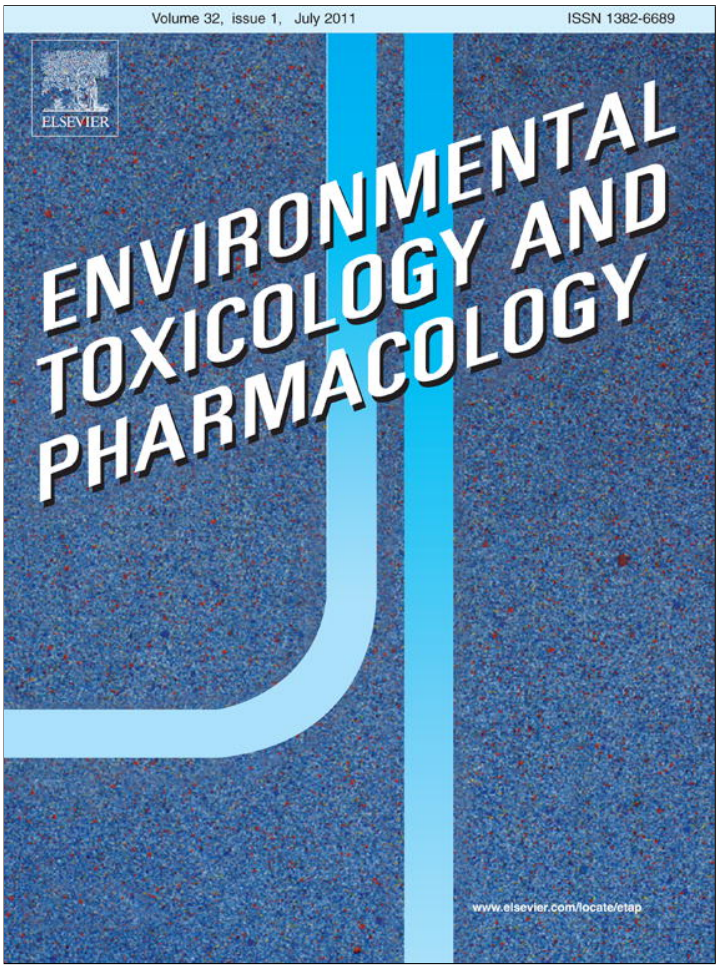

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Asoxime (HI-6) impact on dogs after one and tenfold therapeutic doses: Assessment of adverse effects, distribution, and oxidative stress

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ABSTRACT

Asoxime (HI-6) is a well known oxime reactivator used for counteracting intoxication by nerve agents. It is able to reactivate acetylcholinesterase (AChE) inhibited even by sarin or soman. The present experiment was aimed to determine markers of oxidative stress represented by thiobarbituric acid reactive substances and antioxidants represented by ferric reducing antioxidant power, reduced and oxidized glutathione in a Beagle dog model. Two groups of dogs were intramuscularly exposed to single (11.4 mg/kg.b.wt.) or tenfold (114 mg/kg.b.wt.) human therapeutically doses of HI-6. HI-6 affinity for AChE *in vitro* was evaluated in a separate experiment. Complete serum biochemistry and pharmacokinetics were also performed with significant alteration in blood urea nitrogen, creatine phosphokinase, glucose and triglycerides. Blood samples were collected before HI-6 application and after 30, 60, and 120 min. The overall HI-6 impact on organism is discussed.

1. Introduction

Some artificial toxic compounds known as nerve agents such as sarin, soman, tabun, VX and organophorous pesticides e.g., chlorfevinphos, dichlorvos, paraoxon, parathion are able to irreversibly inhibit the enzyme involved in neurotransmission: acetylcholinesterase (AChE; EC 3.1.1.7). These inhibitors alkylate serine hydroxyl in the active site of AChE and in this way they block splitting of the natural sub-

Abbreviations: AChE, acetylcholinesterase; AChR, acetylcholine receptor nicotinic (n) or muscarinic (m); FRAP, ferric reducing antioxidant power; GSH, reduced glutathione; GSSG, oxidized glutathione; TBARS, thiobarbituric acid reactive substances; T-SH, total free thiol.

