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## DRUG BIOCHEMISTRY

# THE EFFECTS OF COENZYME Q10 AND BAICALIN IN CISPLATIN-INDUCED LIPID PEROXIDATION AND NITROSATIVE STRESS

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**Abstract:** Cisplatin is the alkylating anticancer drug. These drugs show many side-effects including the damage of kidney. The nephrotoxicity of cisplatin is explained mainly by reactive oxygen species (ROS) generation. The increased level of lipids peroxidation was observed in patients treated with this drug. In the toxicity of cisplatin, are also involved reactive nitrogen species (RNS) such as nitric oxide ($NO^{\bullet}$) or peroxynitrite. The lack of cisplatin selectivity and its side effects tend to look for ways to reduce the toxicity in chemotherapy. Our previous studies demonstrated that oxidative stress caused by xenobiotics can sometimes be effectively inhibited by coenzyme Q10 and baicalin. The aim of our research was the evaluation of usefulness of two coenzyme Q10 forms: lipophilic, currently used (QA) and new, produced by nanotechnology, soluble in water, PureSorb-QTM40-P40 (QB). Also the utility of baicalin as free radicals scavenger in reducing the nephrotoxicity of cisplatin was examined. The study was performed on an *in vitro* model, human erythrocytes and serum. Oxidative stress was evaluated by the level of lipid peroxidation (TBARS method). The concentration of nitric oxide ($NO^{\bullet}$) and nitrate ($NO_3$) was estimated in serum [Nitric Oxide Colorimetric Detection Kit (Cat. No. K023-H1) of Arbor Assays], based on reaction with Griess reagent. Cisplatin at concentration: 3.5, 10, 30 and 50 μg/mL significantly increased the level of TBARS in erythrocytes. All antioxidants: baicalin and two forms of coenzyme Q10 significantly inhibited TBARS compared to controls ($p < 0.05$). Both QA and QB studied in a wide range of concentrations (from 1.0 to 120.0 μg/mL) demonstrated their antioxidative effect. In all used doses they statistically significantly decreased TBARS level with the negative correlation ($r = -0.751$; $p = 0.000$). In the study of nitrosative stress, all doses of cisplatin increased $NO^{\bullet}$ and $NO_3$ level in serum ($p < 0.05$). Baicalin and QA showed no statistically significant influence on production of $NO^{\bullet}$ and $NO_3$ in serum, while QB unexpectedly increased these parameters. In joint exposure with cisplatin all three antioxidants, in the most of concentrations, decreased TBARS levels, elevated by cisplatin ($p < 0.05$). In nitrosative stress-induced by cisplatin, the most effective was QB, however, protective influence of all antioxidants varies and the results are ambiguous.

**Keywords**: cisplatin, coenzyme Q10, baicalin, lipid peroxidation, nitric oxide

Cisplatin is classified to the anticancer alkylating agents. Adducts cisplatin-DNA, linking to the p53 protein, Bcl-2 (B-cell lymphoma 2) protein family, caspases, cyclins, CDKs (cyclin-dependent kinases), pRb (retinoblastoma tumor suppressor protein) and MAPK (mitogen-activated protein kinases) activate various signaling cells pathways and ultimately causes cell death (1, 2). The lack of selectivity of alkylating agents, and numerous side effects e.g., nephrotoxicity, tend to look for ways to reduce the side effects of chemotherapy.

In our study on cisplatin it was found that its toxicity is associated largely with the generation of oxygen free radicals (3). This drug stimulates the formation of superoxide anion ($O_2^{\cdot-}$), hydroxyl radical ($^{\cdot}OH$) and hydrogen peroxide ($H_2O_2$) (4). In the toxicity of cisplatin, reactive nitrogen species (RNS) such as nitric oxide ($NO^{\bullet}$) and peroxynitrite are also involved (5). Nitric oxide due to its chemical structure belongs to free radicals family. Cisplatin induces the formation of $NO^{\bullet}$ mainly by activation of NF-κB (family of proteins acting as transcription factor in apoptosis) (6, 7). This, at the reaction with superoxide anion, forms peroxynitrite which damages mitochondrial electron transport chain, disrupting normal cell metabolism (8). Nitric oxide is an unstable form, rapidly metabolized to a more sustainable products (nitrates and nitrites) (9). There is information about the advantageous effects of antioxidants (vitamins C, E, N-acetylcysteine) in the therapy with cisplatin (10, 11). Recently, Found and al. described the amelio-

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ration of acute cisplatin nephrotoxicity by coenzyme Q10 in mice (12).

