Huperzine induces alteration in oxidative balance and antioxidants in a guinea pig model

Miroslav Pohanka [1,2] Martina Hrabinova [1], Filip Zemek [1], Lucie Drtinova [1], Hana Bandouchova [3], Jiri Pikula [3]

1 Faculty of Military Health Sciences, University of Defence, Hradec Kralove, Czech Republic
2 University Hospital Hradec Kralove, Czech Republic
3 Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Czech Republic

Correspondence to: RNDr. Miroslav Pohanka, PhD.
Faculty of Military Health Sciences, University of Defence
Trebesska 1575, 50001 Hradec Kralove, Czech Republic.
TEL: +420973253091; E-MAIL: miroslav.pohanka@gmail.com

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Abstract

OBJECTIVES: Alzheimer's disease (AD) is a neurodegenerative disorder. Symptomatic treatment is available by inhibitors of acetylcholinesterase (AChE) such as rivastigmine, galantamine and donepezil. As huperzine is a promising compound for AD treatment, our study was aimed at evaluating its pertinent implications in oxidative stress.

METHODS: Laboratory guinea pigs were exposed to huperzine A at doses of 0, 5, 25, 125 and 625 μg/kg. The animals were observed for cognitive disorders and sacrificed one hour after exposure. Tonic-clonic seizures were noticed, but only in highly dosed animals. Ferric reducing antioxidant power (FRAP), thiobarbituric acid reactive substances (TBARS), glutathione reductase and glutathione S-transferase were assessed in frontal, temporal and parietal lobes, the cerebellum, liver, spleen and kidney.

RESULTS: Only minimal changes in enzymatic markers were recognized. Huperzine was not implicated in oxidative stress enhancement as the TBARS values remained quite stable. Surprisingly, antioxidants accumulated in the examined brain compartments as the FRAP value was significantly elevated following all doses of huperzine.

CONCLUSIONS: We discuss the potency of huperzine in enhancing the antioxidant capacity of the central nervous system. Huperzine is probably implicated in more processes than cholinesterase inhibition only.

Abbreviations:

AChE	- acetylcholinesterase
AD	- Alzheimer´s disease
BuChE	- butyrylcholinesterase
FRAP	- Ferric reducing antioxidant power
GR	- glutathione reductase
GST	- glutathione S-transferase
NMDA	- N-methyl-D-aspartate
TBARS	- thiobarbituric acid reactive substances

ORIGINAL ARTICLE

INTRODUCTION

Acetylcholinesterase (AChE) inhibition is among the therapeutic options for the symptomatic treatment of Alzheimer's disease (AD). Rivastigmine, galantamine and donepezil are all therapeutically used inhibitors of AChE (Bonner & Peskind 2002). Huperzine is a natural alkaloid from the Chinese lycopod *Huperzia serrata*. Huperzine is produced at quite a high level as *H. serrata* contains more than 0.025% (w/w) of huperzine (Bai 2007). Huperzine is a selective inhibitor of AChE, interacting with its peripheral anionic site. In contrast to AChE, the enzyme butyrylcholinesterase (BuChE) is not sensitive to inhibition by huperzine (Haigh *et al.* 2008). Two types of huperzine are known: huperzine A and B. Although both huperzine types can be used, huperzine A (Figure 1) is the most suitable for pharmacological purposes (Lim *et al.* 2010).

Huperzine is a well-tolerated drug that significantly improves the cognitive ability of AD patients, as indicated by the mini-mental state examination (Wang *et al.* 2009). It is also a potent antagonist of the N-methyl-D-aspartate (NMDA) receptor, and is able to control seizures and *status epilepticus* by blocking NMDA receptors (Coleman *et al.* 2008). Unfortunately, huperzine has been disregarded for a long time. It was mainly employed in Chinese medicine and its impact on basic physiological functions is not well documented (Desilets *et al.* 2009).