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strate, i.e., the neurotransmitter acetylcholine (Barthold and Schier, 2005). The accumulated acetylcholine over-stimulates nicotinic acetylcholine receptors (nAChR) and muscarinic acetylcholine receptors (mAChR) in the neurosynapses as well as neuro-muscular junctions (Bajgar, 2004). Intoxication leads to manifestation of typical symptoms that can be divided into three basic groups: central, muscarinic as mAChR are over stimulated and nicotinic as nAChR become stimulated (Marrs, 1993).

Successful treatment depends on the following principles. First, AChE can be protected by the administration of a reversible inhibitor such as pyridostigmine (Haigh et al., 2010). The reversibly inhibited AChE has the active site occupied so interaction of the irreversible inhibitor with serine in the active site is slowed down. Administration of the reversible inhibitor is suitable as prophylaxis or treatment immediately after intoxication. The second treatment principle is based on administration of anticholinergic drugs such as atropine. It attenuates the effect of acetylcholine on mAChR. Atropine effects can be enhanced by co-administration of anticonvulsants such as benzodiazepine (McDonough et al., 2009). The last group of therapeutics contains oxime reactivators suitable for the causal treatment of organosphosphate intoxication. The oxime reactivators are able to split residua of the organophosphate from AChE and restore the AChE activity in this way (Worek et al., 2007). One of the most powerful oxime reactivators is asoxime (1-(2-hydroxyiminomethylpyridinium)-3-(4-carbamoylpyridinium)-2-oxa-propane dichloride) in literature abbreviated as HI-6. It was first synthesized in 1960s when its good potency for reactivation of AChE inhibited by, e.g., nerve agents sarin and VX, was found. As described, it is even partially efficacious in reactivation of soman-inhibited AChE that is only poorly reactivated by other available drugs (Kepner and Wolthuis, 1978; Kassa, 2002). Due to this fact, HI-6 is currently considered as a drug of choice for the emergency situation following a nerve agent attack in several NATO countries (Lundy et al., 2006).

Apart from the impact as an antidote, HI-6 has probably more side effects as recognized in recent experiments. Non-defined effects such as, e.g., the ability to decrease mortality in tularemic mice (Pohanka et al., 2010a) and suppression of irinotecan side effects (Vrdoljak et al., 2009) were clearly demonstrated. Though the side effects of HI-6 are not fully understood, reversible inhibition of AChE (Pohanka et al., 2010b) and antagonizing of acetylcholine on mAChR (Soukup et al., 2008) were described previously. The antagonizing effect of HI-6 on nAChR was not fully described in literature but can also be expected.

Searching of data about HI-6 adverse effects confirmed limited understanding of the drug implication in body homeostasis. Thus the present experiment is focused on a better description of the HI-6 impact on the body as there are lacking pieces of information. The experiment is focused on assaying standard clinical biochemistry markers as a complex assessment of adverse effects, biochemistry of oxidative stress markers and antioxidants after the HI-6 application. The data were correlated to the residual HI-6 in blood stream. Some other supplementary investigations and *in vitro* assays supporting stated hypothesis were done, too. We chose two doses. The therapeutic one covers standard application. However, the therapeutic dose is determined for health male soldiers or rescue team members in good physical conditions. Women and children have lower weight. Moreover, some medical authorities recommend repeated application of the drug when the symptomatic manifestation hangs on the first application. The ten times of recommended therapeutic dose is investigated for this reason in the second group of animals.