Our *in vitro* study demonstrated that oxidative stress caused by some xenobiotics can be inhibited by coenzyme Q10 or baicalin, however, some interaction were also observed (13, 14). Antioxidative activity of coenzyme Q10 is largely described, also its protective role against reactive nitrogen species. Reduced form, ubiquinol (CoQ10H2) has a capacity to direct reaction with nitric oxide and peroxynitrite (15). Coenzyme Q10, due to its hydrophobicity, has hindered absorption and low bioavailability. However, one produced by nanotechnology (QB), soluble in water, shows an almost 3-fold greater bioavailability than the conventional QA. QB is achieved by closing the molecule of coenzyme Q10 (QA) in the maltodextrin and reducing its size to 0.2 microns. The product, which was developed by Japanese company - Mitsubishi Gas Chemical Company Inc., was described as PureSorb-QTM40 (in short: P40) (16). Baicalin is a flavonoid found in the root of the Chinese medicinal plant, *Scutellaria baicalensis*. Its ability to reduce the oxidative stress, as well as nitrosative stress, was described (17, 18). It significantly inhibited NO production induced by lipopolysaccharide (19). Our previous studies have demonstrated the effectiveness of baicalin in the inhibition of free radical reactions induced by Cr(III) and Cr(VI) (14). The lack of data in the literature about the role of baicalin in cisplatin-induced lipid peroxidation prompted us to study the combined effect of baicalin and cisplatin.

The aim of our research was the evaluation of usefulness of two forms of coenzyme Q10: lipophilic (QA) and new, soluble in water (QB) and baicalin in reducing the formation of free radicals produced by cisplatin. The study evaluated the influence of cisplatin, both forms of coenzyme Q10 (QA and QB) and baicalin on the concentration of lipid peroxidation products (TBARS) in blood and the concentration of nitric oxide (NO) and nitrate ($NO_3$) levels in serum. Then, the effect of these antioxidants in exposure to cisplatin was assessed, in order to determine whether they show protective effect or lead to harmful interaction.

## EXPERIMENTAL

The study was performed on an *in vitro* model: human erythrocytes or serum. Blood was collected on sodium citrate or to clot, from healthy volunteers (the approval of the Ethics Committee, permit KB-158/2010). Blood was centrifuged and plasma discarded. Red blood cells were washed three times with saline (0.9% NaCl), and then 10% cell suspension in PBS (phosphate buffered saline) (pH 7.4) was prepared. The level of hemoglobin (Hb) in blood cell was determined by Drabkin commercial kit. Cisplatin (Ebeve Company) in concentrations: 3.5, 10, 30 and 50 μg/mL in 0.9% NaCl was used. The antioxidants: QA [Coenzyme Q10 – Sigma], QB [soluble in water form PureSorb-QTM40 (40%), Mitsubishi Gas Chemical Co., Inc.] in concentrations 1, 2, 4, 8, 20, 40, 60, 80, 100, 120 μg/mL and baicalin [95%, Sigma-Aldrich] in concentrations: 1, 10, 20, 100, 200 μM were examined. QA and baicalin were dissolved in DMSO, QB in water. To assess the effect of QA, QB or baicalin in cisplatin induced stress, the drug was used in four concentrations: 3.5, 10, 30 and 50 μg/mL; baicalin in four concentrations: 10, 20, 100 and 200 μM but QA or QB were used in five concentrations: 2, 8, 20, 60 and 120 μg/mL. The selection of antioxidants and cisplatin doses was dictated by our experience and literature data. In order to study the possible protective action of various antioxidants in the exposure to cisplatin, the antioxidant was added to red cells suspension, sample was incubated at 37°C for 30 min and then treated with cisplatin and incubated at 37°C for 30 min. After that, concentrations of TBARS or nitric oxide and nitrate were estimated by the appropriate method. TBARS was measured spectrophotometrically according to Stocks, modified by Gawlik and expressed in nmol/g hemoglobin. It is a method involving the reaction of lipid peroxidation products, induced by cumene hydroperoxide, with thiobarbituric acid (20, 21). The concentration of $NO^{\bullet}$ was estimated in serum with Nitric Oxide Colorimetric Detection Kit (Cat. No. K023-H1 of Arbor Assays), based on reaction with Griess reagent. According to this test, the concentration of nitrate ($NO_3$) was also assessed. Nitrate reductase (NR) and NADH before use were kept on ice. Proteins were removed from the test serum by filters with a high resolution of 10 kDa (Biokom). Total nitric oxide content was measured after the sample was incubated with NR and NADH. The reductase in combination with NADH reduces nitrate to nitrite. After 20 min incubation at room temperature, color reagent A (sulfanilamide) and B (N-1-naphthylethylenediamine) were added and incubated at room temperature for 5 min. The colored product absorbance was read at 540 nm. The proper reaction was carried out in microplates (22). To each series of the experiment the control samples were used. The results were evaluated statistically with program Statistica PL 9.0. The variability of distribution was checked with Lilliefors test. Data with a

