THE APPLICATION OF ADSORBENT BENTONITE IN OXIDATIVE STRESS INDUCED BY PARAQUAT

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The protective effect of bentonite (natural and synthetic) in oxidative stress induced by paraquat was studied on 32 adult male Wistar rats. The animals were divided into four groups (n=8). The first group received only paraquat p.o. (via a gastric tube). An hour after administration of paraquat, the second and the third group were treated with natural and synthetic bentonite, respectively, while the fourth group was untreated (control). All three experimental groups were treated once a day, throughout 8 consecutive days. Blood samples were taken 0, 1, 4, 6 and 8 days after the beginning of the treatment.

Oxidative stress induced by paraquat was estimated by means of catalase activity (CAT - mmol/H₂O₂/min/g Hb) and malondialdehyde quantity (MDA - nmol/g Hb).

Oxidative stress proved to be significant an hour and 192 hours after the beginning of chronic intoxication by paraquat.

Both natural and synthetic bentonite expressed a protective action from oxidative stress in the period from 24 h to 144 h post application, acting through the external capacity of cationic exchange, which is known to be approximately 10%. The absorption of paraquat depends on the size of the molecule and its polarisation and is performed by the mechanisms of ion exchange (ionic and electrostatic interaction). Nevertheless, both absorbents developed a significant protective effect (approximately 50 %) 1 h and 192 h after the first application of paraquat, indicating that these protectors act as molecular sieves and thus suppress the process of lipid peroxidation and the development of even more intensive oxidative stress.

Key words: bentonite, oxidative stress, paraquat

INTRODUCTION

Paraquat is a non-selective herbicide highly toxic for both animals and humans. Thousands of deaths occurred after ingestion (in humans often suicidal) or dermal exposure to paraquat (Pesticide Action Network – PAN, 2003). Both in acute and chronic intoxication primary injuries to mammals occur in the lungs,
which selectively accumulate paraquat. Since there are no known pharmacological antagonists of paraquat and no chelating agents capable of binding the poison in the blood or other tissues, the management of paraquat poisoning has been directed towards the modification of the toxicokinetics of the poison by decreasing the absorption or decreasing the oxidant-induced cellular damage (Suntres, 2002).

The aim of the current investigation was to determine the influence of natural and synthetic bentonite as adsorbents in rats poisoned with paraquat. The influence of paraquat on red blood cells of rats in vivo was examined, i.e. the production of certain reactive oxygen species and their influence on lipid peroxidation in the membranes of red blood cells.

The high toxicity of paraquat is achieved by damaging the cell integrity due to highly reactive free radicals which emerge from redox cycling in the presence of molecular oxygen. Superoxide anionic radical (O$_2^-$) produced in the process of redox cycling of this herbicide transforms into hydrogen peroxide (H$_2$O$_2$). The synthesized O$_2^-$ and H$_2$O$_2$ use NADPH, and in reaction with lipids produce lipid hydroperoxides which contribute to further lipid peroxidation. Lipid peroxidation decreases the fluidity of biological membranes, which results in higher permeability for bivalent ions and dramatical changes in ion-dependent processes.

The aim of the study was to investigate the mechanism of toxicity of paraquat after p.o. chronic intoxication in the dose of 25mg/kg. This was achieved by estimation of parameters of oxidative stress, which are the activity of the antioxidative enzyme CAT, and the level of lipid peroxidation via MDA measurements. In addition to this, the research on the protective effect of natural and synthetic bentonite in oxidative stress provoked by paraquat was carried out.

MATERIALS AND METHODS

The investigation was performed on 32 adult male Wistar rats (220-230 g bw). The animals were divided into 3 experimental and a control group (n=8). Animals in experimental groups (I to III) were treated p.o., by a gastric tube, with paraquat ("Gramoxone", Syngenta Crop Protection, Basel, Switzerland, CAS number: 2074-50-2), in a dose of 25 mg/kg b.w. The first experimental group received only paraquat. The second experimental group was treated with 40 % water suspension of natural bentonite in a volume of 10 ml/kg b.w. in the same manner (by gastric intubation), an hour after the administration of paraquat, while the third experimental group was treated with the same volume of synthetic bentonite. The fourth group was the untreated.

All three experimental groups were treated once a day, throughout 8 consecutive days. Blood was collected 1, 24, 96, 144 and 192 hours after the beginning of the treatment and treated with anticoagulant, sodium citrate (3.8 % w/v). Erythrocytes were separated by centrifugation (3000 rpm) and washed in saline three times, immediately followed by the estimation of the enzyme activities.
The CAT activity was estimated by the method of Beutler. CAT degrades hydrogen peroxide, whose concentration can be measured directly by the decrease in the absorbance at 240 nm (Beutler, 1982).

