

**ANTIOXIDANTS MODIFY DIAZEPAM TOXICITY IN MICE**

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**Summary**

Our preliminary studies showed that the chronic application of anthocyanins modified diazepam dependence and inhibited withdrawal signs in rats.

The aim of the present study was to register the effect of anthocyanins and Vit.C on acute intoxication with different toxic doses of diazepam in mice. After application of the substances we determined the 24th hour survival rate in each experimental group and registered mice behavior.

Our results showed that in a low dose anthocyanins potentiated diazepam toxicity, whereas in high doses they displayed antagonistic effect. The combined application of anthocyanins and Vit. C completely prevented the lethal effect of LD<sub>100</sub> diazepam.

**Key words:** anthocyanins, antioxidants, flavonoids, Vit. C, diazepam, drug toxicity

**Introduction**

Drug intoxication is always related to a generation of free radicals that cause membrane damages and alteration of the calcium homeostasis. This seems to be a common unspecific mechanism in toxic responses which may cause cell death [1]. CNS cells are extremely sensitive and vulnerable to the action of free radicals. The impairment of neuronal function may be irreversible.

Benzodiazepines (BDZs) are currently the most commonly prescribed anxiolytics. They are harmless in a wide range of doses but the incidents of intoxication with them (including abuse and attempts to suicide) are not rare. The life-saving medicine against BDZ intoxication is flumazenil which is a concurrent antagonist of BDZ receptors. However, its routine use is not recommended because of a serious side effect - seizures.

Diazepam overdose causes CNS and respiratory depression which may lead to coma and death. Experimental data show that acute administration of high doses of diazepam results in a cascade of oxidative changes and significantly diminishes cell antioxidant defense, especially the intracellular levels of reduced glutathione [2]. In these conditions the role of antioxidants seems predictable.

Both Vit. C and anthocyanins are powerful antioxidants. Anthocyanins are widely spread bioflavonoids known as essential food antioxidants and their therapeutic potential is well documented [3]. They display a variety of pharmacological activities and have neuro- and organoprotective effects [4, 5, 6, 7].

Anthocyanins are also able to enhance the cell antioxidant defense [6], to inhibit apoptosis and to affect transcription factors [5, 8]. Moreover, evidence suggests that anthocyanins have much higher antioxidant activity than common antioxidants such as Vit. C, Vit. E and  $\beta$ -carotene [3, 9].

Our previous experimental data also confirmed the antioxidant and pleiotropic biological effects of anthocyanins [10, 11].

Literature data for the role of anthocyanins alone and in combination with other antioxidants in diazepam acute toxicity are insufficient.

The present study investigated the effects of the antioxidants anthocyanins and vit. C on the 24<sup>th</sup> hour survival of mice treated with different toxic doses of diazepam.

## **Materials and Methods**

### **Animals**

Subjects consisted of white male mice, line H with a body weight 22 - 25 g. Animals were housed in groups of six with free access to food and water and maintained in a temperature and humidity controlled room on a 12-hour light/dark cycle. The experimental procedures were performed during the light phase of the light-dark cycle (between 9:00 and 13:00h).

The experiments were carried out in accordance with the Bulgarian regulations on animal welfare and in conformance to EEC Directive of 1986 (86/609/EEC).

### **Substances**

Anthocyanins – water-soluble powder concentrate, derived from Bulgarian red wine Cabernet Sauvignon and standardized to contain 50 g/l total anthocyanins (70% monomer and 30% phenol polymers). Anthocyanins were given per os in a water solution 0,1 ml/10 g body weight.

Vitamin C – synthetic substance (Sopharma); administered per os in a water solution 0,1 ml/10 g body weight.

Diazepam - synthetic substance (Sopharma), dissolved in Tween 80 and physiological solution; administered as a suspension per os 0,1ml/10 g body weight.

The substances were applied in a 10-min interval.

### **Experimental design**

All animals were handled in the laboratory room for 5 minutes once a day 2-3 days prior to procedures. The experimental mice were separated in 9 groups (n = 6) and treated as follows:

**I gr.** – diazepam in a dose of 750 mg/kg

**II gr.** – diazepam in a dose of 750 mg/kg + anthocyanins 100 mg/kg

**III gr.** – diazepam in a dose of 850 mg/kg

**IV gr.** - diazepam in a dose of 850 mg/kg + anthocyanins 200 mg/kg

**V gr.** – diazepam in a dose of 1000 mg/kg (LD<sub>100</sub>)

**VI gr.** – diazepam in a dose of 1000 mg/kg + anthocyanins 200 mg/kg

**VII gr.** – diazepam in a dose of 1000 mg/kg + anthocyanins 400 mg/kg

**VIII gr.** – diazepam in a dose of 1000 mg/kg + Vit. C 150 mg/kg

**IX gr.** – diazepam in a dose of 1000 mg/kg + Vit. C 150 mg/kg + anthocyanins 200 mg/kg

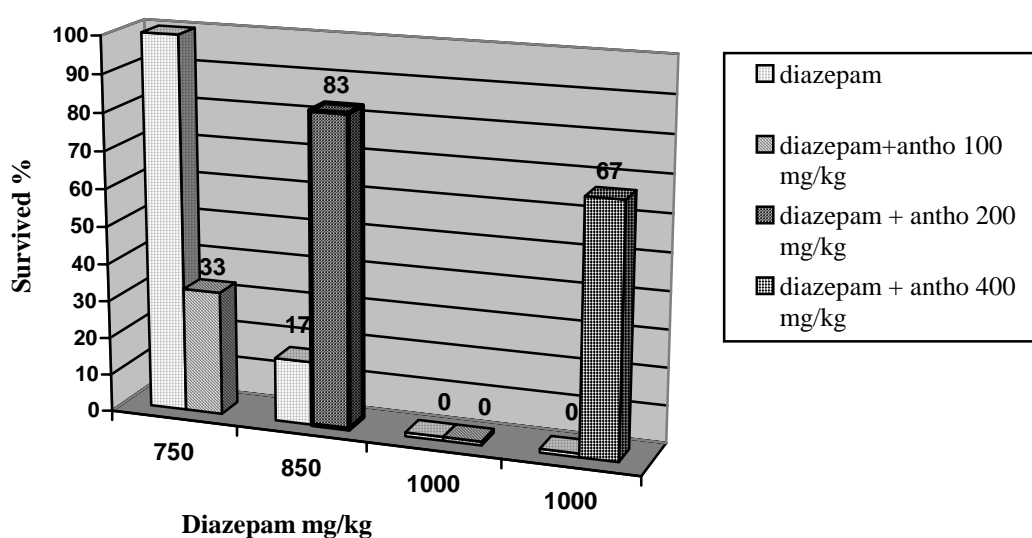
24<sup>th</sup> hours after drug administration we registered the number of survived mice in each group. During the experiment the awakening, behavior, muscle tone and locomotor activity of the survived mice were also evaluated.

The survival rate of mice is expressed in percentage (number of survived mice divided by the total number of mice in the group x 100).

### Results

The experimental results are shown on Fig. 1 and Fig. 2.

Fig. 1 presents the effects of different doses of anthocyanins on the 24<sup>th</sup> hour survival rate of mice, treated with different high doses of diazepam.



**Figure 1.** Effects of anthocyanins on the 24 hour-survival of mice, treated with different doses of diazepam

Our results demonstrated that 750 mg/kg diazepam had no lethal effect on the experimental animals but caused expressed sedation and strongly decreased spontaneous locomotor activity.

Additionally, after administration of the same dose, all mice exhibited decreased spontaneous locomotor activity and expressed sedation but were alive and awake.

When anthocyanins were added in a low dose (100 mg/kg) to the non-lethal dose of diazepam (750 mg/kg) they significantly increased the CNS depressive effect of diazepam. The combined application of diazepam 750 mg/kg + anthocyanins 100 mg/kg caused quick fall into a heavy sleep and in the first 3 hours after drugs administration 33 % death rate was registered; at the 24<sup>th</sup> hour the death rate reached 67 %.

Diazepam in a dose of 850 mg/kg caused depression and decreased activity in the first 10-15 minutes; mice fell into a heavy sleep within the hour.

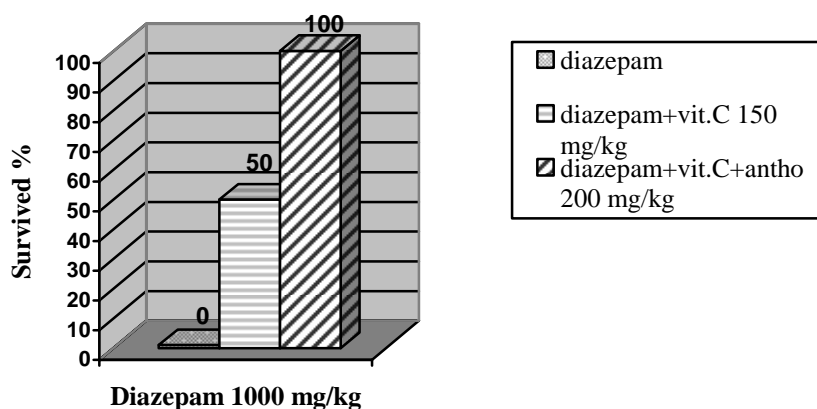
When treated with a dose of 850 mg/kg diazepam mice showed signs of depression and decreased activity in the first 10-15 minutes and fell into a heavy sleep within the hour.

At the 24<sup>th</sup> hour the mortality rate reached 83 % vs 17% survived mice. On the contrary, the combined application of diazepam 850 mg/kg + anthocyanins 200 mg/kg inverted the general diazepam toxicity and led to 17 % lethality vs 83% survived mice.

The administration of 1000 mg/kg diazepam caused a 100 % death rate (LD<sub>100</sub>). Mice receiving the combination of diazepam 1000 mg/kg and anthocyanins (200 mg/400 mg/kg) fell asleep considerably later compared to the group receiving diazepam only. Anthocyanins 200 mg/kg combined with a lethal dose of diazepam had no effect on the survival rate.