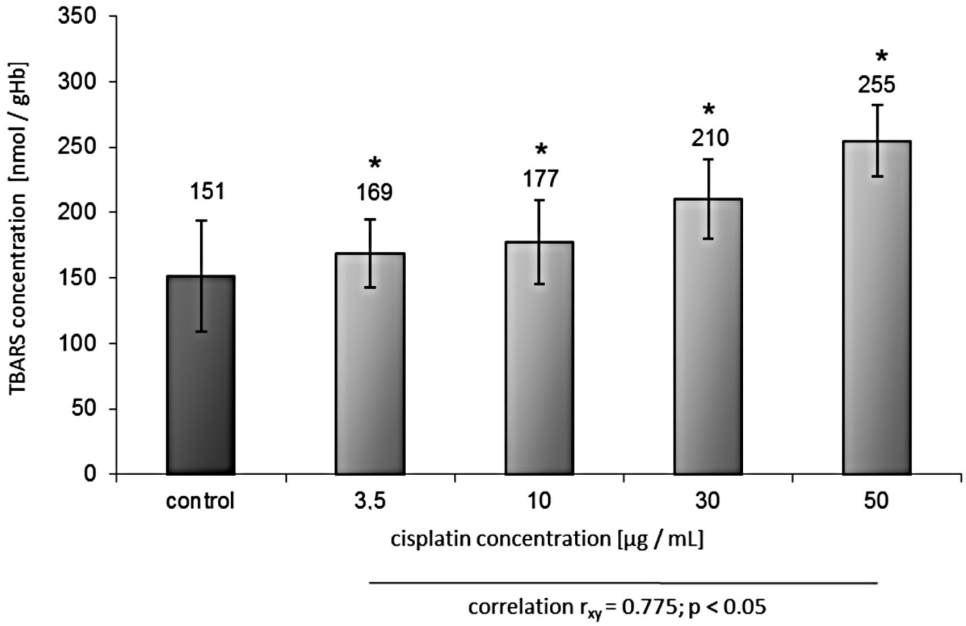


Figure 1. Effect of cisplatin on TBARS concentration in erythrocytes against control
*Statistically significantly higher than control ($p < 0.05$)

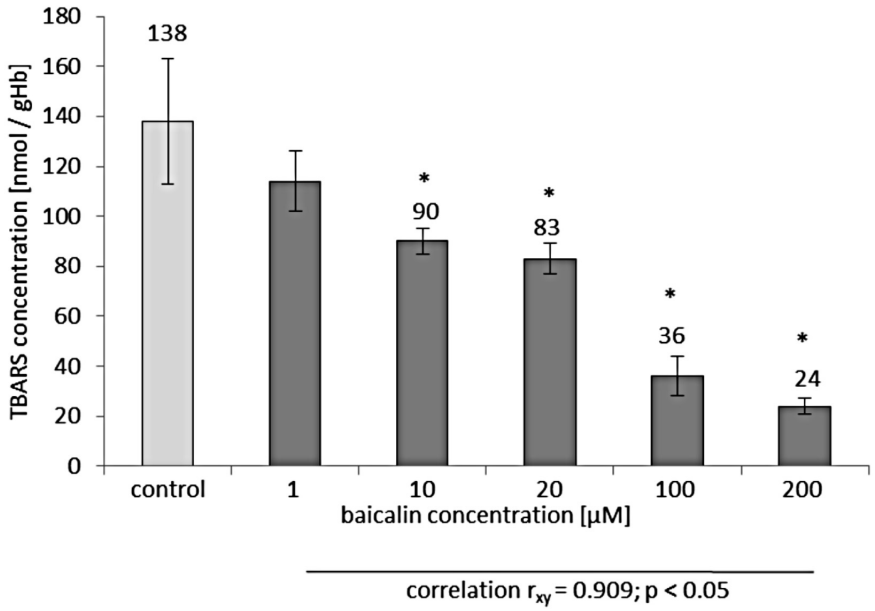


Figure 2. Effect of baicalin on TBARS concentration in erythrocytes against control
*Statistically significantly lower than control ($p < 0.05$)

normal distribution were evaluated using one-factor analysis of variance ANOVA and *post hoc* RIR Tukey's test. Dependence between variables were examined using Pearson and Spearman correlation.