The level of lipid peroxidation (LP) was assayed through the concentration of thiobarbituric acid as the reactive substance (TBARS) in the red blood cells according to Uchiyama and Mihara (Uchiyama and Mihara, 1978). Haemoglobin concentration was determined by the cyanmethemoglobin method (Drabkin and Austin, 1935).

RESULTS

In the current investigations on Wistar rats, after the application of paraquat (p.o., 25 mg/kg), a positive correlation between the changes due to oxidative stress provoked by paraquat (i.e. between the activity of CAT, and the intensity of lipid peroxidation estimated through MDA concentration) was observed 1 h and 192 h after the beginning of the application of paraquat, in all treated groups.

The activity of CAT following intoxication with paraquat was increased, as well as the concentrations of H$_2$O$_2$ (Figures 1, 3 and 5).

A statistically significant increase in CAT activity was observed 1 h, 96 h and 192 h (Figures 1, 3 and 5, respectively) after paraquat application, compared with the activity of the enzyme 24 h (30.88 %) and 144 h (36.27 %) after the beginning of intoxication (Figures 2 and 4, respectively).

Natural and synthetic bentonite administered p.o. a dose of 4 g/kg b.w. significantly decreased the activity of CAT 1 and 192 h after application. Synthetic bentonite was more efficacious in decreasing the activity of the enzyme in comparison with natural bentonite.

An hour after administration of paraquat synthetic bentonite reduced CAT activity by 70.08 % in comparison to animals treated only with paraquat, whilst the efficacy of natural bentonite was approximately half as high as that in the synthetic, decreasing the activity of CAT by 36% (Figure 1).
Figure 2. CAT activity 24 hours after the beginning of treatment

Figure 3. CAT activity 96 hours after the beginning of treatment

Figure 4. CAT activity 144 hours after the beginning of treatment
Figure 5. CAT activity 192 hours after the beginning of treatment

Figure 6. MDA 1 hour after beginning of treatment

Figure 7. MDA 24 hours after beginning of treatment
Both tested adsorbents decreased CAT activity with similar efficacy 192 h after application. Natural bentonite caused a decrease of 51.74 % and the synthetic 46.04 %, compared with the activity of the enzyme in rats treated only with paraquat (Figure 5).

Neither natural nor synthetic bentonite influenced CAT activity significantly 24, 96 and 144 h after the beginning of paraquat application, in comparison to Group 1 (Figures 2, 3 and 4).

Paraquat induces the process of lipid peroxidation in erythrocytes producing various concentrations of MDA over time. In the initial phase of paraquat intoxication (1 h and 24 h after application) the concentration of MDA rose (Figures 6 and 7), which was followed by its decrease (Figures 8 and 9) and another increase in the terminal phase of intoxication (Figure 10).

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**Figure 8.** MDA 96 hours after beginning of treatment

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**Figure 9.** MDA 144 hours after beginning of treatment
DISCUSSION

Being a herbicide, paraquat binds irreversibly and for a long time to the treated soil. Having this in mind, it is logical to predict that certain minerals originating from the soil are capable of antagonizing, at least to some extent, the toxic effects of paraquat in poisoned animals and humans (Cope et al., 2004; Walcarius et al., 2004).

The adsorptive capacity of bentonite after poisoning with paraquat is proved and is approximately 5% (Okonek et al., 1982). However, the bentonite adsorptive capacity cited is not sufficiently protective in paraquat poisoning, thus additional therapy including active coal perfusion is inevitable (Lopez et al., 2002; Idid et al., 1996).

Besides paraquat, bentonites are capable of adsorbing aflatoxin B1 and unorganic anions which contaminate waste waters (Tomašević-Čanović Magdalena et al., 2001; Banat et al., 2000; Vujaković et al., 2003).

Bentonite is an aluminosilicate mineral rich in canals and holes that can adsorb molecules and ions which measure less than the diameters of the holes. The adsorptive selectivity depends mainly on the polarity of the molecules of toxic materials. Bentonites efficiently adsorb H₂O, CO₂, SO₂, NO₂ and H₂S. Besides the internal capacity of cationic exchange, bentonites are characterised by an external capacity of cationic exchange, representing the sum of exchangeable cations on the external surface of bentonite and is approximately 10% of the total capacity for cationic exchange.

The aim of the current investigation was to test the adsorbent-protective effect of bentonite in oxidative stress due to paraquat. Paraquat induces the production of free radicals and thus contributes to cell death. However, the precise mechanism of free radical production induced by this herbicide is unknown. This is the reason for the fact that investigations on metabolism and mechanisms of paraquat toxicity and the possibilities for protection against poisoning with this herbicide are still current.
According to ample research and data it is supposed that the toxic effect of paraquat happens on the molecular level and is a result of free radical production in redox reactions (Bus and Gibson, 1984; Mollace et al., 2003).

