

DRUG BIOCHEMISTRY

THE RESEARCH ON ANTOXID ACTIVITY
IN FREE RADICALS PROCESSES

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Abstract: The joint effect of Antoxid (AX), extract from *Radix Scutellariae baicalensis*, with vitamin C on ferric reducing human plasma ability (FRAP) was examined. In order to explain the mechanism of Antoxid inhibiting effect on lipid peroxidation stimulated by xylene, its influence on hydroxyl radical generation was examined. The preventing and repairing effect of Antoxid was evaluated towards the lipid peroxidation stimulated with xylene by treatment before or after exposition. The results showed that the various combinations of Antoxid with vitamin C possessed antioxidative properties, but the significant synergistic effect was noted for weight ratio 1:1 of Antoxid and vitamin C in concentration 5 µg/mL. Antoxid didn't decrease the hydroxyl radicals level produced by xylene. Both preventing and repairing effect of Antoxid in oxidative stress caused by xylene is not connected with hydroxyl radical scavenging.

Keywords: Antoxid, *Scutellaria baicalensis*, vitamin C, FRAP, hydroxyl radical, xylene

Antoxid (AX) is the water-alcoholic extract obtained from *Radix Scutellariae baicalensis* Georgi according to special procedure. The final step in preparation was the crystallization (1). Antoxid is the main component of BAICADENT-GEL used in stomatology. The flavonoids present in extract from *Radix Scutellariae baicalensis* Georgi are very effective antioxidants (2, 3). It looks that the most important components which determine the FRAP activity of Antoxid are flavonoids baicalein and baicalin (Figure 1) (3, 4).

Our previous investigation showed the stronger effect of Antoxid than vitamin C on ferric reducing plasma ability (FRAP) (5). It was also observed that AX could successfully reduce the toxic effect of xylene on lipid peroxidation (6).

The aim of this study was to examine the joint effect of Antoxid with vitamin C. It was interesting to know whether the common use of those two agents gives harmful interaction or brings some advantages.

The next subject of our study was to explain whether antioxidative properties of AX are connected with its influence on hydroxyl radical generation and whether the mechanism connected with $\cdot\text{OH}$ generation is responsible for biological effect of AX. This radical is very aggressive and could initiate lipids peroxidation. It isn't deactivated with superoxide dismutase or scavenged by other active molecules. So it was interesting to look into mecha-

nism of AX inhibition of lipid peroxidation caused by xylene.

EXPERIMENTAL

The material to FRAP measurement was blood plasma from patients of Surgery Department in Medical University Clinic. The blood was taken on anticoagulant. The patients didn't receive any drugs.

General procedure of FRAP measurement

FRAP was evaluated by the measurements of Fe^{2+} /TPTZ-complex by colorimetric method with spectrophotometer (7).

Three combinations of vitamin C (AC) and Antoxid (AX) mixture were used for examination: M1 (5 µg/mL AX + 5 µg/mL AC); M2 (15 µg/mL AX + 5 µg/mL AC); M3 (5 µg/mL AX + 15 µg/mL AC).

To 450 µL of plasma, 50 µL mixture of methanol/water solution of Antoxid and vit. C. in various concentrations was added. The mixture was incubated for 30 min at 37°C. 20 µL of mixture was added to coupling agents (TPTZ/ FeCl_3) and incubated 4 min. at 37°C, then centrifuged ($4000 \times g$) for 10 min. The absorbance was measured at $\lambda = 593 \text{ nm}$ and compared with the control. Every experiment was repeated 10 times ($n = 10$). The results were evaluated with the Student t-test.

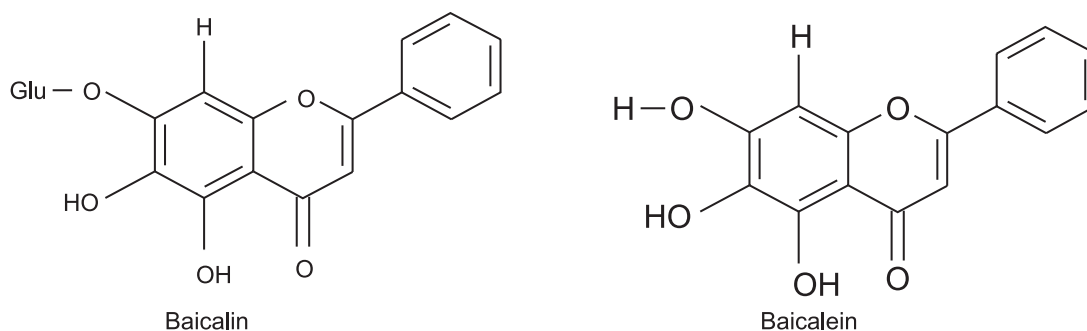


Figure 1. The chemical structure of baicalin and baicalein.

