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Antioxidant markers in guinea pig exposed to obidoxime and HI-6 acetylcholinesterase oxime reactivators containing oxime moiety

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Obidoxime and asoxime (HI-6) are considered to be the most important acetylcholinesterase (AChE) reactivators applicable for treatment of poisoning by nerve agents. Unfortunately, toxicology of the oximes is not well known. For this reason, we decided to investigate the pertinent adverse effects on guinea pigs which are close to humans in toxicological point of view. HI-6 and obidoxime were administered intramuscularly in 5% of the median lethal dose. The animals were sacrificed 15, 30, 60, 120, and 240 min after exposure, and the brain, liver, spleen and kidneys were collected. Ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS), glutathione S-transferase (GST) and glutathione reductase (GR) were measured. Results indicated that obidoxime acted on oxidative stress than HI-6. We found evidence of low molecular weight antioxidants depletion after obidoxime administration. On the other hand, TBARS assay showed significant decrease in brain and little increase in spleen and liver. The effect of HI-6 was more striking than the effect of obidoxime. There was a sign of higher metabolism and production of antioxidants in liver because GR was significantly increased after HI-6 exposure, and it is another sign of ongoing oxidative stress. Owing to the achieved results, obidoxime can be considered as a less toxic drug in counteracting oxidative stress despite its higher toxicity.

Key words: Oxidative stress, obidoxime, HI-6, acetylcholinesterase, butyrylcholinesterase.

INTRODUCTION

Nerve agents and organophosphorus pesticides are organic compounds obtained from phosphorus and characterized by a high toxicity to mammals. Acute organophosphorus pesticide poisoning causes tens of thousands of deaths each year across the developing world (Buckley et al., 2011). Their main toxic effect is inhibition of enzyme acetylcholinesterase (AChE; EC 3.1.1.7); the phosphorous substances covalently bind to hydroxyl group of serine molecule in catalytic site of AChE. When AChE becomes inhibited, it cannot proceed in physiological function - splitting of neurotransmitter acetylcholine (ACh) (Marrs, 1993). ACh as a substrate of AChE become accumulated in the neurosynaptic cleft and causes cholinergic crisis due to the overstimulation of both ACh receptors: muscarinic and nicotinic. The therapy after organophosphorus poisoning is symptomatic (atropine and/or anticonvulsants) and causative (oxime reactivators). Oxime reactivators are a group of antidotes suitable for causal treatment after exposure to these phosphorous compounds (Bajgar, 2010). Oximes react with nerve agents and pesticides bind the AChE active site (Pohanka, 2011). The covalent bound is broken due to nucleophilic substitution (Petroianu et al., 2012). On the other hand, oximes are inhibitors of AChE

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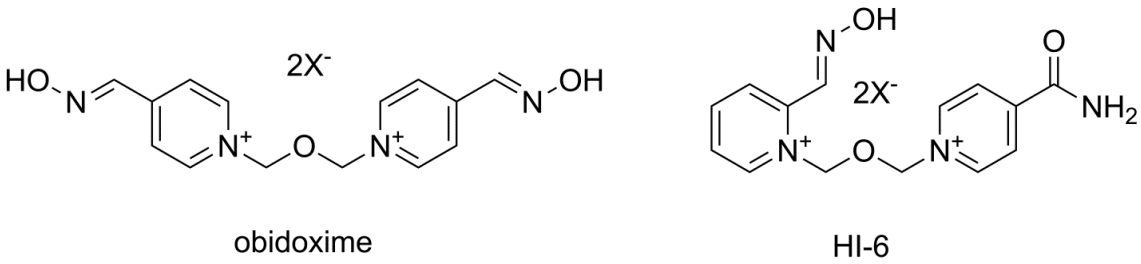

Figure 1. Structures of the oxime reactivators: obidoxime and asoxime (HI-6).

as well and also protect the active site of AChE (Mercey et al., 2012). Oxidative stress is an imbalance between formation and removal of reactive oxygen species. The reactive oxygen species products are free radicals and peroxides. The reactive forms are covered by antioxidants as the reactive forms are scavenged. Redox balance is considered as another parameter of homeostasis. Oxidative stress in humans results in many diseases - including the neuro-degenerative ones such as Parkinson´s disease, Alzheimer´s disease, and Huntington's disease (Guglielmotto et al., 2010); cardiovascular disease – myocardial infarction, atherosclerosis, heart failure (Ramond et al., 2011); and others – fragile X syndrome or chronic fatigue syndrome (Kennedy et al., 2005).

