in cells exposed to 50-250 μM of H_2O_2 CAT activity was unchanged, while significant increase in its activity was shown in cells stressed with doses above 250 μM. Also Fridovich (1998) indicated that catalase is more active at high concentrations of H_2O_2. This enzyme is localized in mitochondria, so can quickly react to oxidative stress and ROS formed on electron transport chain. However, catalase can also act as peroxidase and utilize H_2O_2 for oxidation of small molecules such as methanol, ethanol, and nitrogen.

Saito et al. (2004) had demonstrated that quercetin can generate H_2O_2. It is possible that this phenomenon is responsible for protective effect of quercetin and others antioxidants. The releasing of small amounts of ROS can activate defense mechanisms in cell. In the situation of proper oxidative stress those mechanisms are ready for rapid elimination of harmful molecules and prevent the injury.

Our results had shown that differences in catalase and glutathione peroxidase activity are too small and the protective influence of antioxidants is rather independent on antioxidants enzyme. Similar results obtained Kameoka et al. (1999). In the study with Caco-2 cells genistein, daidzein and kaempferol did not influence the activity of catalase and superoxide dismutase (SOD). They only observed increased activity of metalotionein that protects cells against heavy metal toxicity. Aherne and O'Brien (1999) also proved that 10–200 μM of quercetin, myricetin and rutin had no effect on catalase and SOD activity.

In research performed it was shown that chlorogenic acid, quercetin and epigallocatechin significantly increase expression of anti-apoptotic bcl-2 gene in cells 24 h after H_2O_2-induced stress. In case of quercetin and epigallocatechin the effect was brief and the amount of this transcript decreased after 48 h. It is highly possible that those substances were metabolized by cells and formed derivatives without protective influence. On the contrary, protection by chlorogenic acid was quite stable and in second day the level of bcl-2 was still elevated. It is very interesting that antioxidants alone added to Caco-2 cells caused reduction in bcl-2 mRNA level. It suggests that treatment with antioxidants produces in those cells weak stress, which contributes in the phenomenon of preconditioning.

The results obtained in this study indicated that chlorogenic acid, quercetin and epigallocatechin had acted by different mechanisms, and further research should be performed. Particularly, the rate of antioxidants absorption as well as the enzymatic transformation of those compounds in Caco-2 cells should be evaluated. Then, the potential protective effect of the generated derivatives should be also assayed.

5. CONCLUSIONS

We demonstrated that treatment of Caco-2 cells with chlorogenic acid, quercetin or epigallocatechin at concentration of 100 or 200 μM for 24 h before H_2O_2 addition could activate anti-apoptotic signal in

cells and prevent their apoptosis. Used antioxidants could prevent H_2O_2-induced cell injury, and chlorogenic acid and quercetin were more potent protectors than epigallocatechin. On the basis of the results we found that protective influence of antioxidants is rather independent on antioxidant enzymes activity. The positive impact of antioxidants is probably based on preconditioning phenomenon and depended on polyphenols-induced generation of small amounts of H_2O_2 in medium before the actual stress.

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