The study on hydroxyl radical generation was performed on *in vitro* model of human placental mitochondria. The mitochondria were isolated by Radi method (8) from normal mature placenta obtained after physiological delivery from Medical University Obstetric-Gynecological Clinic. The proteins in mitochondria were measured by Lowry method (9).

General principles of $\cdot\text{OH}$ measurement

Antoxid was dissolved in mitochondrial buffer (TRIS-HCl, pH 7.4) and used in the following concentrations: 1.5; 3.0; 6.0; 12.0 and 30 $\mu\text{g/mL}$. As stimulants of mitochondrial lipid peroxidation 1% *tert*-butyl hydroperoxide (*t*-BOOH) or xylene in concentration 17.64 $\mu\text{g/mL}$ of examined mixtures were used. The hydroxyl radical was measured by deoxyribose degradation (10).

The mitochondrial suspension (0.5 mL) was incubated at 37°C for 15 min with 15 μL of stimulant (*t*-BOOH or xylene in proper concentrations), 15 μL of the examined solution of Antoxid in concentrations as above and 0.5 mL of 20 mmol/L deoxyribose. After incubation, the samples were centrifuged. Then, to the 0.8 mL of supernatant, 0.5 mL of 2.8 % TCA and 0.5 mL of 0.67 % TBA in 0.1 mol/L NaOH were added and the samples were incubated at 100°C for 15 min. The hydroxyl radical level was measured spectrophotometrically at $\lambda = 532$ nm. The results were compared with the controls prepared in the same way without Antoxid (K1 – control with *t*-BOOH, K2 – control with xylene).

RESULTS AND DISCUSSION

Every mixture (M1, M2 and M3) statistically significantly increased the value of FRAP ($p = 0.02 - 0.002$) (Figure 2) what means that the mixtures of Antoxid and vitamin C in various concentrations possess antioxidative properties.

There is no doubt that the most advantageous is the combination of low doses of agents in the weight ratio of 1:1 (equal concentrations). The Antoxid in a dose of 5 $\mu\text{g/mL}$ didn't show the influence on FRAP. The influence of 5 $\mu\text{g/mL}$ of vitamin C on FRAP was also very weak. The mixture of Antoxid in the concentration of 5 $\mu\text{g/mL}$ with 5 $\mu\text{g/mL}$ of vitamin C showed strong synergistic effect on FRAP increasing its value significantly ($p = 0.00002$) (Table 1). There are some observations that the presence of flavonoids and vitamin C in diet is very useful especially in the prophylaxis of cardiac diseases (11-13). The obtained results points on advantageous action of Antoxid mixture and vitamin C on FRAP.

Recently, several methods have been developed to measure antioxidant activity, among them ferric reducing/antioxidant power (FRAP). It is especially important for flavonoids, because they are active as chelators of transition metal ions. In

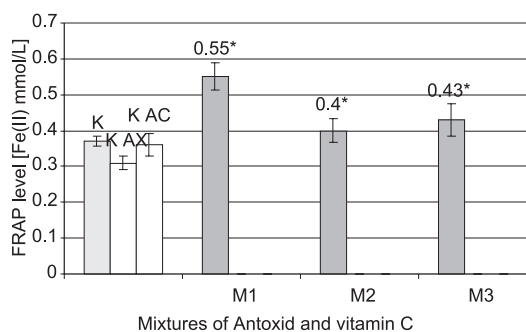


Figure 2. The influence of mixtures M1, M2 and M3 on FRAP level in blood plasma.

M1 – the mixture of 5 $\mu\text{g/mL}$ AX with 5 $\mu\text{g/mL}$ AC; M2 – the mixture of 15 $\mu\text{g/mL}$ AX with 5 $\mu\text{g/mL}$ AC; M3 – the mixture of 5 $\mu\text{g/mL}$ AX with 15 $\mu\text{g/mL}$ AC; K – control with 0.9% NaCl; K AX – control with Antoxid; K AC – control with vitamin C; Data are expressed as the mean \pm SD ($n = 10$)

* significantly different from control value (K); $p < 0,05$

Table 1. The influence of mixtures of Antoxid (AX) and vitamin C on the plasma FRAP level.