The prevention of oxidative stress is based on up-regulation of antioxidant enzymes. We may achieve up-regulation with some trace elements in food supplements, such as selenium, zinc, copper or combination of the elements (Micke et al., 2009). Alteration in the antioxidant enzymes provide information about relationship with some diseases such as Alzheimer´s disease, atherosclerosis and in treatment of diseases linked with chronic inflammation and persisting generation of reactive species (Valko et al., 2005).

Oxidative stress plays a significant role in poisoning with inhibitors of the AChE enzyme. Although the mechanism of inhibition of enzymes is known, but the overall effect on the organism is not completely understood. In previous experiments, we examined some side effects of oxime reactivators. Especially, oxidative stress was plausibly proven to follow administration of oxime reactivators. Unfortunately, complete evidence of the link between oxidative stress and the time pathogenesis starts has not been extensively investigated yet. Hence, in order to recognize scale and bulk of oxidative stress, we decided to investigate it in guinea pigs as a model organism with good extrapolation to humans. Obidoxime (1,3-bis(4-hydroxy-iminomethylpyridinium)-2-oxapropane dichloride) and HI-6 (4-carbamoyl-1-[({2-[(E)-(hydroxyimino)-methyl]pyridinium-1-yl}methoxy)methyl] pyridinium dichloride), as shown in Figure 1, were chosen as the representative oxime reactivators for the experiment purposes.

MATERIALS AND METHODS

Animal exposure

The experiment was done using three months old guinea pig (*Cavia porcellus*) weighing 260 ± 20 g at the start of the experiment. The animals were received from Velaz Company (Prague, Czech Republic) and kept under standard room temperature 22 ± 2°C, humidity 50 ± 10% and light period 12 h per a day. Food and water were provided *ad libitum*. The experiment was permitted and supervised by the Ethical Committee of the Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic. Obidoxime and HI-6 both received from Sigma-Aldrich (Saint Louis, Missouri, USA), were injected intramuscularly (i.m.) in an amount of 100 µL into the rear limb of the animals. The animals were divided into 5 groups each of 5 specimens, while 8 animals were disposed for control purposes. The control group was used for both reactivators and it was exposed to saline solution in the same volume: 100 µl. Animals in the 5 groups were i.m. exposed to either obidoxime or HI-6 in doses 5% of LD_{50}; it was 4.15 mg/kg for obidoxime and 45.1 mg/kg for HI-6. The animals were sacrificed by light CO_2 narcosis 15, 30, 60, 120, and 240 min after the start of the experiment for both reactivators.

Preparation of samples for antioxidant markers assessment

The frontal lobe, cerebellum, spleen, liver and kidney were collected after euthanasia from the animals. In total, 100 mg of freshly collected tissue was used for sample preparation. The tissue sample was placed in 1 ml of saline solution (0.9 w/v of sodium chloride in deionized water) and processed immediately. The individual samples were homogenized using a T10 basic ULTRA-TURRAX® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) mechanical homogenizer for 1 min.

Methods of antioxidants markers assessment

Ferric reducing antioxidant power assay (FRAP), thiobarbituric acid reactive substances (TBARS), glutathione S-transferase (GST) and glutathione reductase (GR) were assessed using standard protocols previously reported (Pohanka et al., 2011).

Statistical analysis

The experimental data were processed by the statistical software Origin 8 SR2 (OriginLab Corporation, Northampton, MA, USA). The results were tested for their significance. Significant differences between the experimental groups were estimated by one-way analysis of variance (ANOVA) with Bonferroni's test, considering

Table 1. Results on the ferric reducing antioxidant power (FRAP) for obidoxime.