In the presence of an electron donor, NADPH, and the enzyme cytochrome P-450 NADPH reductase, the PQ$^{2+}$ ion being highly electrophilic, is metabolised by monoelectron reduction in the cell into stable monoionic cation radical PQ$^{+*}$.

\[
PQ^{2+} + \text{NADPH} \rightarrow PQ^{+*} + \text{NADP}^+\]

In aerobic conditions, PQ$^{+*}$ reacts with oxygen and in the reaction of monoelectronic reduction it delivers its odd electron, thus leading to superoxide anionic radical (O$_2$$^{\cdot-}$) formation and PQ$^{+*}$ oxidation into PQ$^{2+}$.

\[
PQ^{+*} + O_2 \rightarrow PQ^{2+} + O_2^{\cdot-}\]

In the presence of reduction equivalents, the regenerated PQ$^{2+}$ ions are again reduced to monocation PQ$^{+*}$ radicals that repeatedly react with oxygen producing O$_2^{\cdot-}$.

The metabolic path of O$_2^{\cdot-}$, highly depends on pH and can go towards reduction or oxidation expressing either its oxidative or antioxidative properties.

\[
O_2 \rightleftharpoons O_2^{\cdot-} \rightarrow H_2O_2\]

In protonic conditions (high concentrations of H$^+$ ions) in the presence of Fe$^{3+}$, Cu$^{2+}$, the superoxide anionic radical O$_2^{\cdot-}$ is oxygenized into molecular oxygen (O$_2$) (Liochev and Fridovich, 1994).

In aprotonic conditions (low concentration of H$^+$ ions), when oxidative stress is developed and the reserve of reduction equivalents of NAD(P)H is spent, i.e. when there is too much NAD(P)$^{+*}$, the reduction of O$_2^{\cdot-}$ into H$_2$O$_2$ is favoured.

The synthesized peroxide is degraded by CAT. CAT (EC 1.11.1.6) is located in peroxisomes, quickly transforming high concentrations of H$_2$O$_2$ into molecular oxygen and water.

\[
\text{CAT} \quad 2H_2O_2 \rightarrow O_2 + 2H_2O\]

This is a two-stage reaction and involves the binding of two molecules of hydrogen peroxide to CAT, thus there is a slight probability for the reaction to occur when the concentration of peroxide is low. CAT is a tetramer protein, with the active centre bound to four subunits comprising the haem group (Fe$^{3+}$protoporfin). Besides the haem, each subunit includes NADPH that stabilizes the enzyme (Mates et al., 1999).

The activity of CAT in paraquat intoxication accompanies the changes of hydrogen peroxide concentration. Statistically significant decrease in CAT activity was detected 24h (30.88 %) and 144 h (36.27 %) after the beginning of paraquat administration, in comparison with the enzyme activity 1 h from the beginning of poisoning. The enzyme was possibly in some way inhibited or its capacity may...
have been significantly exhausted due to intensive elimination of H$_2$O$_2$ synthesized as a result of conjoined activity of PQ$^{2+}$ and O$_2$. In order to maintain the homeostatic mechanisms in cells after intoxication with PQ. The exhaustion of the enzymes involved in antioxidative protection (SOD, CAT, GR, GPx, etc.) that sequester the reactive products, as well as the decrease in NADPH and glutathione (electron donor), disturb the redox state in cells and promotes oxidative stress (Kono and Fridovich, 1982; Halliwell and Gutterdge, 1999).

Oxidative stress due to continuous O$_2$ production in redox reactions developed 192 h after the beginning of intoxication, consequently increasing the activity of CAT to 68.25±5.16 mmol/min/g Hb (Figure 5). The processes happening during paraquat redox cycling are irreversible and lead to cell death (Bus and Gibson, 1984).

In the presence of transition metals (Fe or Cu), superoxide anion radical O$_2$ transforms into hydrogen peroxide (H$_2$O$_2$), a molecule with pronounced oxidative properties. The product of homolytic break of unstable connection in H$_2$O$_2$ (HO- OH) is the hydroxil radical (HO·), the most potent reactive radical, that instantly reacts with numerous cell macromolecules, including lipids, nucleic acids and proteins (Valko et al., 2006a; Valko et al., 2006b).

Hydrogen peroxide (H$_2$O$_2$) via HO· leads to peroxidation of polyunsaturated fatty acids (PUFAs), the constituents of cell membranes and membranes of cell organeles. In the terminal phase of lipid peroxidation malondialdehyde (MDA) is produced as one of the products of degradation of PUFAs. In the reaction of TBA with MDA a pink coloured complex is produced, whose absorbance is measured at 535 nm. The concentration of MDA indicates the level of lipid peroxidation (Uchida, 2003; Traverso et al., 2004).