2. Experimental design

2.1. *Assay of AChE activity and calculation of HI-6 affinity to AChE*

AChE (isolated from human erythrocytes) was purchased from Sigma–Aldrich (St. Louis; MO; USA). An adapted photometrical Ellman's method was used for AChE activity evaluation (Pohanka et al., 2008). A cuvette was filled with 0.4 ml 0.4 mg/ml of 5,5′-dithiobis(2-nitrobenzoic acid), abbreviated as DTNB hereinafter in the text. Consequently, 25 μl of AChE solution in phosphate buffered saline (PBS) with overall activity 0.5 μkat, 25 μl of tested HI-6 concentration and 450 μl of PBS were injected into the cuvette. The reaction was started by addition of acetylthiocholine chloride (ATChCl) in concentration of 10 μM–10 mM. Absorbance was measured at 412 nm against mixture with AChE replaced by PBS. AChE activity was calculated using extinction coefficient for thionitrobenzorate: $\varepsilon = 14{,}150\ M^{-1}\ cm^{-1}$. The measurements were carried out in triplicate. The biochemistry of inhibitory impact was evaluated using Lineweaver–Burk plot. Inhibitory constants K_{i1} (HI-6 affinity for AChE alone) and K_{i2} (HI-6 affinity for complex AChE–ATChCl) were calculated. The physical meaning of constants is following:

$$K_{i1} = \frac{[E][I]}{[EI]}$$

$$K_{i2} = \frac{[ES]\,[I]}{[ESI]}$$

where E is concentration of enzyme, I concentration of inhibitor (HI-6), ES concentration of complex enzyme substrate, ESI concentration of complex enzyme, substrate and inhibitor.

2.2. *Dogs exposure to HI-6*

The experiment was carried out using two-year-old Beagle dogs under anaesthesia, weighing 10–15 kg. For anaesthesia, we used butorphanol 0.2 mg kg^{-1} (Butomidor, Richter Pharma, Austria) + xylazin 2 mg kg^{-1} (Xylapan, Vetoquinol, Poland) + ketamine 10 mg kg^{-1} (Narketan, Vetoquinol, Poland) + atropin 0.01 mg kg^{-1} (Atropin sulphate, Fatro, Italy). A total of ten dogs were divided into two groups of five animals. Animals were kept at the vivarium of Faculty of Military Health Sciences (FMHS). All the experiments were performed under permission and supervision of the Ethical Committee of the Faculty of Military Health Sciences, Hradec Kralove, Czech Republic.

Blood was collected from vena jugularis through an i.v. cannula just prior to the experiment. After that, HI-6 dichloride

(Sigma–Aldrich) was applied intramuscularly in the human therapeutic dose (i.e., 11.4 mg/kg of body weight – kg.b.wt.; the first group) or in tenfold human therapeutic dose (i.e., 114 mg/kg.b.wt.; the second group). Due to difficult manipulation with dogs, the second group was processed one week after the first. The human therapeutic dose was re-calculated for the dog using the following reference (Lundy et al., 2005). Blood was collected again 30, 120, and 180 min after HI-6 administration. Non-heparinized blood was allowed to clot in order to collect serum; heparinized blood was centrifuged at 3000 × *g* and plasma was separated into another tube. Blood mass was homogenized by osmotic disruption by mixing with four volumes of deionized water and kept at laboratory temperature for 5 min. Membranes were separated by centrifugation 3000 × *g* for 10 min. The samples were kept in a freezer (−80 °C) until assay.

2.3. Ferric reducing antioxidant power assay

Ferric reducing antioxidant power (FRAP) was used to estimate the total antioxidant capacity in tested samples. Protocols were slightly modified from the reference (Pohanka et al., 2009a). 2.5 ml of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) were mixed with another 2.5 ml of 20 mM $FeCl_3$ and 25 ml of 0.1 M acetate buffer pH 3.6. After heating at 37 °C for 10 min, 200 μl of mixture was split with 770 μl of deionized water and 30 μl of sample. Ten minutes of incubation followed. After mixture centrifugation at 10,000 × *g* for 10 min, absorbance at 593 nm was read against blank (mixture with sample replaced by the saline solution). The molar value of FRAP is calculated using the extinction coefficient $\varepsilon = 26{,}000\ M^{-1}\ cm^{-1}$.