Although huperzine seems to be a perspective drug for the symptomatic treatment of AD, the available toxicological data are scarce. There is a tight link between AD and oxidative stress (Su *et al.* 2008). Huperzine has not yet been extensively investigated regarding oxidative stress and its antioxidant properties. Clinical searches confirmed good tolerability of huperzine, low toxicity, and low adverse effects when administered in therapeutic doses (Little *et al.* 2008). Huperzine is quite stable in the body. On the other hand, it can be degraded by monooxigenases as epoxidation and hydroxylation can be expected. E.g. Garcia *et al.* (2004) reported production of 13,14-epoxy huperzine.

Due to the scare data of link between oxidative stress and huperzine, we decided to investigate the influence of huperzine on oxidative stress and protective antioxidant barriers in multiple organs. The main aim of the experiment was to judge whether huperzine could enhance or weaken the protection of organs, including the central nervous system, against oxidative stress. We selected guinea pig model as these animals are not able to produce ascorbic acid like human beings (Kaplan *et al.* 2010). Owing to oxidative stress and antioxidant protection, the guinea pig model is well representative to humans. The experiment was aimed to research of pertinent huperzine toxic effect so a wide scale doses was chosen. The dosages started from the reported human therapeutic doses to the toxic doses where excitotoxicity can be expected.

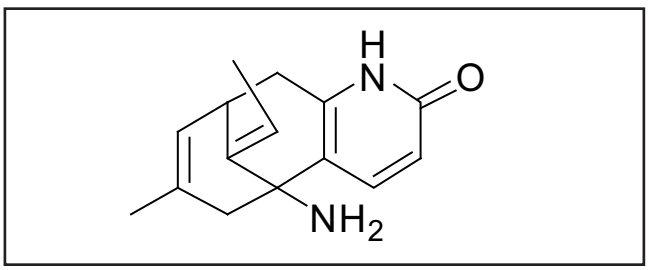

Fig. 1. Structure of huperzine A.

MATERIAL AND METHODS

Animal exposure

Guinea pigs (*Cavia porcellus*) were purchased from the Velaz Company (Prague, Czech Republic). They weighed 250±10 g and were three months old at the start of the experiment. The animals were kept under room temperature at 22±2 °C, humidity 50±10% and 12 hours- light per day. Food and water was 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. Huperzine A (Sigma-Aldrich, Saint Louis, Missouri, USA) was intramuscularly injected into the pelvic limb of the experimental animals (the animals were injected with a total of 100 μl of solution). The first group of animals, i.e. the control group, only received saline solution administered in the same way as huperzine. The second group received an amount of huperzine that approximately corresponded to the median reported therapeutic dose of 5 μg/kg (Myers *et al.* 2010). The next three groups received doses that were fivefold greater compared to each previous dose (i.e. 25, 125 and 625 μg/kg). The elevated dosage was chosen for estimation of adverse effects and initiate excitotoxicity caused by AChE inhibition. The animals were sacrificed by light CO_2 narcosis one hour after the start of the experiment.

Tissue processing and marker assessment

The frontal, temporal and parietal lobes, and the cerebellum, liver, spleen and kidney were collected from each animal. The tissues were homogenized using an Ultra-Turrax mill (Ika Werke, Staufen, Germany). A total of 100 mg of freshly collected tissue was mixed with 1 ml of saline solution (0.9 w/v of sodium chloride in deionized water) for one minute.

Ferric reducing antioxidant power (FRAP), glutathione reductase (GR), glutathione S-transferase (GST) and thiobarbituric acid reactive substances (TBARS) were assessed using previously published protocols (Pohanka *et al.* 2010; Pohanka *et al.* 2011a–c).

Descriptive and inferential statistics

All experimental data were processed using the mathematical software Origin 8 SR2 (OriginLab Corporation, Northampton, MA, USA). The significance of differences between individual groups was estimated by one-way ANOVA with Scheffe's test, considering both $0.01<p\leq0.05$ and $p\leq0.01$ probability levels for each group, which containing eight specimens.

