Early brain magnetic resonance imaging can predict short and long-term outcomes after organophosphate poisoning in a rat model

Shai Shrot a,b,1,*, Maya Tauber c,1, Arthur Shiyovich a, Nadav Milk a, Yossi Rosman a, Arik Eisenkraft a,d,e, Tamar Kadar f, Michael Kassirer a, Yoram Cohen c

a Medical Corps HQ, IDF, P.O. Box – 02149, Tel-Hashomer Base, Ramat-Gan, Israel
b Department of Diagnostic Imaging, Tel Hashomer, Affiliated to Sackler Faculty of Medicine, Tel-Aviv University, Tel Aviv, Address: 2 Sheba Rd, Ramat-Gan, 52621, Israel
c School of Chemistry, The Raymond and Beverly Sackler Faculty of Exact Sciences, Tel Aviv University, Ramat Aviv, Tel Aviv 69978, Israel
d NBC Protection Division, Israel Ministry of Defense, Hakiria, Tel-Aviv 69699, Israel
e Institute for Research in Military Medicine, The Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 91120, Israel
f The Israel Institute for Biological Research, P.O. Box 19, Ness Ziona 74100, Israel

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A B S T R A C T

Introduction: Magnetic resonance (MR) imaging is a sensitive modality for demonstrating in vivo alterations in brain structure and function after acute organophosphate (OP) poisoning. The goals of this study were to explore early imaging findings in organophosphate-poisoned animals, to assess the efficacy of centrally acting antidotes and to find whether early MR findings can predict post-poisoning cognitive dysfunction.

Methods: Sprague–Dawley rats were poisoned with the agricultural OP paraoxon and were treated with immediate atropine and obidoxime (ATOX) to reduce acute mortality caused by peripheral inhibition of acetylcholinesterase. Animals were randomly divided into three groups based on the protocol of centrally acting antitodal treatment: group 1 – no central antitodal treatment (n = 10); group 2 – treated with midazolam (MID) at 30 min after poisoning (n = 9); group 3 – treated with a combination of MID and scopolamine (SCOP) at 30 min after poisoning (n = 9) and controls (n = 6). Each animal had a brain MR examination 3 and 24 h after poisoning. Each MR examination included the acquisition of a T2 map and a single-voxel 1H MR spectroscopy localized on the thalamus, to measure total creatine [Cr], N-acetyl-aspartate [NAA] and cholines [Cho] levels. Eleven days after poisoning each animal underwent a Morris water maze to assess hippocampal learning. Eighteen days after poisoning, animals were euthanized, and their brains were dissected, fixed and processed for histology.

Results: All paraoxon poisoned animals developed generalized convulsions, starting within a few minutes following paraoxon injection. Brain edema was maximal on MR imaging 3 h after poisoning. Both MID and MID + SCOP prevented most of the cortical edema, with equivalent efficacy. Brain metabolic dysfunction, manifested as decreased NAA/Cr, appeared in all poisoned animals as early as 3 h after exposure (1.1 ± 0.07 and 1.42 ± 0.05 in ATOX and control groups, respectively) and remained lower compared to non-poisoned animals even 24 h after poisoning. MID and MID + SCOP prevented much of the 3 h NAA/Cr decrease (1.22 ± 0.05 and 1.32 ± 0.1, respectively). Significant correlations were found between imaging findings (brain edema and spectroscopic changes) and clinical outcomes (poor learning, weight loss and pathological score) with correlation coefficients of 0.4–0.75 (p < 0.05).

Conclusions: MR imaging is a sensitive modality to explore organophosphate-induced brain damage. Delayed treatment with midazolam with or without scopolamine provides only transient neuroprotection with some advantage in adding scopolamine. Early imaging findings were found to correlate with

Abbreviations: ADC, apparent diffusion coefficient; OP, organophosphate; ACh, acetylcholine; NAA, N-acetyl aspartate; SE, status epilepticus; BZD, benzodiazepines; MRS, magnetic resonance spectroscopy; MRI, magnetic resonance imaging; DWI, diffusion weighted MR imaging; MWM, Morris water-maze; CNS, central nervous system; SPM, statistical parametric mapping; Cr, creatine; Cho, choline; IM, intramuscular; MID, midazolam; SCOP, scopolamine; ATOX, Atropine and toxogonin (obidoxime); PRESS, Point Resolved Spectroscopy.

* Corresponding author at: 2 Sheba Road, Ramat Gan 52621, Israel. Tel.: +972 3 5302530; fax: +972 3 5357315.
E-mail address: shashrot@gmail.com (S. Shrot).