2.4. Thiobarbituric acid reactive substances assay

Thiobarbituric acid reactive substances (TBARS) assay was adopted from the protocol by Pohanka et al. (2011). 67 mg of thiobarbituric acid was suspended into 1 ml of dimethylsulfoxide and consequently pour with 9 ml of deionized water. 200 μl of tested sample was purified from proteins by precipitation with 400 μl of 10% trichloroacetic acid for 15 min on ice. Proteins were separated by centrifugation at 3000 × *g* for 15 min. 400 μl of the supernatant or saline solution (for blank purposes) were poured with the same volume of thiobarbituric acid mixture and heated at 100 °C for 10 min. After cooling, absorbance was measured at 532 nm against blank and TBARS level was calculated using extinction coefficient of $156{,}000\ M^{-1}\ cm^{-1}$.

2.5. Evaluation of HI-6 residual levels

Samples were analysed by reversed phase HPLC with electrochemical detection. The chromatographic system consists of an isocratic pump LC-10AD (Shimadzu, Duisburg, Germany), a 7125 injection valve – 10 μl loop (Rheodyne, Cotati, USA), a Coulochem II detector with analytical cell model 5011 (ESA, Bedford, USA) and a Clarity software (DataApex, Prague, Czech Republic).

An analytical column LiChrospher® 60, 250 × 4.6 (5 μm) was used in our study, with a guard column 4 × 4 (RP-select B) (Merck, Darmstadt, Germany). The mobile phase was 22% acetonitrile (gradient grade)/double distilled and deionized water. The mobile phase contained 3 mM/l octane sulfonic acid, 1 mM/l tetramethylammonium chloride. The pH was adjusted at 2.2 with H_3PO_4. The flow rate of the mobile phase was 1.4 ml/min (Karasova et al., 2010). Measurement cells had the respective potentials of +350 mV and +650 mV. The potential of the guard cell was +1000 mV. The detector gain was set at 5 μA for higher concentrations (first group) and 1 μA for lower concentrations (second group). All chromatograms were obtained at air-conditioned room temperature of 22 °C.

2.6. Assay of glutathione and total thiols

Reduced (GSH) and oxidized (GSSG) glutathione as well as total serum thiols (T-SH) were assayed throughout experiments as described below. Electrochemical measurements were performed with 747 VA Stand instrument connected to 746 VA Trace Analyzer and 695 Autosampler (Metrohm, Switzerland) using a standard cell with three electrodes and cooled sample holder (4 °C). A hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2 was the working electrode. An Ag/AgCl/3 M KCl electrode was the reference and glassy carbon electrode was auxiliary electrode. The Brdicka supporting electrolyte containing 1 mM $Co(NH_3)_6Cl_3$ and 1 M ammonia buffer ($NH_3(aq) + NH_4Cl$, pH = 9.6) was used and changed after five measurements; surface-active agent was not added. Brdicka reaction parameters were as follows: an initial potential of −0.35 V, an end potential of −1.8 V, a modulation time of 0.057 s, a time interval of 0.2 s, a step potential of 1.05 mV, a modulation amplitude of 250 mV, Eads = open circuit, time of adsorption: 240 s. All experiments were carried out at 4 °C (Julabo F12, Germany). GPES 4.9 supplied by Eco-Chemie was employed.

2.6.1. High performance liquid chromatography coupled with CoulArray (coulometric) detector

HPLC-ED system consisted of two solvent delivery pumps operating in the range of 0.001–9.999 ml min^{-1} (Model 582 ESA Inc., Chelmsford, MA), Metachem Polaris C18A reverse-phase column (150.0 mm × 2.1 mm, 5 μm particle size; Varian Inc., CA, USA) and a CoulArray electrochemical detector (Model 5600A, ESA, USA). The electrochemical detector includes three flow cells (Model 6210, ESA, USA). Each cell consists of four working carbon porous electrodes, each one with auxiliary and dry H/Pd reference electrodes. Both the detector and the reaction coil/column were thermostated. The sample (5 μl) was injected using an autosampler (Model 542 HPLC, ESA, USA).