RESULTS AND DISCUSSION

We used a wide scale of huperzine doses. The lowest doses respond to the reported therapeutic doses in human subjects (Zhang *et al.* 1991; Rafi *et al.* 2011). The upper doses were chosen for investigation of huperzine toxic effect, induction of excitotoxicity and the other oxidative stress related adverse effects (Milatovic *et al.* 2008). Administration of the high (625 μg/kg) dose of huperzine was accompanied by a symptomatic manifestation of cholinergic crisis in these animals. The animals who received the lower doses of huperzine and the controls showed no such manifestation. Fasciculation started quite early and culminated 30 minutes after exposure. After this, fasciculation changed into tonic-clonic seizures that lasted until the animals were sacrificed.

We recognized a strong potency of huperzine for enhancing the level of low molecular weight antioxidants, as demonstrated by the FRAP method (Table 1). The most extensive effect was recognized in the central nervous system, including the frontal, temporal and parietal lobes and the cerebellum, with significantly ($0.01<p\leq0.05$) increased FRAP values even after the huperzine dose of 5 μg/kg. The dose of 25 μg/kg caused a further increase of low molecular weight antioxidants that was double the amount found in the controls. The further increase of the huperzine dosage above 25 μg/kg had only a minimum effect on a further increase of low molecular weight antioxidants in the central nervous system. The liver, spleen and kidney were not extensively influenced by huperzine and a slight increase of low molecular weight antioxidants was only observed in animals who received the high doses of huperzine. The experimental data point to a potentially protective effect of huperzine in the brain via a pathway other than the inhibition of AChE or NMDA receptor antagonism. The observed effect was not caused by the pertinent antioxidant properties of huperzine as the molar antioxidant level of accumulated antioxidants exceeded the molar level of the applied huperzine. Moreover, the effect was selective for the central nervous system rather than peripheral organs and points to a disproportional effect mainly implicated in the brain. The fact that antioxidants were extensively produced by all of the brain compartments examined enhances the significance of the present experiment and is promising in terms of AD treatment.

The accumulation of antioxidants is not clearly understood. We found only a limited amount of data in the known scientific databases; however, the positive effect of huperzine on antioxidants has also been discussed by some other authors (Little *et al.* 2008; Rafi *et al.* 2011). Huperzine is suspected to be able to modulate the cholinergic anti-inflammatory pathway and thus to suppress the expression of inducible nitric oxide synthase, nuclear factor κB, cyclooxygenase 2 and other enzymes (Wang *et al.* 2007). This phenomenon can be also beneficial for antioxidant homeostasis.

The TBARS value indicated damage to membranes by reactive oxygen species and it responded to the molar level of the fragment of oxidized lipids, i.e. malondialdehyde. The level of TBARS is typically increased when oxidative stress is uncovered by antioxidants (Greggio *et al.* 2009). As seen in Table 2, there was no significant production of malondialdehyde, even in association with the high huperzine dose of 125 μg/kg. The temporal lobe was one exception with sensitivity to huperzine at the dose of 125 μg/kg. The present experimental data point to the harmlessness of huperzine. Pertinent oxidative stress can be expected at an overdose level many times above the recommended huperzine intake. The aetiology of malondialdehyde production in the animals exposed to 625 μg/kg huperzine could be based on seizures combined with contemporary hypoxia.

Tab. 1. Ferric reducing antioxidant power (FRAP) in tissue samples ± standard errors.