## RESULTS

It was observed that cisplatin at concentrations: 3.5, 10, 30 and 50 µg/mL significantly

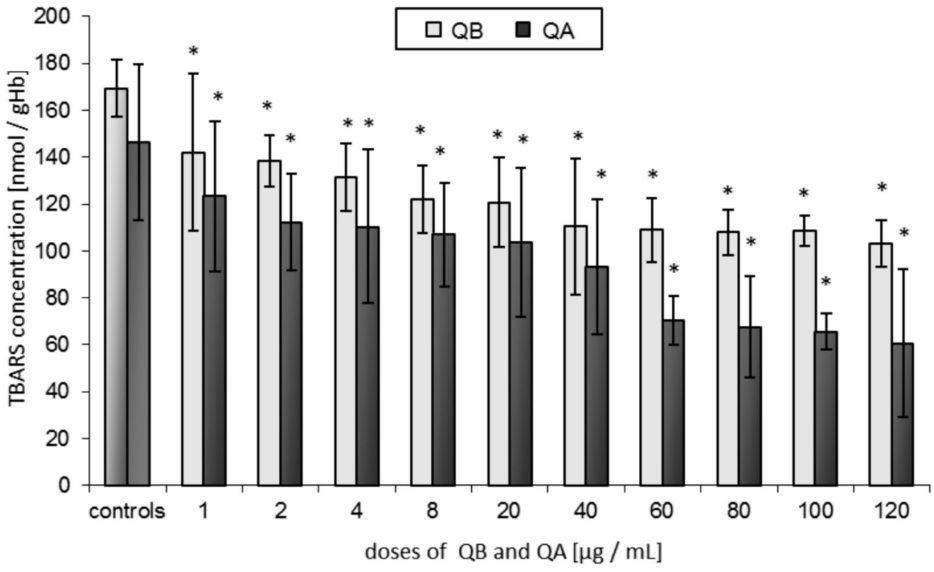


Figure 3. The comparison of two forms of coenzyme Q10: QA and QB on TBARS concentration against controls
*Statistically significantly lower than controls ($p < 0.05$)

increased the level of TBARS ($p < 0.05$) (Fig. 1). The positive correlation between TBARS and the concentration of cisplatin with Pearson's coefficient $r = 0.775$ ($p < 0.05$) was noted. Baicalin at the concentrations: 10, 20, 100 and 200 µM significantly inhibited TBARS induced by cumene hydroperoxide, compared to controls, with the negative, statistically significant dose-response correlation ($r = -0.909$; $p < 0.05$) (Fig. 2). The lipophilic QA in all doses statistically significant decreased TBARS level with the negative correlation ($r = -0.751$, $p < 0.05$). The water-soluble coenzyme Q10 (QB) showed similar effects as QA ($p < 0.05$) (Fig. 3). The negative linear relationship between concentrations of QB and TBARS level ($r = -0.4508$, $p < 0.05$) was observed. In the second stage, the effect of cisplatin and three antioxidants on $NO^{\cdot}$ and nitrate concentration was estimated. All doses of cisplatin increased $NO^{\cdot}$ and $NO_3$ level ($p < 0.05$) (Table 1). Unexpectedly, similar effect was observed for QB, because in concentration: 2.0; 8.0 and 40.0 µg/mL increased $NO^{\cdot}$ and $NO_3$ level as compared to the control ($p < 0.05$) (Table 1). Both baicalin and QA didn't influence statistically significantly on $NO^{\cdot}$ and $NO_3$ production in serum (Table 1). Then, the effectiveness of antioxidants against the influence of cisplatin on TBARS in red blood cells and $NO^{\cdot}/NO_3$ level in serum was estimated. The positive, statistically significant effects of two forms of coenzyme Q10 (QA and QB) and baicalin on lipid peroxidation process stimulated by cisplatin was noted. All three antioxidants decreased TBARS levels, increased by cisplatin in the order: QB > QA > baicalin (Figs. 4-6). Baicalin decreased TBARS levels induced only by low concentrations of cisplatin: 3.5 and 10.0 µg/mL (Fig. 6). There was no occurrence of any harmful interaction (e.g., synergistic action). Some differences were noted in nitrosative stress. Although baicalin alone had no effect on $NO^{\cdot}$ and $NO_3$, in combined action with cisplatin decreased them in a statistically significant way in comparison to controls (action of cisplatin alone) (Fig. 7). The action of QB is different than QA in nitrosative stress. In contrary to QA, the water soluble QB reduced the $NO^{\cdot}$ and $NO_3$ level increased by cisplatin at the highest dose of 50 µg/mL (Fig. 7). QA is not as beneficial in nitrogen stress as it was in oxidative stress, caused by cisplatin. The higher QA concentration (40 µg/mL) is needed to decrease $NO^{\cdot}/NO_3$ level in serum. The form QB is more active. It looks that also in nitrosative stress, as in oxidative stress caused by cisplatin, soluble form (QB) in more effective than lipophilic one (QA).