1 These authors contributed equally to this work.

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0161-813X © 2015 Elsevier Inc. All rights reserved.
1. Introduction

Organophosphates (OPs) are toxic compounds commonly used in agriculture, but can be used in the battlefield as well. In parts of the developing world, pesticide poisoning, mainly of OPs, causes more deaths than infectious diseases (Eddleston et al., 2002). The warfare related OPs, such as sarin, soman and VX, also known as nerve agents, are extremely toxic and are considered to be among the deadliest chemical warfare agents. Recent use of the nerve agent sarin on civilians in Syria indicates their potential threat (Rosman et al., 2014). Acetylcholine (ACh) accumulation in cholinergic synapses, due to irreversible inhibition of the ACh-degrading enzyme (acetylcholinesterase), is thought to be their main mechanism of toxicity. Clinically, respiratory failure is the main cause of death in severe OP poisoning and is attributed to a combination of hypersecretion, bronchoconstriction, respiratory muscle paralysis and damage to the medullary respiratory centers (Eddleston et al., 2006). Central nervous system (CNS) effects of OPs include nonspecific symptoms, such as irritability, restlessness, disorientation and confusion, which can evolve into generalized seizures and statues epileptics (SE) (Marrs et al., 2007). It has been shown that SE may lead to significant brain injury, mainly in the cortex, hippocampus, amygdala and in the thalamus (Britt et al., 2000; Kadar et al., 1995; McDonough and Shih, 1997). It was shown that following seizure initiation there are prominent pathological changes in brain tissue including pronounced perineuronal and perivascular edema as well as swelling of astrocytes (McDonough and Shih, 1997; Petras, 1994). T2-weighted magnetic resonance imaging (MRI) and diffusion-weighted MRI (DWI) are particularly sensitive to recognize areas of cerebral edema (Brant-Zawadzki et al., 1987; Moseley et al., 1990). T2 prolongation and reduced apparent diffusion coefficient, ADC, were found in brain tissue of rats exposed to the OP soman. Similarly to pathological studies, OP induced cerebral edema was prominent in the hippocampus and in the temporal and piriform cortices (Bhagat et al., 2001, 2005; Carpentier et al., 2008; Testylier et al., 2007). Magnetic resonance spectroscopy (MRS) enables the measurement of levels of various metabolites in vivo. The main metabolites which can be measured by 1H MRS are the total choline which represent different choline-containing compounds (used to make cell membranes), total creatine, which represent creatine and phosphor-creatine (chemicals involved in energy metabolism) and N-acetyl aspartate (NAA). NAA is thought to be present predominantly in neuronal cells and is considered to be a neuronal marker. Indeed, most neurological disorders involving neuronal loss or dysfunction result in reductions in brain NAA levels (Burscher and Holtas, 2001). The ratios of peak integrals of metabolites are commonly used to analyze changes in 1H MR spectroscopy in clinical setting (de Seze et al., 2010). In many cases NAA/Cr ratios are computed when studying neuronal disorders and insulin in which it is believed that the changes in total creatine are marginal. Recently, we have shown that there is significant early and long lasting spectroscopic evidence of neuronal loss or dysfunction, i.e. decreased NAA/Cr ratios, following acute high dose poisoning with the OP paraoxon which continue long after acute brain edema has resolved (Shrot et al., 2012).

In case of poisoning, immediate treatment with an anticholinergic drug, such as atropine, antagonizes the cholinergic hyper-activation at muscarinic receptor sites, and oximes, such as obidoxime or pralidoxime, are used to reanimate inhibited enzyme (Marrs et al., 2007). Although increasing survival, the combination of atropine and oxime is not sufficient to prevent major brain injury following OP poisoning, especially if it is delayed (McDonough and Shih, 1997). Interestingly, adding the muscarinic antagonist scopolamine, which is far more potent in the CNS compared to atropine, to the common treatment protocol (Ketchum et al., 1973), does not provide any neuroprotection compared with the “conventional” treatment of atropine and oxime alone (Weissman and Raveh, 2008). Extensive research has revealed a complex picture consisting of an involvement of several neurotransmitter systems, following an early cholinergic crisis, in which the glutamatergic system plays a pivotal role (McDonough and Shih, 1997). If the initial cholinergic crisis is not stopped, the levels of glutamate and other excitatory amino acid increase reinforcing the seizure activity and neuropathology ensues. It is assumed that most of the neuronal damage can be avoided if seizures are controlled promptly. Benzodiazepines (BZDs), potent GABA<sub>α</sub> receptor agonists, are highly effective in stopping OP-induced SE when administered early after SE initiation (McDonough and Shih, 1997). However, delayed BZD treatment impedes SE only temporarily and prevents brain damage only partially (de Araujo et al., 2012; Gilat et al., 2005).

Spatial learning and memory impairments are well known consequences of OP intoxication, with ensuing neuronal degeneration in CNS structures (e.g. hippocampus). Indeed, cognitive incapacitation, such as impaired learning and memory resulting from exposure to OPs, has been reported in humans and animals (Brown and Brix, 1998). The Morris water maze test is known for its sensitivity to hippocampal damage (Brandeis et al., 1989).

In this work, we used clinical and imaging parameters (T2-weighted imaging and single voxel 1H MRS) to evaluate the efficacy of delayed treatment with midazolam and scopolamine, compared with midazolam alone, in attenuating paraoxon-induced brain damage and preventing late behavioral deficits. As acting through different mechanisms, we assumed that adding scopolamine to delayed BNZ treatment will increase its attenuated efficacy. We have also looked for a correlation between early MR findings and clinical outcomes.