Parameter (µmol/g)	Control	15 min	30 min	60 min	120 min	240 min
Liver	1.46±0.28	1.09±0.14	1.21±0.27	1.09±0.16	1.11±0.18	1.29±0.18
Spleen	1.78±0.17	1.38±0.25	1.44±0.34	1.30±0.18*	1.27±0.08*	1.48±0.34
Kidney	0.885±0.215	0.863±0.253	0.885±0.311	0.829±0.223	0.843±0.202	0.862±0.098
Cerebellum	0.800±0.280	0.614±0.222	1.33±0.05 (*)	0.476±0.045	0.548±0.197	0.493±0.051
Frontal lobe	0.655±0.188	0.411±0.031**	0.384±0.025**	0.411±0.024**	0.374±0.022**	0.408±0.020**

*=p<0.05. **=<0.01.

Table 2. Results on the thiobarbituric acid reactive substances (TBARS) for obidoxime.

Parameter (µmol/g)	Control	15 min	30 min	60 min	120 min	240 min
Liver	0.567±0.101	0.600±0.039	0.717±0.147	0.722±0.088	0.569±0.043	0.509±0.030
Spleen	0.491±0.066	0.409±0.051	0.389±0.059	0.559±0.079	0.510±0.076	0.499±0.045
Kidney	0.800±0.113	0.679±0.032	0.654±0.043*	0.586±0.040**	0.597±0.111**	0.495±0.041**
Cerebellum	0.873±0.135	0.575±0.066**	0.577±0.023**	0.639±0.030**	0.571±0.026**	0.630±0.032**
Frontal lobe	0.816±0.084	0.643±0.052**	0.611±0.049**	0.660±0.070**	0.642±0.060**	0.667±0.066**

*=p<0.05. **=<0.01.

Table 3. Results on glutathione reductase (GR) for obidoxime.

Parameter (µmol/g)	Control	15 min	30 min	60 min	120 min	240 min
Liver	0.128±0.052	0.253±0.021**	0.265±0.025**	0.221±0.017**	0.225±0.041**	0.219±0.059**
Spleen	0.146±0.059	0.196±0.017	0.177±0.090	0.218±0.075	0.186±0.100	0.259±0.017
Kidney	0.129±0.031	0.217±0.105	0.250±0.029	0.227±0.092	0.312±0.134*	0.247±0.017
Cerebellum	0.164±0.046	0.114±0.018	0.140±0.058	0.145±0.024	0.168±0.047	0.184±0.029
Frontal lobe	0.114±0.114	0.239±0.059	0.166±0.059	0.238±0.086	0.215±0.028	0.282±0.068*

*=p<0.05. **=<0.01.

Table 4. Ferric reducing antioxidant power (FRAP) for HI-6.

Parameter (µmol/g)	Control	15 min	30 min	60 min	120 min	240 min
Liver	1.46±0.28	1.02±0.206**	0.790±0.050**	1.03±0.060**	0.925±0.123**	0.999±0.203**
Spleen	1.78±0.17	0.889±0.111**	0.934±0.344**	1.05±0.18**	0.968±0.170**	0.932±0.272**
Kidney	0.885±0.211	0.562±0.133	0.436±0.120*	0.456±0.050*	0.339±0.020*	0.591±0.333
Cerebellum	0.800±0.282	0.432±0.181	0.504±0.165	0.342±0.323	0.418±0.275	0.672±0.243*
Frontal lobe	0.655±0.180	0.499±0.221	0.481±0.244	0.346±0.162	0.364±0.160	0.362±0.201

*=p<0.05. **=<0.01.

probability level p=0.05 (0.01<p≤0.05) and p=0.01 (p≤0.01) probability levels for each group.

RESULTS

The animals were in a good condition and they exerted no symptoms of poisoning. Moreover, no convulsions occurred during the whole experiment. FRAP was used for the assessment of antioxidants content (Tables 1 and 2), lipid peroxidation was represented by TBARS (Tables 3 and 4) and enzymatic marker by GR (Tables 5 and 6). All the experiments were assessed on liver, spleen, kidney, cerebellum and frontal lobe for the both reactivators. FRAP value was extensively altered for the both reactivators. Beside the difference in the reactivators exposed animals, the tested organs had variation in the

Table 5. Thiobarbituric acid reactive substances (TBARS) for HI-6.