Huperzine dose	0 μg/kg	5 μg/kg	25 μg/kg	125 μg/kg	625 μg/kg
Frontal lobe (nmol/g)	278±47	348±27 (**)	491±47 (**)	555±19 (**)	523±32 (**)
Temporal lobe (nmol/g)	241±24	316±25	481±37 (**)	545±39 (**)	517±14 (**)
Parietal lobe (nmol/g)	250±32	360±43 (**)	489±30 (**)	541±12 (**)	505±16 (**)
Cerebellum (nmol/g)	301±28	364±66 (**)	509±30 (**)	592±38 (**)	461±36 (**)
Liver (μmol/g)	2.81±0.26	2.86±0.16	2.85±0.19	3.13±0.25 (**)	3.26±0.24 (**)
Spleen (μmol/g)	3.39±0.79	3.71±0.25	3.33±0.23	3.54±0.29	3.85±0.24 (*)
Kidney (μmol/g)	1.47±0.15	1.68±0.11	1.65±0.24	1.60±0.16	1.79±0.21 (*)

* $p<0.05$, ** $p<0.01$, n=8 specimens in each group

These findings correspond with the facts known from multiple toxicological experiments (Greggio *et al.* 2009; Gasparova *et al.* 2010). It should be emphasized that toxic malondialdehyde is only detoxified by low molecular weight antioixidants (Agarwal *et al.* 2010). The pro-oxidative effects of huperzine were reduced due to its abovementioned ability to accumulate antioxidants in guinea pig organs.

Glutathione reductase is an enzyme that reduces previously oxidized glutathione (Rahaman *et al.* 1999). It is expressed at a high level in response to several causes; however, the accumulation of oxidized glutathione with the requirement for its reduction is the main cause. In brief, the higher activity of GR in tissues corresponds to oxidative stress and it is a hallmark of the protective effect of the glutathione system against the applied drug (Limon-Pacheco & Gonsebalt 2011). Nevertheless, the data should be compared with FRAP and TBARS values as the activity of GR alone does not reveal whether or not oxidative stress was covered by the antioxidant mechanisms. The activities of GR in the selected tissues are summarized in Table 3. We found different impacts of huperzine on the examined tissues. The most extensive expressions of GR were observed in the frontal and parietal lobes (significant at $p \leq 0.01$) for the doses of 5–125 μg/kg. Surprisingly, GR was decreased ($p \leq 0.01$) in the frontal, temporal and parietal lobes after the huperzine dose of 625 μg/kg. The

Tab. 2. Thiobarbituric acid reactive substances (TBARS) in tissue samples ± standard errors.

Huperzine dose	0 μg/kg	5 μg/kg	25 μg/kg	125 μg/kg	625 μg/kg
Frontal lobe (nmol/g)	263±35	262±25	279±35	274±28	324±25 (**)
Temporal lobe (nmol/g)	267±31	282±25	299±34	308±22 (**)	353±42 (**)
Parietal lobe (nmol/g)	228±32	243±10	246±31	266±22	292±19 (**)
Cerebellum (nmol/g)	298±35	287±17	311±35	327±26	355±31 (**)
Liver (μmol/g)	231±20	229±21	241±22	218±1	246±19
Spleen (μmol/g)	339±32	387±21	380±26	379±37	361±34
Kidney (μmol/g)	237±27	234±21	254±31	264±26	271±16 (**)

** $p<0.01$, n=8 specimens in each group

Tab. 3. Glutathione reductase (GR) in tissue samples ± standard errors.

Huperzine dose	0 μg/kg	5 μg/kg	25 μg/kg	125 μg/kg	625 μg/kg
Frontal lobe (nmol/g)	33.2±4.0	40.5±3.0 (**)	38.9±2.7 (**)	39.9±3.0 (**)	29.7±2.8 (**)
Temporal lobe (nmol/g)	36.1±2.9	41.6±2.7	35.4±2.4	34.6±1.7	29.9±2.3 (**)
Parietal lobe (nmol/g)	30.9±3.2	42.7±2.9 (**)	37.3±3.5 (**)	35.8±2.2 (**)	26.6±2.5 (**)
Cerebellum (nmol/g)	32.9±4.6	39.5±3.6 (*)	33.5±2.7	36.7±3.3	34.9±3.7
Liver (μmol/g)	93.1 ±9.7	97.9±5.8 (*)	96.3±4.6	95.6±7.8	94.1±3.4
Spleen (μmol/g)	60.1±7.2	46.4±6.4 (**)	50.8±6.1 (**)	48.2±5.3 (**)	46.4±5.2 (**)
Kidney (μmol/g)	56.9±3.7	53.8±3.4	64.8±4.3 (*)	60.8±4.3	59.8±3.1