24 hours after receiving a combination of diazepam 1000 mg/kg + anthocyanins 400 mg/kg the survived mice (67%) were awake and active, although with ataxia. At the 29<sup>th</sup> hour their spontaneous movements were restored. At the 48<sup>th</sup> hour we registered normal muscle tone of the mice using the “net/rod hanging” tests. Furthermore, at the 48<sup>th</sup> hour after the treatment all survived mice showed signs of a withdrawal syndrome: wet dog shakes, teeth chattering, grooming, burrowing, tremor, piloerection, but no seizures. These signs, although with diminishing intensity, were observed up to the 5<sup>th</sup> day after the treatment.

Fig. 2 presents the effects of Vit. C 150 mg/kg administered separately and in a combination with anthocyanins 200 mg/kg on the 24<sup>th</sup> hour survival rate of the mice after treatment with diazepam 1000 mg/kg (LD<sub>100</sub>).



**Figure 2.** Effects of vit.C alone and in combination with anthocyanins on the 24 hour-survival of mice, treated with lethal doses of diazepam

Mice treated with the combination of diazepam 1000 mg/kg + Vit. C 150 mg/kg fell into a heavy sleep in one hour. At the 24<sup>th</sup> hour after drug administration we recorded 50% survival rate in mice. At the 34<sup>th</sup> hour the mice woke up with an extremely limited locomotor activity.

The combined application of Vit. C 150 mg/kg + anthocyanins 200 mg/kg led to enhanced independent effect of each of the antioxidants and to complete antagonization of diazepam lethality. At the 24<sup>th</sup> hour after the combined administration of Vit. C 150 mg/kg + anthocyanins 200 mg/kg and LD<sub>100</sub> diazepam, all mice were alive and awake; at the 30<sup>th</sup> hour they began moving. However, their activity restoration was incomplete, with apparent ataxia.

The revival of the survived mice was much slower compared to that of the mice treated with diazepam 1000 mg/kg + anthocyanins 400 mg/kg.

### **Discussion**

Diazepam is a widely used in the clinical practice benzodiazepine. As the other BDZs it has low toxicity. In our experiment a dose of 750 mg/kg diazepam caused no death in the treated mice. Additionally, the combined application of this dose diazepam and anthocyanins 100 mg/kg enhanced diazepam toxicity and at the 24<sup>th</sup> hour 67% mortality was registered.

This effect of anthocyanins could be explained with some studies reporting a synergetic interaction between flavonoids and diazepam and determining flavonoids as ligands of benzodiazepine receptors [12, 13, 14]. Thus, in a dose of 100 mg/kg anthocyanins potentiated diazepam inhibitory effects on CNS and led to high mortality rate in experimental groups.

However, our data showed that high doses of anthocyanins protect brain functions and have significant positive effects on the survival rate of mice, treated with lethal doses of diazepam.

Flavonoids can protect the brain by some distinctive mechanisms. These include increasing intracellular levels of reduced glutathione, directly lowering levels of free radicals, preventing the Ca<sup>2+</sup> influx despite high levels of free radicals [15] and modulating different transduction mechanisms [4, 5, 7].

Anthocyanins are known to have strong antioxidant potential. On the other hand, it is suggested that their organoprotective and antitoxic effects are due not only to their antioxidant properties. In a case of intoxication the CNS excitatory mediator glutamate activity is very high and leads to excessive generation of free radicals that enhance excitability. Several investigators report that some flavonoids inhibit glutamate excitotoxicity by acting on the NMDA receptors [16, 17, 18, 19, 20]. We hypothesize that except for their antioxidant activities anthocyanins might have displayed antagonizing effect on diazepam toxicity by suppressing the action of glutamate.

In addition, evidence suggests that anthocyanins prevent catecholamine oxidation [21, 22]. Dopamine controls the motor activity and noradrenaline is known as the "mediator of alertness". Thus, the protective effect of anthocyanins on catecholamines maybe accelerated the signs of general condition improvement of the mice - waking up, muscle tone, locomotor activity, etc.

Vit. C is a well known antioxidant, essential for the human health. A few studies report that Vit. C cumulates in neurons and its presynaptic release is exchanged for the neuronal reuptake of glutamate [23, 24]. Taken together these data may explain the effect of Vit. C on diazepam lethality.

The result of the combined application of Vit.C + anthocyanins 200 mg/kg on the survival of mice, treated with diazepam LD<sub>100</sub>, is very intriguing. In this dose anthocyanins had no effect

on the survival rate but their combination with Vit.C resulted in a 100% mice survival. This result illustrates the synergetic action of the two antioxidants and suggests the possibility for their combined usage in drug intoxication.

Our results demonstrated that both anthocyanins and Vit. C antagonized diazepam acute toxicity without causing serious side effects.

We suggest that the combined application of Vit. C and anthocyanins in appropriate doses may be used without any risk in the treatment of benzodiazepine intoxication.

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