2. Materials and methods

2.1. Animals

The experimental protocol was examined and approved by the Tel-Aviv University institutional committee for animal experimentation as required by local law (approval number L-12-051). Adult (8 weeks old) male albino Sprague–Dawley rats (Harlan-biotech, Jerusalem, Israel), weighing 290–335 g at the beginning of the experiment, were housed under standard laboratory conditions in plastic cages, two per cage in a controlled environment with constant temperature of 21 ± 2 °C and a 12 h light/dark cycle. Food and water were available ad libitum. Care and maintenance were in accordance with the principles described in the “guide for care and use of laboratory animals” (NIH publication 85-23, 1985).

2.2. Drugs

Paraoxon, atropine sulfate, obidoxime (toxogonin®), midazolam and scopolamine were purchased from Sigma–Aldrich, Jerusalem, Israel. Paraoxon was diluted in a vehicle containing...
40% propylene glycol (Sigma–Aldrich, Jerusalem, Israel). All other drugs were diluted in normal saline.

Anesthesia was induced with ~3% isoflurane (Vetmarket Ltd., Petah Tikva, Israel) and maintained with 1–2% isoflurane in 95% O2 at a flow rate of 0.3–0.5 l/h. Respiratory rate was monitored throughout the entire MR examination and was maintained between 40–60 breaths/min. Body temperature was maintained by a feedback system of circulating water at 38 °C.

2.3. Study design

This study included 42 rats divided into 5 groups: 3 test groups, a control group and a baseline group. The 3 test groups were exposed to 1.4 LD50 of paraaxon (450 μg/kg, IM). One minute after poisoning, all rats were treated with ATOX (Atropine [3 mg/kg, IM] and obidoxime [TOXogonin°, 50 mg/kg, IM]) in order to lower mortality but enabling brain damage. The first test group (ATOX group; 10 rats) was treated only with ATOX and was not treated with any additional neuroprotective treatment. The second test group (ATOX + MID group; 9 rats) was treated in addition to ATOX with midazolam (1 mg/kg) 30 min after paraaxon poisoning. The third test group (ATOX + MID + SCOP group; 9 rats) was treated on top of ATOX with midazolam (1 mg/kg) and with scopolamine (0.1 mg/kg) 30 min after paraaxon poisoning. The control group (6 rats) was not poisoned with paraaxon and was treated only with ATOX as described above. The baseline group (8 rats) was neither poisoned nor treated with antidotes and was used to compare the imaging and spectroscopy parameters of the different tests and control groups with the baseline parameters of untreated rats. Due to technical reasons, there were four batches of poisoning, each included representatives of all 3 test groups and control group (7–9 animals each). Study timeline is shown in Fig. 1. Each animal in the test and control groups was anesthetized and had MR examination of its brain 3 and 24 h after paraaxon poisoning. Eleven days post-exposure or treatment, each rat in the tests and control groups was submitted to a Morris water maze (MWM) task, which was conducted during 5 days and included 5 sequential learning trials and a probe trial. Each animal in the ‘baseline group’ had only one MR examination. Summary of the experiment’s groups is shown in Table 1.

2.4. Clinical assessment

Animals were observed continuously for at least one hour after poisoning and scored for motor manifestations of seizure activity on 5, 30 and 60 min. Scores were assigned as follows: (0) no convulsions, (1) chewing and facial clonus, (2) tremor and focal convulsions, and (3) tonic–clonic generalized convulsions. Repeated weighing once per day was initiated prior to paraaxon poisoning and continued for 10 days post-exposure.

2.5. MR imaging – acquisition

MRI experiments were performed on a 7 T/30 cm horizontal bore Bruker BioSpec (Bruker, Ettlingen, Germany) MRI scanner equipped with a BGU20 gradient system capable of producing pulse gradients of 40 G cm−1 in each of the three dimensions (x, y and z). A body coil was used as the transmit coil and a 15 mm quadrature coil (Bruker, Ettlingen, Germany) dedicated for the rat brain was used as the receiving coil. The MRI experiments consisted of T2 maps and single-voxel localized 1H MR spectroscopy (MRS).

The T2 maps were collected with the following parameters: Twelve continuous 1.35 mm coronal slices were acquired with a field of view (FOV) of 2.56 cm × 2.56 cm and 256 × 128 digital resolution reconstructed to a 256 × 256 matrix, resulting in an in-plane resolution of 100 × 100 [μm]2. The number of echoes was set to 16 with TR/TE of 3000/10–160 ms and two averages. The total imaging time was ~13 min for each rat.

The single-voxel 1H MRS study was performed by water suppressed PRESS sequence with TR/TE of 2000/135 ms, voxel size of 4 × 4 × 4 mm3, 256 scans, TD = 4096 and spectral width of 4 kHz. The total acquisition time was 8 min and 32 s. The voxel was located along three slices, and it mostly consisted of the thalamus in both hemispheres (Fig. 2). VAPOR water suppression scheme was used, and the voxel of interest was shimming selectively by a volume selective PRESS sequence. In the PRESS sequence, the echo delay was set so that the lactate peak will appear in antiphase.