Parameter (µmol/g)	Control	15 min	30 min	60 min	120 min	240 min
Liver	0.567±0.101	0.687±0.062	0.651±0.107*	0.647±0.084	0.680±0.061	0.682±0.035
Spleen	0.491±0.066	0.471±0.015	0.561±0.054	0.584±0.057	0.584±0.049	0.548±0.042
Kidney	0.800±0.114	0.888±0.033	0.911±0.059	0.979±0.091	0.937±0.039	0.916±0.118
Cerebellum	0.873±0.135	1.13±0.05**	1.29±0.13**	1.36±0.06**	1.22±0.15**	1.25±0.08
Frontal lobe	0.816±0.084	1.09±0.07**	1.04±0.06**	1.16±0.10**	1.00±0.10**	1.12±0.03**

*=p<0.05. **=<0.01

level of low molecular weight antioxidants. However, the effect was unequal in the different organs. Obidoxime caused a decrease of FRAP (significant on p=0.01) in frontal lobe from 15 min and in spleen after 60 min (significance at p=0.05). A significant decrease (p=0.01) was also observe in the liver and in the spleen from 15 min after HI-6 exposure as well. The second decrease were evident about half of the value beside control group (p=0.05) in kidney after 30 min.

TBARS levels were also detected by the same organs for the both reactivators. The significance was the same (p=0.01) for the reactivators in cerebellum and in frontal lobe from 15 min. It is interesting to note that the level of lipid peroxidation was reduced for obidoxime, but increased for HI-6 compared to the control. In obidoxime exposed animal, one more decrease was found in kidney after 30 min (significant on p=0.05) respective from 60 min (significant on p=0.01). Furthermore, GR significantly (p=0.01) increased when compared to controls in obidoxime group in liver after 15 min. In the HI-6 exposed animals, GR activity was significantly altered in kidney on probability level p=0.05 after 30 min and on p=0.01 after 60 min. Beside the kidney, HI-6 caused significant alteration of glutathione reductase activity in spleen after 30 min (p=0.05). Compared to the other markers, GST activity in the examined organs has no tendency and shift in GST activity appears to be random. The marker fluctuated in a range 283 to 325 µmol/g in kidney, 480 to 553 µmol/g in liver, and 11.5 to 28.4 µmol/g in the other organs. No statistically significant result was provided for the marker.

DISCUSSION

Obidoxime and HI-6 are bispyridinium oximes. They are used in the treatment of acute nerve agents poisoning. The oxime substances are called reactivators and they are known for their ability to reverse the binding of organophosphorus compounds to the enzyme AChE. At present, we know that the effectiveness of the oximes is too low to resolve the acute toxic effects of some organophosphorus nerve agents. This finding is supported by a long term research resumed by Kassa (2002). Our main interest was to find out adverse effect after intramuscular administration in guinea pig model.

Our results indicated that the level of low molecular weight antioxidants decreased in all the organs after administration of obidoxime with the exception of kidneys. Based on the FRAP measurement results, we can infer that variation in metabolism took place in the guinea pigs after administration of obidoxime. Liver is obviously more sensitive to the impact of oxime reactivators in terms of the FRAP value compared to brain (Pohanka et al., 2011). However, it was observed that the decrease of low molecular weight antioxidants was significant in frontal lobe tissue in the obidoxime case. This is sign of non-normal homeostasis or antioxidant imbalance in the brain. In clinical studies, evidence of initiated pathological processes is linked to alteration of antioxidant balance and antioxidants are typically depleted in the brain of patients that suffers from neurodegenerative disease (Fujita et al., 2012).

The TBARS value indicates damage to membranes by reactive oxygen species and in response to the molar fragment of oxidized lipids (malondialdehyde) (Pohanka et al., 2011). The increase of malondialdehyde is common once oxidative stress is uncovered by antioxidants (Jomova et al., 2010). From the results of TBARS, it was clearly evident that the lipid peroxidation significantly decreased in kidney, cerebellum and frontal lobe. On the other hand, we observed a little increase in the liver and spleen in 30, 60 and 120 min after the administration of the oximes. Though the TBARS level was not extensively altered after application of the oxime reactivators, depletion of antioxidants evident from the FRAP level point to increased sensitivity to a pro-oxidant effect. Moreover, liver is a source of low molecular weight antioxidants such as reduced glutathione (GSH) distributed by blood system to the other organs where it helps to maintain oxidative homeostasis (Hermes-Lima et al., 2012; El-Demerdash et al., 2012). Depletion of antioxidants in liver causes aggravation of antioxidant balance in the other organs. GR is a ubiquitous enzyme that catalyses the NADPH-dependent reduction of oxidized glutathione (GSSG) to GSH. GSH is an important molecular antioxidant; it is dominantly involved in the destruction of free reactive oxygen species and in the metabolism of exogenous and endogenous compounds (Rana et al., 2002). As seen in the experi-

Table 6. Glutathione reductase (GR) for HI-6.