## DISCUSSION AND CONCLUSION

The study examined the effectiveness of two forms of coenzyme Q10 and baicalin in oxidative stress induced by cisplatin. This drug caused the formation of superoxide anion, hydroxyl radical and hydrogen peroxide. There are three intracellular levels at which cisplatin may initiate the formation of ROS: cell membrane, cytoplasm and cell organelles. It was demonstrated that administration of cisplatin results in a marked decrease in activity of antioxidative enzymes: catalase, SOD and GPx with concomitant significant increase in TBARS level (23). Cisplatin induced also the reactive nitrogen species (RNS) - peroxynitrite (ONOO), nitric oxide ($NO^{\cdot}$) or nitric dioxide ($NO_2^{\cdot}$) (24, 25). Except positive role, nitric oxide increases inflammation and oxidative processes. Produced in excess, raises peroxynitrite synthesis (6, 26). The side effect of ROS produced by cisplatin is widely described, while the contribution of reactive nitrogen species (RNS) in cisplatin toxicity is raised less.

The present study confirmed cisplatin involvement in oxidative stress in red blood cells. Moreover, the participation of cisplatin in nitrosative stress was shown, because it raised the formation of nitric oxide and nitrates in serum. In studies of Rybak et al. (23), during cisplatin treatment, the increase in iNOS activity and $NO^{\cdot}$ synthesis in the middle ear occurred, leading to hearing dysfunction in rats (27). Furthermore, recent studies on guinea pigs showed that the toxic mechanism of cisplatin involves both the oxidative and nitrosative stress. The level of MDA, NO and peroxinitrite in the middle ear tissue increased significantly (28). The influence of peroxynitrite is due to its strong oxidative properties, thus initiating lipid peroxidation, DNA and protein damage. The reaction of peroxynitrite with -SH groups of proteins lead to disulfides formation (29). So in agreement with previous studies, the present one confirmed that oxidative and nitrosative stress is implicated in the side effects of cisplatin

Coenzyme Q10 effects as a powerful antioxidant which scavenges free radicals, prevents the ini-

Table 1. The mean value of $NO^{\cdot}$ and $NO_3$ in serum in exposure to cisplatin, baicalin, QA and QB.