2.6. Morris water maze (MWM)

Eleven days post poisoning, spatial learning and memory were evaluated by MWM. The MWM was based on previous described protocols (Raveh et al., 2002; Vorhees and Williams, 2006). Briefly, the maze consists of a 1.2-m diameter pool filled with water (22 °C), which was made opaque by the addition of powdered milk, with an escape platform mounted 2 cm below the surface of the water. Several distal visual cues were placed on the walls of the water maze room. Each rat was trained for 5 days, four trials (one block per day), in which the platform position remained constant and was located in the center of the south–east quadrant of the pool. Within each block of four trials, each rat started at each of the starting locations, but the sequence of locations was randomly selected. Each trial consisted of placing a rat by hand into the water facing the wall of the pool at one of four starting locations, i.e. north, south, east or west, around the pool’s perimeter. On the first day, prior to training, the rat was placed on the platform for 60 s. If, on a particular trial, a rat found the platform, it was permitted to remain on it for 15 s. A trial was terminated after 120 s if a rat failed to find the platform, and the rat was placed on the platform for additional 15 s before starting the next trial. Escape latency (the time to find the platform) was recorded on each trial. For each animal, daily average escape latency time was calculated from the four trials of each block. For each animal, overall decrease in latency time (%), average decrease in latency time per day (%) and average improvement in latency time compared with initial latency time (%) were also calculated. A probe test was carried out 3 h after the last trial. For the probe test, all rats were assigned to the same, new starting position. During the probe test, the platform was removed from the pool and the rats were allowed to swim for 30 s. The path of each rat in the pool was recorded. The percentage of time and path the animal spent in each quadrant of the pool were calculated.

**Fig. 1.** A timeline of the experimental design.
2.7. Histological evaluation

Eighteen days following poisoning, four rats from each group were sacrificed by intracardiac perfusion with ice cold PBS for 5 min, followed by 15 min of 4% paraformaldehyde. Brains were removed and immersed in 4% paraformaldehyde for 24 h and cryoprotected in 30% sucrose PBS for another 48 h. Later, the brains were incubated and stored in PBS solution of 0.01% sodium azide (in 4°C).

Coronal sections, 7-μm-thick, were cut serially at the levels of the striatum and the hippocampus and selected sections were stained with hematoxylin and eosin (H&E) for light microscopy examination. Brain damage was semiquantitatively analyzed according to a scoring scale ranging from 0 (intact brain) to 5 (severe damage in the typical vulnerable brain regions, i.e. hippocampus, piriform cortex, fronto-parietal cortex, thalamus) as previously described (Shrot et al., 2012).

2.8. Data analysis

Weight changes were analyzed by a two-way ANOVA for repeated measurements. Testing for homogeneity of variance was conducted with non-parametric Levene’s test. Generally, due to small samples in our study, non-normality was assumed and the unequal variance t-test was performed on ranked data for comparison analysis (as described by Ruxton (2006)). MWM parameters were analyzed using a two-way repeated measures analysis of variance (ANOVA) test which included two independent variables (day and group in the case of the learning trial, platform and group in the case of the probe trial) and one dependent variable (escape latency in the case of the learning trial, percentage of time/path spent in each quarter in the case of the probe trial). In addition, non-parametric Kruskal–Wallis one-way analysis of variance was performed on the learning trial since the escape latencies in the different groups did not have equal variances.

Pearson’s correlation test was used for evaluation of the correlation between imaging and clinical parameters. Generally, significance was defined as p < 0.05.

2.9. MR analysis

2.9.1. T2-maps analysis

After the MRI protocol was completed, the data was analyzed by using both an in-house Matlab program tool (www.cs.tau.ac.il/~ofertas/Fistuk) and the statistical parameter mapping (SPM2) program to compare the different groups in the study. First, all images were rotated and cropped (with the T2 map of one representative baseline brain as a template) using the Fistuk tool. Later, all images were normalized with SPM program. After these procedures, we used the voxel-based analysis procedure to compare the different groups in the study at each time point, and in the two time points within each group. Regions that expressed a statistical difference (p < 0.005) after a one-way ANOVA test between the groups were highlighted on the T2 map template.

2.9.2. T2 region of interest analysis (ROI)

T2 maps were transferred onto an external computer for data processing. A representative axial slice at the level of the hippocampus and thalamus was selected to be analyzed for T2 values. Six ROIs were selected in the left hemisphere and drawn manually on the axial slice. The ROIs corresponded to fronto-parietal cortex(1), hippocampus(2), thalamus(3), parieto-temporal cortex(4), piriform cortex(5), and hypothalamus(6) (see Fig. 2 for the definition of the ROIs). ROI values were analyzed using Matlab software. The T2 ROI’s values were used for the correlation analysis.