2.7. Serum biochemistry

Dog sera were analysed using an automated analyzer (SPOTCHEMTM EZ SP-4430, ARKRAY, Japan) for Alb (g/l) – albumin; ALP (μkat/l) – alkaline phosphatase; ALT (μkat/l) – alanine aminotransferase; AMY (μkat/l) – amylase; AST (μkat/l) – aspartate aminotransferase; BUN – blood urea nitrogen; CPK (μkat/l) – creatine phosphokinase; CRE (μmol/l) – creatinine; Glu (mmol/l) – glucose; GST (nkat/protein mg) – glutathione S-transferase; IP (mmol/l) – inorganic phosphorus; T-Cho

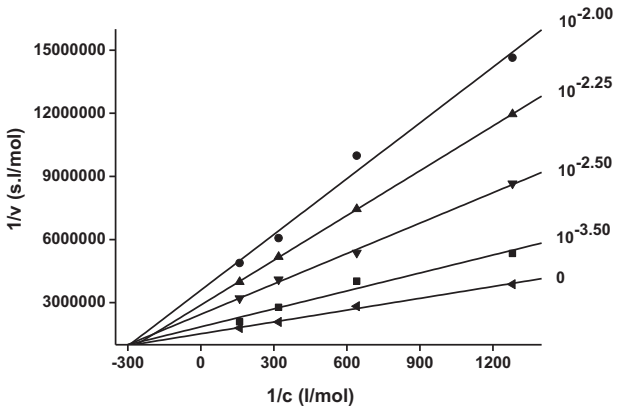

Fig. 1 – Double reciprocal graph of enzyme rate versus ATChCl concentration. Numbers at the right side indicate the molar concentration of HI-6.

(mmol/l) – total cholesterol; T-Pro (mmol/l) – total protein; TG (mmol/l) – triglycerides.

2.8. *Mathematical and statistical analysis*

Software Origin 8 SR4 (OriginLab Corporation, Northampton, MA, USA) was used for data processing and significance testing. ANOVA with Tukey test was used for significance consideration between the groups. The significance was recalculated for two probabilities levels $P = 0.05$ and $P = 0.01$ for the group size $n = 5$.

3. Results

The investigation of enzymes appoints at the non-competitive mechanism of AChE inhibition by HI-6 (see Fig. 1). The inhibitory constants for the free AChE (K_{i1}) was $(2.39 \pm 0.28) \times 10^{-4}$ mol/l. The affinity of HI-6 for AChE bearing ATChCl in the active site was the constant (K_{i2}) equal to $(0.97 \pm 0.16) \times 10^{-4}$ mol/l.

The residual level of HI-6 in dog plasma was evaluated chromatographically. The final data are shown in Table 1. The HI-6 highest level was recognized in samples collected 30 min after application. The therapeutically dose of HI-6 provided maximum concentration of 4.21 ± 2.79 mg/l but the tenfold HI-6 therapeutic dose provided maximum plasma level of 27.04 ± 11.71 mg/ml. After 240 min, HI-6 decreased to 1.20 ± 0.24 mg/ml and 9.10 ± 3.57 mg/ml for therapeutically and tenfold therapeutic doses, respectively.

The next effort was appointed at following stress markers and low-molecular-weight antioxidants in blood mass and plasma as well. FRAP and TBARS levels are shown in Tables 2 and 3, respectively. In both cases, there were found significant shifts of FRAP and TBARS levels due to the HI-6 application. Plasma FRAP was significantly elevated 120 min after the HI-6 application in the therapeutic dose on the probability level of $0.01 < P \leq 0.05$ against the initial level. However, the FRAP level was turning back 240 min after experiment beginning. The tenfold therapeutic dose provided elevated plasma FRAP in both 120 and 240 min on the probability level of $0.01 < P \leq 0.05$ against the initial level. There were found no significant changes in the blood mass FRAP values in both therapeutic as well as tenfold therapeutic doses. HI-6 was also tested *in vitro* whether it acts as an antioxidant in the FRAP assay. Though the concentration of HI-6 was tested up to 100 mM, no significant antioxidant properties were found. It means that HI-6 neither affects the FRAP assay nor physiologically interferes with oxidative stress markers.