| Cisplatin conc. [µg/mL] | Mean conc. $NO^{\cdot}$ [µM/L] ± SD | Mean conc. $NO_3^-$ [µM/L] ± SD |
|---|---|---|
| control | 7.47 ± 1.23 | 6.87 ± 1.22 |
| 3.5 | 11.20 ± 0.082 * | 10.67 ± 0.08 ** |
| 10 | 11.14 ± 0.72 * | 10.33 ± 0.76 ** |
| 30 | 11.60 ± 0.099 * | 10.81 ± 0.24 ** |
| 50 | 13.81 ± 0.24 * | 12.96 ± 0.30 ** |
| Baicalin conc. [µM] | Mean conc. $NO^{\cdot}$ [µM/L] ± SD | Mean conc. $NO_3^-$ [µM/L] ± SD |
| control | 11.19 ± 1.19 | 10.32 ± 1.07 |
| 10 | 11.16 ± 0.05 | 10.17 ± 0.06 |
| 20 | 11.74 ± 0.38 | 10.77 ± 0.30 |
| 100 | 10.48 ± 0.42 | 9.76 ± 0.55 |
| QA conc. [µg/mL] | Mean conc. $NO^{\cdot}$ [µM/L] ± SD | Mean conc. $NO_3^-$ [µM/L] ± SD |
| control | 11.19 ± 1.19 | 10.32 ± 1.07 |
| 2 | 11.66 ± 0.20 | 11.07 ± 0.20 |
| 8 | 11.02 ± 0.57 | 10.27 ± 0.49 |
| 40 | 10.82 ± 0.89 | 10.11 ± 0.77 |
| QB conc. [ µg/mL] | Mean conc. $NO^{\cdot}$ [µM/L] ±SD; | Mean conc. $NO_3^-$ [µM/L] ± SD |
| control | 8.46 ± 0.046 7.91 ± 0.06 | |
| 2 | 12.88 ± 0.71 * | 12.11 ± 0.72 ** |
| 8 | 11.59 ± 0.46 * | 10.86 ± 0.44 ** |
| 40 | 12.56 ± 0.45 * | 11.81 ± 0.43 ** |

*Statistically significant against control for NO ($p < 0.05$). **Statistically significant against control for $NO_3$ ($p < 0.05$); SD - standard deviation.

tiation and propagation of lipid peroxidation in cellular biomembranes and helps regeneration of α-tocoferol (30, 31). Also baicalin was active in our study, decreasing TBARS level statistically significantly. Flavonoids of *Scutellaria baicalensis* such as baicalin or baicalein are effective scavengers of free radicals, because they release a proton from its phenol group and scavenge superoxide anions and nitric oxide (32). Some studies of baicalin showed its greater ability to scavenge free radicals by this flavonoid than tocopherol or allopurinol in mitochondrial membranes. The protective effect of baicalin on fibroblasts and epidermal cells of glomerular mesangial against reactive forms of oxygen, including the ability to reduce the production of hydroxyl radical, was proved (33). Also Bochorakova et al. (34) showed the baicalin ability to scavenge ·OH radicals formed during UV photol-

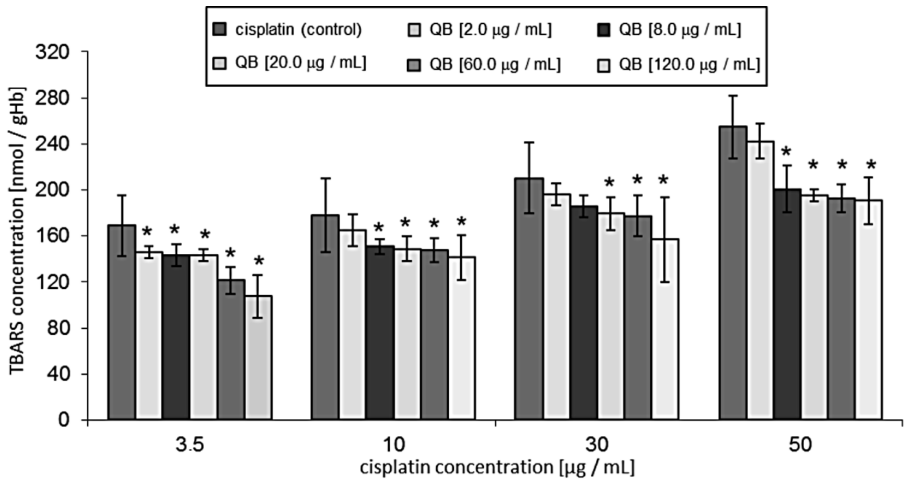


Figure 4. Effect of QB in five concentrations (2.0, 8.0, 20.0, 60.0 and 120.0 µg/mL) on TBARS level in erythrocytes exposed to cisplatin against control sample (cisplatin alone)
*Statistically significantly lower than control ($p < 0.05$)