2.9.3. Single voxel 1H MRS analysis

The MRS spectra were zero filled to 8k, multiplied by a line broadening factor of 10 Hz, then phase-corrected. The spectra were imported to Mestre-C software. The NAA, total creatine (Cr, which in fact represents creatine and phosphocreatine) and cholines (Cho) peaks were then fitted to a Lorentzian function, and the area under each peak was generated from the fitted function. The NAA/Cr, NAA/Cho and Cho/Cr ratios of the different groups in the study in each time point and of the two time points within each group were compared using unequal variance t-test.

<table>
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<th>30 min treatment</th>
<th>Imaging</th>
<th>Behavioral assessment</th>
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<td>–</td>
<td>–</td>
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<td>10</td>
<td>+</td>
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<td>3 and 24h</td>
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<tr>
<td>ATOX + MID</td>
<td>9</td>
<td>+</td>
<td>IM MID (1 mg/kg)</td>
<td>3 and 24h</td>
<td>+</td>
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<tr>
<td>ATOX + MID + SCOP</td>
<td>9</td>
<td>+</td>
<td>IM MID (1 mg/kg) + IM SCOP (0.1 mg/kg)</td>
<td>3 and 24h</td>
<td>+</td>
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<tr>
<td>Control</td>
<td>6</td>
<td>–</td>
<td>–</td>
<td>3 and 24h</td>
<td>+</td>
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Fig. 2. MR image of a rat brain showing the different ROIs used for analysis. The red square represents the voxel used in the single-voxel 1H MRS experiment. Different ROIs used for T2 analysis are also shown in yellow ellipses: (1) fronto-parietal cortex, (2) hippocampus, (3) thalamus, (4) parieto-temporal cortex, (5) piriform cortex, and (6) hypothalamus. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3. Results

3.1. Clinical assessment

All OP-poisoned animals developed generalized tonic–clonic convulsions, starting within 1–2 min following paraoxon injection. Only animals not treated with central acting antidotes (MID with or without SCOP) showed significant convulsive activity 60 min post paraoxon poisoning. Mean clinical scores are presented in Fig. 3A. Although a significant transient decrease in weight was found in all paraoxon poisoned animals compared to control animals (unequal variance t-test, \( p < 0.05 \)), no significant difference was found between the different therapy groups (Fig. 3B). On MWM, all experimental groups had significant learning through the 5 days test, with no significant difference found between the test groups and the control group in escape latency time (Fig. 4) and in percentage of time or path in probe test (data not shown).

3.2. Imaging results

3.2.1. T2 maps

When comparing T2 measurements in different ROIs, poisoned animals showed increased values 3 h post poisoning mainly in the thalami and cortical areas (mean increase of 2–5%, \( p < 0.05 \)). The effect of MID and MID + SCOP on T2 prolongation was complex. For example, Fig. 5 demonstrates ROI analysis of the thalami and the parieto-temporal areas. Three hours post exposure, a significant increase in T2 values was found in both thalami and parieto-temporal cortices (in ATOX group). Much of the edema subsided 24 h post exposure in the thalami but persisted to some extent in the parieto-temporal cortices. In the thalami, addition of MID with or without SCOP did not prevent 3 h T2 prolongation, whereas it significantly attenuated the T2 prolongation in parieto-temporal cortices. At 24 h post exposure, T2 values of the both ATOX + MID and ATOX + MID + SCOP groups were not different from the T2 values of the control or baseline groups.

Fig. 3. (A) Clinical scores of animals in different experimental groups in the first hour after paraoxon poisoning. No convulsions were seen in non-poisoned animals (Control, purple bars). Data is shown as mean score and standard deviation. \( * p < 0.05 \), \( ** p < 0.005 \) (unequal variance t-test), when the comparison is relative to the bar in the same time-point whose color is identical to the color of the asterisk. (B) The effect of different treatment protocols on weights of rats exposed to paraoxon. No statistical difference was found between different weight curves (one-way ANOVA with repeated measures). On day 1, significant weight loss was found in paraoxon poisoned animals compared with control (\( p < 0.05 \) ATOX, ATOX + MID, ATOX + MID + SCOP compared with control animals, unequal variance t-test).

Fig. 4. Escape latency (mean ± standard error) measured 8 days after treatment with paraoxon and various antidotal treatments, compared to control rats as described in Section 2. No difference found between poisoned groups compared to control (two-way repeated measures analysis of variance (ANOVA) test.)
Voxel-based analysis of the T2 values in the different time points after intoxication compared to baseline levels found significantly elevated T2 signal in cortical and subcortical gray matter early as 3 h post poisoning in the ATOX group (Fig. 6A[II]). The cortical edema became more diffuse 24 h post poisoning, whereas thalamic edema decreased (Fig. 6B[II]). The addition of MID or MID + SCOP 30 min post-poisoning decreased the 3-h cortical and the thalamic edema (Fig. 6A[II] and A[III]) and nearly totally eliminated brain edema 24 h post-poisoning (Fig. 6B[II] and B[III]). On the voxel-based analysis, only the ATOX group showed significant brain edema 24 h post poisoning and no significant differences in the 24-h cerebral edema were found between ATOX + MID compared ATOX + MID + SCOP groups.