Parameter (µmol/g)	Control	15 min	30 min	60 min	120 min	240 min
Liver	0.128±0.052	0.169±0.036	0.120±0.023	0.156±0.038	0.155±0.028	0.162±0.031
Spleen	0.146±0.059	0.247±0.053	0.266±0.060*	0.284±0.036*	0.203±0.033	0.284±0.080*
Kidney	0.129±0.031	0.156±0.026	0.199±0.034*	0.254±0.036**	0.258±0.034**	0.324±0.030**
Cerebellum	0.164±0.046	0.142±0.035	0.108±0.054	0.125±0.058	0.152±0.055	0.145±0.022
Frontal lobe	0.114±0.114	0.153±0.026	0.183±0.060	0.142±0.047	0.134±0.051	0.164±0.096

*=p<0.05. **=<0.01.

mental data, there was significantly increased GR activity only in liver, but the non-significant increase was found in all the organs of the exposed animals. Hence, it can be concluded that oxidative stress took place after the administration of oxime reactivators.

The examination of low molecular weight antioxidants were conducted in all selected organs using the FRAP test. The significant variations of the marker in animals treated by HI-6 are interesting when compared with each other. For both reactivators, we detected a decrease of low molecular weight antioxidants in organ's tissues. In HI-6 exposed animals, we found significantly decreased low molecular weight antioxidants in the liver, spleen and in kidney. The decrease was almost double compared to the controls. And although the depletion of low molecular weight antioxidants is not necessary a pathological process, however, the depletion increases vulnerability of organism to the other pathological processes such as degradation of macromolecules (Valachova et al., 2010).

In TBARS assay, we found increased value of malondialdehyde in all the organs. The sensitivity of the brain to impact of oxime reactivators was a surprising fact. Owing to the obidoxime, the increase of lipid peroxidation uncovered oxidative stress in organism. On the other hand, HI-6 initiated a depletion of antioxidants in the examined organs. The depletion was more extensive for HI-6 than was evident in obidoxime-exposed animals. We must emphasize that the more extensive effect of HI-6 could be caused by application of the equitoxic doses into the animals. The animals treated with HI-6 received larger mass than the animals treated with obidoxime. Application of equitoxic doses have drawback in the more complicated investigation of some side effects of the tested compounds. However, the animals had similar adverse effects as toxic doses were the same. Therefore, further researches should be aimed at providing a link between the equitoxic and equimolar doses for oxime reactivators. Considering chemical structure of the oxime reactivators, obidoxime has two oxime moieties, while HI-6 has only one oxime and one carbamoyl moieties. The presence of the carbamoyl group would be considered as a risk factor for the reported depletion of antioxidants.

In the GR assessment, we obtained an increase of enzyme activity in all organs. Though GR activity was increased in all of the examined organs, the alteration was significant in kidneys only. It confirms that the organs met reactive oxygen species evoking GR expression as GR is considered as a plausible marker of appeared oxidative stress (Anand et al., 2012). This finding can be interpreted that the two tested reactivators acted as pro-stressogenic factors in the kidney and the detrimental processes would be initiated once the reactivators administered as a prophylactic. The link between oxidative stress in kidneys and nephropathy has been reported by several studies (Yiu et al., 2010). The fact that kidneys are influenced by the oxime reactivators is therefore not surprising, considering the renal elimination and temporal retention of the compounds (Ligtenstein and Kossen, 1983).

Conclusion

It can be approved that both reactivators have significant effect on oxidative homeostasis in the body. Thus far, we have proven that obidoxime is less implicated in oxidative stress than HI-6. This is an interesting fact because HI-6 is commonly considered as a low toxic oxime reactivator when taking acute toxicity into account. In contrast, HI-6 has potential adverse effects when administered repeatedly.

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