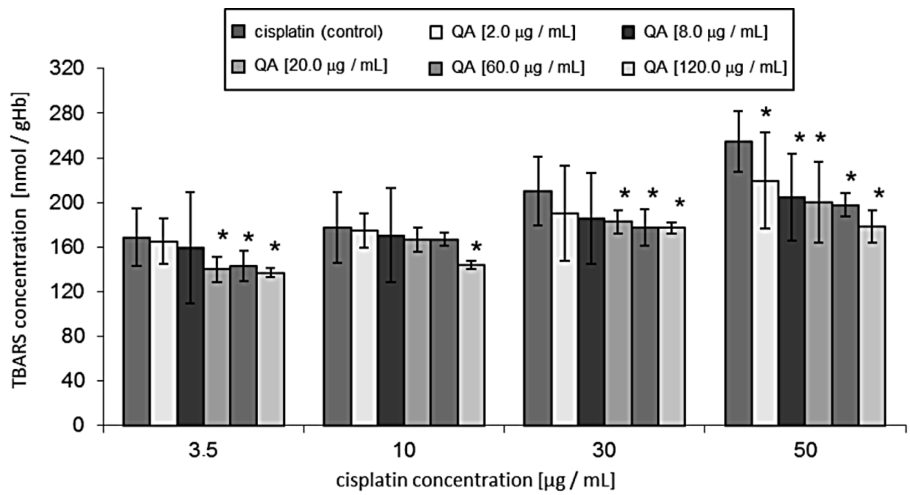


Figure 5. Effect of QA in five concentrations (2.0; 8.0; 20.0; 60.0 and 120.0 µg/mL) on TBARS level in erythrocytes exposed to cisplatin against control sample (cisplatin alone)
*Statistically significantly lower than control ($p < 0.05$)

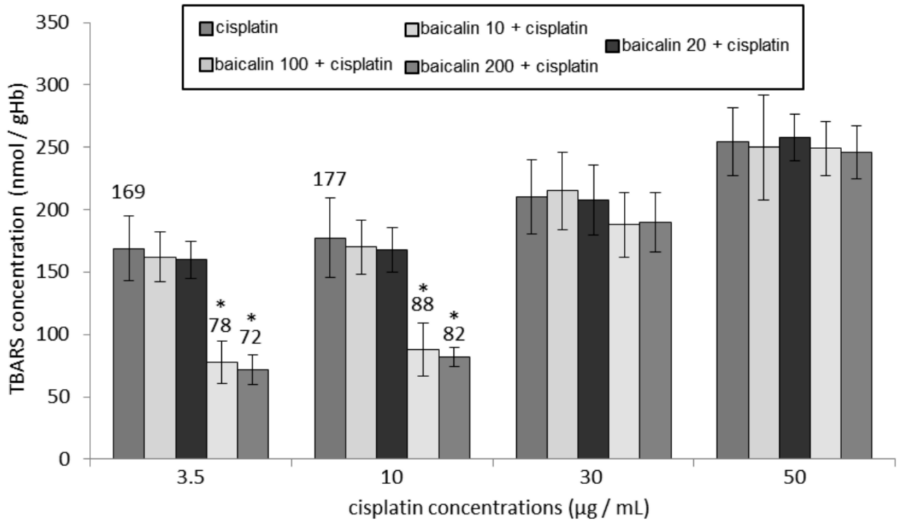


Figure 6. Effect of four concentrations of baicalin (10, 20, 100 and 200 µM) on TBARS level in erythrocytes exposed to cisplatin against control sample (cisplatin alone)
*Statistically significantly lower than control ($p < 0.05$)

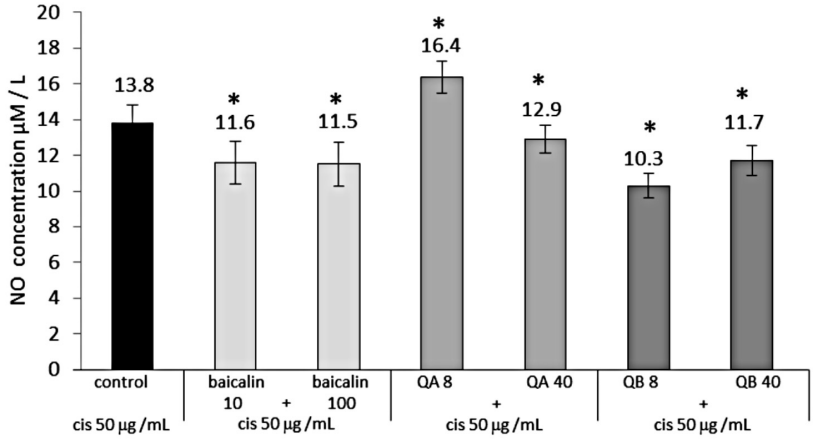


Figure 7. Effect of baicalin in concentrations: 10.0 and 100.0 µM; QA in concentrations: 8.0 and 40.0 µg/mL; QB in conc. 8.0 and 40.0 µg/mL on $NO^{\cdot}$ level in serum at combined exposure with cisplatin at dose 50.0 µg/mL in comparison to sample with cisplatin alone (control)
*Statistically significant against control for $NO^{\cdot}$ ($p < 0.05$)

ysis of $H_2O_2$. Baicalin has also the ability to chelate metals (Fe, Cu). The mentioned data indicate that flavonoid interacts positively with ROS.