3.2.2. Spectroscopy analysis

Fig. 7A and 7B shows the NAA/Cr and the NAA/Cho levels 3 and 24 h post poisoning for all groups. We found a significant early 3 h post poisoning decrease in NAA/Cr and in NAA/Cho levels in the ATOX group compared with baseline levels. The addition of MID with or without SCOP attenuated 3 h NAA/Cr decrease. However, 24 h NAA/Cr ratios were significantly low in all paraoxon treated groups compared with control or baseline groups. For the NAA/Cho levels, we found that 3 h post-poisoning, both the ATOX + MID and ATOX + MID + SCOP groups were comparable to the baseline and showed higher values than that of the ATOX group. Interestingly, at 24 h post treatment, these NAA/Cho ratios decrease and no difference was found between ATOX + MID or ATOX + MID + SCOP groups compared with ATOX alone group. Twenty-four-hour Cho/Cr ratios significantly increased in all paraoxon-treated groups compared with 3-h measurements, with no significant difference between the different treatment groups and the control group (data not shown).

3.3. Histology

Brain lesions with varying degrees of severity were noted in all the paraoxon treated groups. The most severe changes appeared in the ATOX group including vacuolar degeneration in the piriform cortex, as well as acidophilic cells in the hippocampus and in the fronto-parietal cortex (mean score of 2.75). Calcification and gliosis were observed in the dorso-lateral thalamic nuclei in one animal out of four (Fig. 8). Milder changes were found in ATOX + MID (mean score of 2) and ATOX + MID + SCOP (mean score of 1.25) groups.

3.4. Clinical–pathological and imaging correlations

3.4.1. Early weight loss

The cerebral edema at 3-h post exposure in the thalamus, piriform and parietal-temporal cortexes were found to be in a
significant correlation with 24-h weight loss (correlation coefficients of 0.63, 0.57 and 0.49, respectively, \(p < 0.005\), Pearson’s correlation test). Moreover, 24-h spectroscopic values were found to be in a significant strong negative correlation with the 24-h weight loss (NAA/Cr, Cho/Cr and NAA/Cho ratios with correlation coefficients of \(-0.71\), \(-0.54\), \(-0.5\), respectively, \(p < 0.005\), Pearson’s correlation test). Representative scatterplots are presented in Fig. 9.

### 3.4.2. Cognitive decline (MWM)

Although no group effect was found in the MWM, individual animals in various treatment groups had significant deficits with significant decreased scores in the MWM parameters. The cerebral edema 24 h after exposure in parieto-temporal and piriform cortices were found to be in a significant negative correlation with relative latency time decrease in the MWM (Pearson's correlation coefficients of \(-0.47\), \(p = 0.005\) and \(-0.42\), \(p = 0.014\), respectively).

The edema 3 h after exposure in the thalamus and parieto-temporal cortices were found also in a significant negative correlation with 5th day relative time and path in expected quarter of the pool (correlation coefficients of \(0.36\)–\(0.39\), \(p = 0.03\)–\(0.04\)).

### 3.4.3. Imaging-pathology correlation

Overall pathological score was found to be in a significant positive correlation with 3- and 24-h cerebral edema (3 h edema in the piriform \([r = 0.76, p < 0.005]\), hypothalamus \([r = 0.63, p < 0.05]\), thalamus \([r = 0.62, p < 0.05]\); and 24 h edema in the piriform cortex \([r = 0.77, p < 0.005]\)). Moreover, there was a significant correlation between spectroscopic ratios and the

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**Fig. 6.** Results from VBA preformed on the 3-h (A) and 24-h (B) T2 maps of the poisoned rats treated with ATOX (I), ATOX + MID (II), ATOX + MID + SCOP (III) and control (IV) groups, compared with baseline T2 maps. Highlighted regions represent regions with significantly elevated T2 values (\(p < 0.005\)). The color scale is in units of the \(t\) statistics. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
Fig. 7. MR spectroscopy analysis: (A) NAA/Cr and (B) NAA/Cho levels for 3- and 24-h scans after paraaxon poisoning. ATOX and ATOX + MID groups show significantly lower 3-h NAA/Cr compared to ATOX + MID + SCOP or control. In animals treated with scopolamine a significant decrease appeared in 24-h NAA/Cr compared with the 3-h ratio. Treatment with MID with or without scopolamine transiently prevented early decrease in NAA/Cho levels compared to control. Data is shown as mean and standard deviation of the mean, when the comparison is relative to the bar in the same time-point whose color is identical to the color of the asterisk. * represents p < 0.05, the symbol ** represents p < 0.005 (unequal variance t-test).

Fig. 8. Representative H&E staining of coronal brain sections at the level of the dorsal hippocampus (A) following paraaxon exposure and treatment with ATOX without any central acting antidotes. Brain sections taken from the fronto-parietal cortex (A[I], B and C), hippocampus (A[II], D), CA1 (E) and dorso-lateral thalamus (A[III], F) showing necrotic cells and vacuoles in cortical and hippocampal regions and gliosis and calcification in the thalamus (arrows). Scale bar indicates 100 μm.
pathological score (3-h NAA/Cho, $r = 0.59$, $p < 0.05$; and 24-h NAA/Cr and 24-h NAA/Cho, $r = 0.56$, $p < 0.05$).