In the present investigations, 1h after the beginning of intoxication with paraquat, the process of lipid peroxidation was noticeable and statistically significant (p<0.001). The increase in lipid peroxidation immediately after the beginning of intoxication was an expected indicator of activated aggressive, oxidative mechanisms (Kehrer, 1993). In the following 96 h from the beginning of poisoning, the concentration of MDA, the parameter that describes the intensity of lipid peroxidation, decreased (but not significantly in comparison with the untreated animals), what was obviously the consequence of the activation of the antioxidative system.

The process of lipid peroxidation itself is capable of increasing the process of free radical production, using NADPH as the electron donor.

Lipid peroxidation of cell membranes or the membranes of the endoplasmic reticulum, results in changes in the permeability for Ca$^{2+}$ ions leading to the increase in their concentration in the cell, which eventually results in remarkable changes in Ca$^{2+}$ dependent processes (Comporti, 1989).

Being an electron acceptor, PQ$^{2+}$ builds complexes with numerous molecules, electron donors, neutral organic molecules, metal compounds, etc. The building of complexes between paraquat and poliphenol huminic acids strongly contributes to the deactivation of herbicides adsorbed in the soil (Gevao et al., 2000; Pacheco et al., 2003). Helate complexes between paraquat and heavy metal salts can be formed, e.g. AgCl, Hg$_2$Cl$_2$, HgCl$_2$, PbJ, as well as with anions
Cu$_2$Cl$_6^{2-}$, MnCl$_4^{2-}$, FeCl$_4^{2-}$. Certain complexes with anions enable the longstanding herbicidal activity of paraquat (Amondham et al., 2006).

In the present research, an hour after application, natural and synthetic bentonite decreased the activity of CAT by 36 % and 70.08 %, respectively, while their influence 192 h after administration is quite similar, being 51.74 % and 46.04 %, respectively. In addition to this, both bentonites decreased lipid peroxidation in the first hour from the beginning of poisoning by 55 % (natural bentonite) and 45 % (synthetic bentonite). On the other hand, 192 h from the beginning of the intoxication the difference in their adsorbent efficacy was almost negligible (Bn=41.24 % and Bs=43.75 %).

By comparison of the relations catalase/lipid peroxidation (CAT/LP) in the group administered paraquat only and the control group, it was remarkable that the relation in the former group (CAT/LP=0.78) 24 h after the beginning of intoxication was 3.37 times lower than in the latter (CAT/LP=2.63); this indicates a more intensive process of lipid peroxidation. This proves our hypothesis that paraquat induces the synthesis of peroxides that are not degraded by CAT, but that cooperate with superoxide anionic radical (O$_2^-\cdot$) leading to increased of lipid peroxidation and thus to synthesis of larger quantities of MDA.

It can be concluded that paraquat provokes oxidative stress that is considerable 1 h and 192 h after the beginning of intoxication with this herbicide.

Both natural and synthetic bentonite showed a protective activity in the period from 24 to 144 h after application, acting via external capacity of cationic exchange that is considered to be approximately 10 %. The absorption of paraquat is conditioned by the size of the molecule and its polarity, and is performed by the mechanisms of ionic exchange (ionic interaction and electrostatic interactions - van der Waals forces) (Pradas et al., 1996). The mechanism of adsorption is complicated and has yet to be described.

Both adsorbents expressed significant protective effects (approximately 50 %) 1 h and 192 h after application, which indicates that both natural and synthetic bentonite acted as molecular sieves and thus suppressed the process of lipid peroxidation and the development of more intensive oxidative stress.

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PRIMENA BENTONITA KAO ADSORBENTA TOKOM OKSIDATIVNOG STRESA INDUKOVANOG PARAHVATOM

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SADRŽAJ

U ovom radu su izneti rezultati proučavanja protektivnog efekta prirodnog i sintetskog bentonita tokom oksidativnog stresa indukovanog aplikacijom parahvata pacovima soja Wistar. Ogleđene životinje su bile podeljene u četiri jednake grupe. Prva grupa pacova tretirana je samo parahvatom pomoću želudacne sponde. Jedan sat nakon aplikacije parahvata, pacovi druge i treće grupe su bili tretirani prirodnim ili sintetskim bentonitom, aplikovanim per os. Četvrta grupa pacova nije tretirana i služila je kao negativna kontrola. Eksperimentalne grupe životinja su bile tretirane jednom dnevno tokom osam uzastopnih dana. Uzorci krvi su uzimani 0, 1, 4, 6 i 8 dana nakon prvog tretmana.

Stepen oksidativnog stresa je procenjivan na osnovu aktivnosti enzima katalaze i na osnovu količine malondialdehida. Prirodni i sintetski bentonit ispoljili su protektivnu aktivnost smanjenjem parametara oksidativnog stresa u periodu od 24 do 144 sata nakon aplikacije. Autori predpostavljaju da bentonit deluje kao molekularno sito suprimirajući proces lipidne peroksidacije.