In comparison with FRAP, TBARS were significantly elevated in only blood mass and no significant changes were found in plasma. For the HI-6 therapeutically dose, the TBARS level was significantly ($0.01 < P \leq 0.05$) elevated 30 min after experiment beginning then the TBARS decreased to the initial value. The tenfold HI-6 therapeutic dose significantly ($0.01 < P \leq 0.05$) elevated the TBARS level in all three time intervals after the experiment beginning. GSH, GSSG and T-SH were not significantly altered. GSH was increased after therapeutic dose of HI-6 and decreased after ten therapeutic doses of HI-6. The change was not significant. GSSG and T-SH levels were stable without any significant shift. The insignificant data are not depicted.

Biochemistry of 17 standard markers was done. The relevant markers levels are depicted solely for the therapeutically dose (Table 4) and tenfold therapeutically dose (Table 5) of HI-6. In concern of the therapeutically dose, only two markers were significantly elevated. First, blood

Table 1 – Residual level of HI-6 in plasma sample ± standard deviation. Standard deviation was calculated for $n = 5$.

Time of exposure (min)	0	30	120	240
1 × TD HI-6 (mg/l)	0.00 ± 0.00	4.21 ± 2.79	1.82 ± 0.58	1.20 ± 0.24
10 × TD HI-6 (mg/l)	0.00 ± 0.00	27.04 ± 11.71	14.62 ± 3.21	9.10 ± 3.57

Table 2 – Ferric reducing antioxidant power (FRAP) in packed cells and plasma samples. One or two asterisks indicate significance level at the probability level $P = 0.05$ or $P = 0.01$, respectively. Standard deviation (s_d) was calculated for $n = 5$. TD – therapeutically dose.

FRAP ± s_d (mmol/l)				
Time of exposure (min)	0	30	120	240
Blood mass 1 × TD	19.4 ± 1.7	19.6 ± 0.3	19.4 ± 0.9	20.0 ± 0.5
Blood mass 10 × TD	18.2 ± 1.9	18.7 ± 1.3	19.6 ± 1.4	18.4 ± 2.2
Plasma 1 × TD	0.294 ± 0.043	0.334 ± 0.066	0.392 ± 0.101 (*)	0.359 ± 0.052
Plasma 10 × TD	0.316 ± 0.030	0.338 ± 0.025	0.365 ± 0.018 (*)	0.362 ± 0.023 (*)

Table 3 – Thiobarbituric acid reactive substances (TBARS) in packed cells and plasma samples. One or two asterisks indicate significance level at the probability level $P = 0.05$ or $P = 0.01$, respectively. Standard deviation (s_d) was calculated for $n = 5$. TD – therapeutically dose.

TBARS ± s_d (μmol/l)				
Time of exposure (min)	0	30	120	240
Blood mass 1 × TD	5.35 ± 0.78	7.81 ± 0.67 (*)	5.72 ± 0.97	5.26 ± 0.56
Blood mass 10 × TD	5.20 ± 0.51	6.70 ± 0.63 (*)	7.60 ± 1.03 (*)	6.40 ± 0.60 (*)
Plasma 1 × TD	1.82 ± 0.35	1.72 ± 0.87	1.73 ± 0.31	2.17 ± 1.08
Plasma 10 × TD	1.66 ± 0.35	1.55 ± 0.44	1.63 ± 0.71	1.63 ± 0.55

Table 4 – Serum biochemical analysis in dogs exposed to therapeutically dose of HI-6. Standard deviation calculated for $n = 5$. One or two asterisks indicate significance level at the probability level $P = 0.05$ or $P = 0.01$, respectively. Abbreviations: BUN – blood urea nitrogen; CPK – creatine phosphokinase; Glu – glucose; TG – triglycerides.