4. Discussion

4.1. Imaging findings

In our study, three hours after a single high dose exposure to paraoxon, significant T2 prolongation appeared in susceptible brain areas, i.e. cerebral edema ensued. These changes were found mainly in the thalamus and in the temporal cortices and subsided as early as 24-h post-poisoning. A similar finding was reported by Gullapalli et al. (2010). They found that increased T2 values in the piriform cortex, hippocampus, thalamus and amygdala of guinea pigs treated with 1 LD$_{50}$ of the OP soman correlated well with convulsive activity (Gullapalli et al., 2010). Transient T2 increase in susceptible brain areas was also evident on other rat models of status epilepticus (SE), including pilocarpine and kainic acid induced SE as well as electrical stimulation of the amygdala, which also induces SE (Fabene et al., 2003; Grohn and Pitkanen, 2007; NairisMagi et al., 2004; Wall et al., 2000). Histological analysis suggested that regions with T2 prolongation in the acute period following seizure activity reflect cerebral edema (Fabene et al., 2003; Tanaka et al., 1993). Interestingly, Bhagat et al. described an early transient decrease in T2 values in the hippocampus and in the thalamus in soman poisoned rats which resolved after 24 h (Bhagat et al., 2001, 2005). Possible explanations for this decrease in T2 values were either a transient decline in extracellular water or an increased concentration of paramagnetic deoxyhemoglobin, which is elevated in situations in which the metabolic requirements are not met by the delivery of oxygen, as in the case of SE (van Eijden et al., 2004).

In the brain, NAA is thought to be present predominantly in neurons, where it acts as an important biomarker for cell viability and function. A decrease in NAA levels has been described in various conditions in which there is neuronal loss or dysfunction (Burtscher and Holtas, 2001). However, it is unknown if the changes are etiological or merely secondary to various brain insults. We found that NAA/Cr ratio also appears to be a sensitive marker for severity of OP induced neuronal injury. Although much of the cerebral edema subsided 24 h after paraoxon poisoning, spectroscopic parameters such as NAA/Cr and NAA/Cho, were more sensitive for neuronal dysfunction or stress and were found to be also decreased 24 h after poisoning. These findings are in concordance with our previous results which demonstrated decreased NAA/Cr levels even 7 days after OP poisoning, long after cerebral edema subsided (Shrot et al., 2012). Moreover, we found that 24-h NAA/Cr ratios correlated well with early (weight loss) and late outcomes (pathological score). Fauvelle et al. found a decrease in NAA levels in the piriform cortex of soman-poisoned mice, beginning 4 h after poisoning and lasting for seven days (Fauvelle et al., 2010). Decreased NAA/Cr ratio, as a marker of neuronal dysfunction or neuronal injury, was also described in other models of seizures such as the kainic acid and the lithium-pilocarpine models of epilepsy (Lee et al., 2012; Najm et al., 1997). The choline peak is attributed to choline-containing compounds, which are used to make cell membranes. Elevated choline levels appear in conditions with high turnover of cell membranes (Burtscher and Holtas, 2001). We found that Cho/Cr ratios 24 h after poisoning were higher than those measured at 3 h in the ATOX group suggesting a significant delayed elevation in the choline levels at 24 h after poisoning. Similarly, choline was also found to be increased after soman or sarin poisoning (Fauvelle et al., 2010; Flynn and Wecker, 1986). This increase in choline might represent high turnover of cell membrane due to apoptosis, necrosis or severe inflammation (Bertholdo et al., 2013). Nevertheless, Lee et al. did not show any change in the Cho/Cr levels in the lithium-pilocarpine rat model of SE (Lee et al., 2012).

4.2. Clinical-imaging correlation

Although MR findings in SE induced brain damage, and specifically following OP poisoning, is well studied, there is a paucity of data regarding the correlation between these imaging findings and clinical outcomes. In our study the magnitude of cerebral edema in various brain areas, although transient, was found to correlate with early weight loss, late cognitive decline and pathological score. A similar correlation was found between spectroscopic evidence of neuronal dysfunction and clinical outcomes. Engelhorn et al. found a significant correlation between maximal decrease in ADC during the acute phase of SE (i.e. cytotoxic edema) and neuronal cell loss seen at 2 weeks after
pilocarpine-induced SE (Engelhorn et al., 2007). Interestingly, significantly lower ADC values were found in animals that died following the induction of SE compared with surviving animals. A significant correlation was also reported between early T2 increase in the piriform and entorhinal cortices and chronic epilepsy development (Roch et al., 2002). In patients with temporal lobe epilepsy, reduced temporal NAA/Cr and NAA/Cho ratios were associated with frequent generalized tonic–clonic seizures and poorer seizure control (Bernasconi et al., 2002; Fabene et al., 2003; Mendes-Ribeiro et al., 1998). Interestingly, in our study no correlation was found between spectroscopic evidence of neuronal injury and learning deficits. We believe that this finding was probably due to voxel localization on the thalamus which is not involved in task performance tested by MWM which mainly tests hippocampus functioning. We believe that our findings emphasize the role of quantitative imaging in assessing brain damage following an insult.