* $p<0.05$, ** $p<0.01$, n=8 specimens in each group

Tab. 4. Glutathione S-transferase (GST) in tissue samples ± standard errors.

Huperzine dose	0 μg/kg	5 μg/kg	25 μg/kg	125 μg/kg	625 μg/kg
Frontal lobe (nmol/g)	13.7±1.7	14.7±2.6	15.7±1.8	17.4±1.9 (**)	23.6±3.5 (**)
Temporal lobe (nmol/g)	17.2±1.4	20.0±2.2	20.3±2.3	22.8±3.4 (*)	26.3±4.0 (**)
Parietal lobe (nmol/g)	23.3±3.0	25.7±2.7	22.2±2.4	25.8±2.6	33.2±4.0 (**)
Cerebellum (nmol/g)	39.1±1.9	36.4±4.8	37.2±3.4	37.1±3.1	37.7±5.1
Liver (μmol/g)	520±20	499±32	548±35	539±27	541±30
Spleen (μmol/g)	23.1±3.7	21.8±2.5	21.2±2.2	19.3±2.4	20.1±2.1
Kidney (μmol/g)	288±33	278±22	310±34	334±54	304±26

* $p<0.05$, ** $p<0.01$, n=8 specimens in each group

GR levels in the cerebellum, liver and kidney were not extensively altered; however, the spleen showed significantly ($p \leq 0.01$) down-regulated levels of GR due to all tested doses of huperzine. The data obtained correspond with the unequal impact of huperzine on different organs. When considering TBARS, FRAP and GR, it can be concluded that huperzine acts as a weak pro-stress agent that triggers potent antioxidant protection.

Glutathione S-transferase was the last biomarker to be assessed. The experimental data are clearly summarized in Table 4. Glutathione S-transferase was only expressed at significant levels due to huperzine exposure in the central nervous system. The peripheral organs (liver, spleen and kidney) showed stable levels of GST. On the other hand, the activity of GST in the peripheral organs was higher than in the central nervous system. Glutathione S-transferase increased activity in the frontal and temporal lobes after the application of both 125 and 625 μg/kg huperzine. The parietal lobe had a lower sensitivity than the frontal or temporal lobes as GST was increased after the huperzine dose of 625 μg/kg. No effect of huperzine was recognized in cerebellum. Glutathione S-transferase should be considered not only as an enzyme that participates in protection from oxidative stress-induced pathology, but also as a protein that plays an important role in detoxification processes (Alabarse *et al.* 2011; Hoque *et al.* 2010). Its action is necessary for the maintenance of homeostasis; GST dysfunction can be fatal in many circumstances, including the increased incidence of cancer (Kiran *et al.* 2010). In accordance with this, a higher sensitivity of frontal and temporal lobes to huperzine should be expected. On the other hand, the induction of GST expression can be beneficial in AD patients for preserving the functions of the central nervous system.

It can be concluded that huperzine does not initiate oxidative stress at doses hundreds of times higher than therapeutic doses. On the contrary, huperzine is able to initiate the accumulation of low molecular weight antioxidants in the central nervous system. The described effect points towards a beneficial pathway in AD treatment other than the inhibition of AChE combined with enhancement of the cholinergic system. Though the molecular mechanism of the effect of huperzine is not clearly understood, its pro-antioxidant effect seems to be plausible as it was recognized in several brain compartments and showed a dose-response characteristic.

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