Concerning nitrosative stress, our experiments showed varied influence of examined antioxidants on nitric oxide and nitrates in serum. The effect was not as obvious as in the study of TBARS. Baicalin and QA didn't act on these parameters, while QB raised the level of nitric compounds. Literature data reported that coenzyme Q10 can suppresses excess $NO^{\cdot}$ and prevents nitrosative stress. The reduced coenzye Q10 form, ubichinol, directly reacts with nitric oxide and peroxynitrite. The reaction is associated with the oxygen consumption (14). The participation of baicalin in nitrosative stress was also reported. In a study of murine macrophage cell line, baicalin significantly inhibited $NO^{\cdot}$ production, induced by lipopolysaccharide. The experiment also noted inhibitory effect of baicalin on iNOS gene expression *in vitro* (18).

The presented research demonstrated that both forms of coenzyme Q10 reduced oxidative and nitrosative stress caused by cisplatin in red blood cells or serum. Although coenzyme Q10 properties are well known, only one scientific report on the effect of coenzyme Q10 in cisplatin treatment was published. In that study, coenzyme Q10 significantly decreased lipid peroxidation, induced by cisplatin in mice, by preventing the deficit of reduced glutathione, superoxide dismutase and glutathione peroxidase activity. The authors argued that coenzyme Q10 has significant, protective effect in cisplatin-induced nephrotoxicity in mice (12). Similarly, in our *in vitro* study, coenzyme Q10 (both QA and QB) decreased lipid peroxidation induced by ciplatin. The antioxidative properties of coenzyme Q10 may appear from direct reaction with superoxide radicals, produced by cisplatin. Coenzyme Q10 acts as a free radical scavenger, suppress the generation of ROS by blunting the expression of NADPH oxidase and scavengers lipid peroxidation products (13, 35). Indirect effect of coenzyme Q10 is connected with the regeneration of another antioxidant, vitamin E (36). In the current study, baicalin showed weaker antioxidant properties than QA and QB in exposure to higher doses of cisplatin. Probably, in view of the strong prooxidative effect of cisplatin, baicalin was ineffective and not decreased TBARS level. Regarding the joint effect of antioxidants on $NO^{\cdot}$ and $NO_3$ in serum exposed to cisplatin, we observed that QB and baicalin acted contrary to QA, because decreased production of nitric oxide elevated by cisplatin. Nitric oxide could play a dual role, both as prooxidant and antioxidant. In contrast to the unquestionably toxic ROS, e.g., $^{\cdot}OH$, nitric oxide radical reacts slowly with the biological molecules. It is reported that nitric oxide has the astonishing ability to remove hydroxyl radical induced by cisplatin (26, 36). Nitric oxide also increases glutathione levels in cells. By blocking the iron ions, it prevents superoxide anion formation (26). Literature reports the participation of baicalin in nitrosative stress but there is no data about the protective effects of baicalin in exposure to cisplatin. Information obtained from our *in vitro* studies indicate that this flavonoid hadn't significant effect on $NO^{\cdot}$ and $NO_3$ level but in combined action with cisplatin inhibited generation of nitrogen products in serum.

The presented study evaluated the following antioxidants *in vitro*: two forms of coenzyme Q10 - lipophilic (QA) and soluble in water (QB), and baicalin with high inhibiting effect on lipid peroxidation induced by cisplatin. In assessing the utility of these antioxidants in nitrosative stress induced by cisplatin, efficiency showed QB and baicalin. However, the results were differentiated and not unequivocal. More than a few strategies and methods have been used to study the role of oxidative/nitrosative damage in humans treated with cisplatin and *in vitro* and *in vivo* experimental models of cisplatin toxicity. These strategies included administration of several antioxidants. Our experiments are a sort of complement research and attempt to explain the influence of the new product (QB), compared to known compounds such as QA and baicalin, moreover, confirm the lack of harmful interaction between cisplatin and studied antioxidants.

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