Samples (a)	Concentration [$\mu\text{g/mL}$]		FRAP values [mmol/L] (b)
	Antoxid	Vitamin C	
M1	5.0	5.0	$0.5513 \pm 0.03858^{***}$
M2	15.0	5.0	$0.3987 \pm 0.03414^*$
M3	5.0	15.0	$0.4263 \pm 0.04435^{**}$
K	0	0	0.3683 ± 0.01426
K AX	5.0	0	0.3144 ± 0.01973
K AC	0	5.0	0.3672 ± 0.03131

a – M1 – M3 – mixtures of Antoxid and vitamin C; K – control with 0.9% NaCl; K AX – control with Antoxid; K AC – control with vitamin C. b – Data are expressed as the mean \pm standard deviation (\pm SD); number of samples = 10. Significance (in comparison to control K): * $p < 0.03$; ** $p < 0.005$; *** $p < 0.0001$

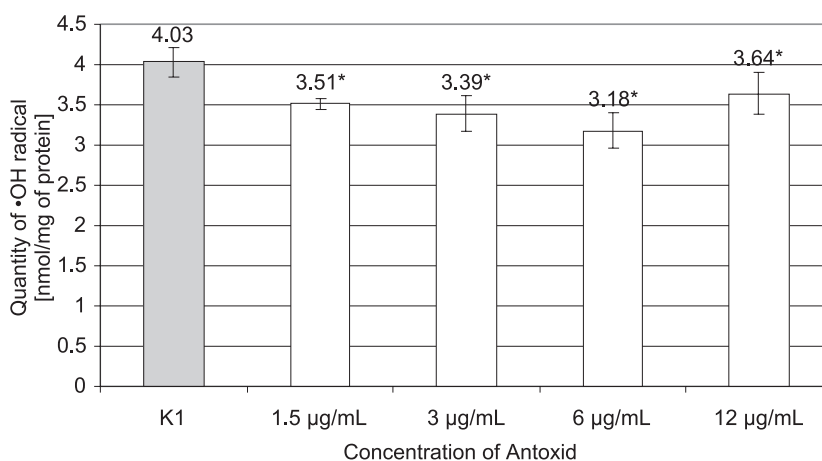


Figure 3. Antoxid influence on $\cdot\text{OH}$ radical generation in mitochondrial suspension stimulated with 1% *t*-BOOH (K1). Data are expressed as the mean \pm SD (n = 10); K1 – control with *t*-BOOH. * significantly different from control value (K1); $p < 0,05$

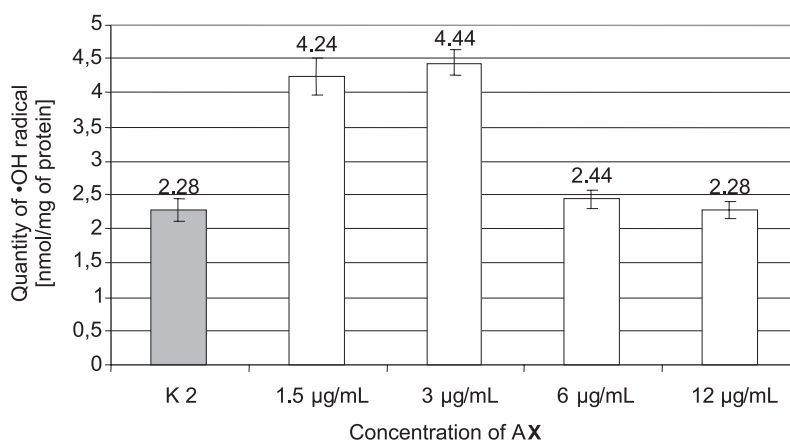


Figure 4. Antoxid influence on $\cdot\text{OH}$ radical level in mitochondrial suspension exposed to xylene in concentration of 17.64 $\mu\text{g/mL}$. Data are expressed as the mean \pm SD (n = 9-10); K2- control with xylene.

general, the antioxidants can be classified into two categories dependent on the mechanism of their action: preventive antioxidants or chain-breaking antioxidants (14). Preventive antioxidants, such as superoxide dismutase or catalase, inhibit formation of reactive oxygen species (ROS). Vitamin C belongs to the second class, which scavenges oxygen radical and thereby breaks oxidative stress.

When the effect of AX on $\cdot\text{OH}$ generation in mitochondria by *t*-butyl hydroperoxide was measured, it was noted that a statistically significant ($p < 0.001$) decrease in $\cdot\text{OH}$ level after AX treatment in doses 1.5–12.0 $\mu\text{g/mL}$ (Figure 3).