Time of exposure (min)	0	30	120	240
BUN (mmol/l)	2.62 ± 0.73	2.63 ± 0.52	2.40 ± 0.53	3.88 ± 0.87 (*)
CPK (μkat/l)	7.52 ± 2.28	7.29 ± 3.47	5.26 ± 3.64	5.67 ± 3.24
Glu (mmol/l)	3.14 ± 1.46	4.20 ± 1.93	7.12 ± 2.85	12.6 ± 4.9 (**)
TG (mmol/l)	0.455 ± 0.163	0.343 ± 0.093	0.350 ± 0.060	0.460 ± 0.156

Table 5 – Serum biochemical analysis in dogs exposed to tenfold therapeutically doses of HI-6. Standard deviation calculated for $n = 5$. One or two asterisks indicate significance level at the probability level $P = 0.05$ or $P = 0.01$, respectively. Abbreviations: meaning same as in the previous table.

Time of exposure (min)	0	30	120	240
BUN (mmol/l)	3.44 ± 0.84	3.52 ± 0.86	3.64 ± 0.94	4.66 ± 0.99 (*)
CPK (μkat/l)	6.07 ± 3.31	7.72 ± 2.08	9.20 ± 1.10 (*)	9.59 ± 2.77 (*)
Glu (mmol/l)	4.58 ± 0.65	6.38 ± 2.00	11.2 ± 3.8 (**)	15.9 ± 5.5 (**)
TG (mmol/l)	0.332 ± 0.080	0.316 ± 0.070	0.448 ± 0.113	0.578 ± 0.180 (*)

urea nitrogen ($0.01 < P \leq 0.05$) and, second, glucose ($P \leq 0.01$) were elevated 240 min after HI-6 administration. The HI-6 administration in tenfold therapeutically dose triggered multiple shifts of biochemical markers. Blood urea nitrogen ($0.01 < P \leq 0.05$), creatine phosphokinase ($0.01 < P \leq 0.05$), glucose ($P \leq 0.01$) and triglycerides ($0.01 < P \leq 0.05$) were significantly elevated 240 min after HI-6 administration. Moreover, glucose ($P \leq 0.01$) and creatine phosphokinase ($0.01 < P \leq 0.05$) were already elevated 120 min after experiment starting. Albumin, alkaline phosphatase, alanine aminotransferase, amylase, aspartate aminotransferase, creatinine, glutathione S-transferase, high-density lipoprotein, inorganic phosphorus, total cholesterol and total plasma protein were steady without any significant changes.

4. Discussion

HI-6 was tested *in vitro* as well as *in vivo*. Two major findings were observed during the *in vitro* tests. First, HI-6 does not play a role of an antioxidant though it has an impact on endogenous antioxidants as proved by *in vivo* experiments. Second, HI-6 non-competitively inhibits AChE with medium association constant to the enzyme. However, the fact that HI-6 is preferably bound on AChE rather than free in the medium was quite surprising. On the other hand, reversible inhibition was clearly described in previous experiments, e.g., in the reference (Pohanka et al., 2010b). Moreover, oxime reactivators and its derivatives can interact with acetylcholine receptors due to structural similarities with acetylcholine (Fossier et al., 1990; Loke et al., 2002). Similar findings were clearly recognized for HI-6 on both mAChR and nAChR, too (Chen et al., 1996; Soukup et al., 2008). Pharmacokinetics and distribution through the blood brain barrier is another specific point of view in the HI-6 impact (Sakurada et al., 2003). The peak of HI-6 level was not focused in this work. The fastness of HI-6 elimination was in compliance with the expected velocity (Klimmek and Eyer, 1986). Similar conclusions were done on pigs (Nyberg et al., 1995) and dogs (Jovanovic et al., 1992). Human volunteers proved similar peak of HI-6 like proved in here presented experiments. In a clinical research, 24 male volunteers had average HI-6 maximum after 0.67 h (Clement et al., 1995). This time interval supports here found levels of HI-6 in plasma.