4.3. Cognitive evaluation

MWM results represent deficits in spatial learning and memory originating from hippocampal pathology and are considered to be a sensitive test for assessment of neurologic damage resulted from OP poisoning (Brandeis et al., 1993; Raveh et al., 2002). MWM was also shown to be a sensitive tool for demonstrating paraoxon-induced long-lasting cognitive deficits (Sanchez-Santre et al., 2004). However, in our study, no group effect of learning deficit was found in the paraoxon-treated animals. The immediate treatment with atropine and obidoxime which was necessary to prevent early mortality due to peripheral acetylcholinesterase inhibition probably provided some protection against paraoxon-induced hippocampal injury. Atropine sulfate, but not the quaternary salt atropine methyl bromide was found to provide a protective effect on the performance of paraoxon-poisoned rats (Chambers and Chambers, 1989). Moreover, atropine and various oxime compounds were able to inhibit epileptiform activity in guinea pig hippocampal slices in the presence of paraoxon or soman (Endres et al., 1989; Harrison et al., 2004). Although the clinical picture of the poisoned animals was relatively homogeneous, learning deficits were found in individual animals. A similar finding was reported by Kadar et al. after poisoning rats with 1 LD50 of sarin, a potent military OP, i.e. only 70% of the poisoned animals showed brain lesions on histological studies (Kadar et al., 1995). Moreover, brain lesions were seen only in 9 of 15 soman-exposed monkeys after clinical convulsions despite the fact that only 1 of 21 did not convulse (Britt et al., 2000).

4.4. Pharmacological neuroprotective treatment

Early administration of central acting antidotes, such as anticholinergic or GABAergic agents, is highly effective in terminating OP-induced seizures and in preventing the neuropathology (McDonough and Shih, 1997). When delayed, the efficacy of these neuroprotective agents is decreased significantly and long-term brain damage and behavioral deficits are common (McDonough and Shih, 1997). This is true especially in regard to the central anticholinergic agents (Gillat et al., 2005; McDonough et al., 2000). In our study, we showed that even delayed administration of central acting neuroprotective agents, such as midazolam or a combination of midazolam and scopolamine can prevent most of the cerebral edema. However, metabolic evidence of neuronal stress (i.e. NAA/Cr decrease) was found up to 3 h after poisoning. No significant advantage was found for adding scopolamine over midazolam alone in preventing these changes, probably due to predominantly non-cholinergic mechanisms. 30 min post OP poisoning (McDonough and Shih, 1997). Unfortunately, both treatment protocols provide only temporary neuroprotection and at 24 h neuronal insult is comparable with ‘conventional’ treatment protocol (atropine and oxime). Our hypothesis is that when delayed, continuous neuroprotection agents might be necessary to prevent further neuronal injury. Similarly, the repeated doses of NMDA antagonist, ketamine, were needed to provide neuroprotective effects, when given 30 min after soman exposure in a mouse model (Dhote et al., 2012) and a combination of ketamine, midazolam and atropine provides significant neuroprotection even when given two hours after severe soman poisoning (Dorandeau et al., 2005).

4.5. Limitations

Extrapolating expected effects in humans from animal data has been one of the basic problems in animal research for many years. Indeed, although our model found OP-induced severe brain damage and in vivo imaging to correlate with early and late clinical effects, quantitative extrapolation of the toxicological and pharmacological parameters to humans (e.g. OP dose, route of exposure, time up to antitodal treatment, antitodal dosage, etc.) must be done carefully and with proper uncertainties factors.

In our study, convulsive activity ceased after anesthesia was administered during first MR imaging. Volatile anesthetic agents including isoflurane have been well recognized for their potential neuroprotective properties since the 1960s (Matchett et al., 2009). Deep anesthesia probably slows the metabolic activity of the brain, stops seizures and provide some neuroprotection. Therefore, the use of anesthesia may have confounded our results. Additional limitation is the control group, which was treated with saline and ATOX alone but not with MID or SCOP. Both MID or SCOP are neuro-active drugs which might have effect on brain imaging and on cognitive performances.

4.6. Conclusions

In conclusion, MR is a sensitive noninvasive technique for early detection, monitoring and prediction of OP-induced brain damage. Cerebral edema and neuronal dysfunction appear as early as 3 h after poisoning. A significant correlation was found between these early MRI and MRS parameters and both early and late clinical outcomes; this correlation could be used in the future for early evaluation of severity of OP poisoning. Delayed treatment with midazolam with or without scopolamine provides only transient neuroprotection. Thus, continuous or repeated administration of neuroprotective agents in addition to the ‘conventional’ treatment protocol for OP poisoning (consisting of atropine and oximes) may be necessary for effective long-term neuroprotection.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

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