It looks that the inhibiting effect of AX on lipid peroxidation provoked by *t*-BOOH, expressed as MDA level, could be caused by inhibition of hydroxyl radical generation by AX.

Quite different seems the mechanism of AX effect towards lipid peroxidation provoked by xylene. It looks that the pathway connected with $\cdot\text{OH}$ generation is not responsible for MDA decrease resulted after AX treatment. When mitochondria exposed to xylene in dose 17.64 $\mu\text{g/mL}$ were treated with AX in doses 1.5, 3.0, 6.0 or 12.0 $\mu\text{g/mL}$ any decrease in $\cdot\text{OH}$ generation was observed (Figure 4).

Contrary, the low doses (1.5 and 3.0 $\mu\text{g/mL}$) even stimulated the $\cdot\text{OH}$ formation, when the higher doses (6.0 or 12.0 $\mu\text{g/mL}$) did not show any effect.

The mechanism of AX preventing action was also examined by treatment of mitochondria with AX, 30 min before exposition to xylene (Figure 5). The results point that also the preventing activity towards lipids peroxidation caused by xylene is not connected with inhibition of $\cdot\text{OH}$ generation by AX. The results point that $\cdot\text{OH}$ generation was even increased by AX, but it didn't correlate with MDA increase.

Maybe the mechanism concerned with other reactive oxygen species e.g. hydrogen peroxide production is involved in the activity of AX. It was shown that baicalein protects rat cardiomyocytes from hypoxia/reoxygenation damage via the prooxidant mechanism (15). Neonatal rat cardiomyocytes pretreatment with baicalein reduced lactate dehydrogenase release, while pretreatment with baicaline was ineffective. The results show the important role of hydrogen peroxide produced during the autooxidation of baicalein in its cardioprotective effect. The study demonstrates H_2O_2 -dependent mechanism of baicalein. Probably, the same mechanism is involved in xylene-stimulated lipid peroxidation in mitochondria.

Baicalin and baicalein were found to prevent human cell damage, especially liver cells, induced

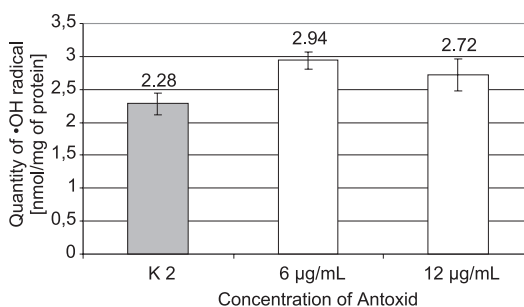


Figure 5. Protective effect of Antoxid on $\cdot\text{OH}$ radical generation in mitochondrial suspension exposed to xylene in concentration of 17.64 $\mu\text{g/mL}$. Data are expressed as the mean \pm SD ($n = 8-9$) K2 – control with xylene.

by ROS, which makes them hepatoprotective. It was suggested that the mechanism of action is connected with radicals scavenging ability of main flavonoids from *Scutellaria baicalensis* and their interaction with iron ions. The phenolic groups of flavonoids are good hydrogen atom donors and peroxy radicals (ROO \cdot) or peroxy nitrite scavengers. Antioxidative activity of baicalin is based on hydroxyl radical and superoxide anion radical scavenging and inhibition of xanthine oxidase, 12-lipoxygenase and nitric oxide synthase. It was observed that three hydroxyl groups present in A ring of baicalein have great influence on antioxidant capacity (3, 16).

There are some reports that total phenolic and vitamin C contents in fruits and vegetables correlates with antioxidant activity (14). It can be connected with regeneration mechanism based on interaction of various antioxidants. Both baicalein and baicalin can form semiquinone radicals which can be autooxidized producing superoxide anion radical. A recent study has reported the H_2O_2 -generating ability of some flavonoids (15). Vitamin C probably inhibits the auto-oxidation of flavonoids.

CONCLUSIONS

1. The mixture of Antoxid and vitamin C showed antioxidative properties measured by an increase of human plasma FRAP value in various concentrations.

2. The synergistic action of components was observed for the weight ratio of 1:1 of Antoxid with vitamin C in concentration 5 $\mu\text{g/mL}$.

3. Antoxid decreased hydroxyl radical generation in exposure to *t*-BOOH but not to xylene.

4. The preventing and repairing effect of Antoxid in oxidative stress caused by xylene is not connected with hydroxyl radical scavenging.

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