We recognized that the TBARS and FRAP levels were altered after HI-6 application. The increase of FRAP was more intensive in animals exposed to one therapeutic dose of HI-6. Concerning to it, TBARS was more increased in animals after exposure to ten therapeutic doses of HI-6. The logical explanation of this phenomenon is that HI-6 triggers oxidative stress. The therapeutic dose is low enough to be covered by low molecular weight antioxidants represented by FRAP. Oxidative stress after HI-6 ten therapeutic doses was not covered by antioxidant barrier enough. The shift of oxidative stress markers is not clearly understood. Some separate experiments were carried out and confirmed that oxidative stress could play a yet neglected role in the organophosphorous compound toxicity as well as antidotes application (Pohanka et al., 2009b). The TBARS level found in dogs exposed to HI-6 clearly correlates with the HI-6 residual level. The maximal TBARS value was in the same interval, i.e., 30 min, as the maximal HI-6 level in dogs exposed to an HI-6 therapeutic dose. The TBARS level became elevated for all time intervals after the

HI-6 application in the tenfold therapeutic dose. The HI-6 residual level remained quite high when applied in the higher dose. It appoints at the fact that HI-6 can contemporary trigger lipid oxidation processes. However, the stress is overcome immediately when HI-6 is excreted. The significantly elevated FRAP levels follow the TBARS in the next time interval. It can be explained as a self-protective mechanism suppressing the triggered oxidative stress. It is noteworthy to emphasize that dogs, in comparison with humans, are able to produce endogenous ascorbic acid (Linster and Van Schaftingen, 2007).

The biochemical examination provided unique data. We recognized shifts in levels of biochemical markers when compared the first and second groups. This phenomenon was caused by performance of experiment in two time intervals. Owing to the probable reason, the markers were altered by feeding and actual physical condition. On the other hand, the relative changes of assessed markers in the individual groups are reliable and not influenced when compared each to the other. Though the lower HI-6 dose was quite safe, changes in urea and glucose metabolism arose 240 min after the HI-6 application. This effect is quite surprising in view of the fact that the HI-6 residual level was low due to the elimination processes at this moment. Moreover, oxidative stress was counteracted. The metabolic deterioration was more extensive in dogs exposed to the higher HI-6 dose as glucose, urea and triglyceride levels were elevated. Creatine phosphokinase serum level responds to the muscular dystrophy and/or acute renal failure. On the other hand, aspartate aminotransferase level was unchanged. Pertinent renal dysfunctions should be examined in a separate experiment in the future. When renal functions deteriorate, alteration in the oxime reactivator pharmacokinetics can be expected in a similar way as proved for 2-pralidoxime by Kayouka et al. (2009a,b). Muscular irritation by HI-6 was described by Moore et al. (1991) in sheep. They also found differences in HI-6 and 2-pralidoxime impact in sheep and the data were confirmed by the histopathological examination. Muscular dystrophy initiated by HI-6 in combination with quite fast elimination can be considered as an adverse process limiting the HI-6 application for prophylactic purposes. On the other hand, there were no symptoms appointing at liver failure or hemolysis as common for e.g., nerve agents prophylactic pyridostigmine (Hamaoui and Mercado, 2009; Prozorovski et al., 1994). Good parameters of liver functional tests after the HI-6 application were positive and in strong compliance with the experiments carried out for 2-pralidoxime (Garcia et al., 2010).

5. Conclusions

The oxime reactivator HI-6 was tested *in vitro* as well as *in vivo*. It was found that the therapeutically dose is relatively safe; however, it can trigger mild oxidative stress and metabolic disorders. The tenfold therapeutically dose can induce oxidative stress and metabolic disorder in an extensive scale. The most significant HI-6 impact on body is hyperglycemia both in a therapeutically as well as a tenfold dose. The hyperglycemia remains even after HI-6 elimination.

Conflict of interest

None.

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