Distortion Product Otoacoustic Emissions as Potential Non-Invasive Predictors and Biomarkers of Soman-Induced Central Neurotoxicity

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SUMMARY

The neurotransmitter acetylcholine is involved both in soman intoxication and in central regulation (efferent system) of cochlear outer hair cells. It was then assumed that exploring the hearing function may provide markers of the central events triggered by soman intoxication. The organophosphorus nerve agent soman is an irreversible cholinesterase (ChE) inhibitor that can produce long-lasting seizures and brain damage. In the present study, distortion product otoacoustic emissions (DPOAEs), a non-invasive audiometric method, were used to monitor cochlear functionality in rats administered with a moderate dose of soman (45 µg/kg). DPOAEs were investigated either 4 h or 24 h post-challenge. In parallel, the effects of soman on brain ChE activity and on brain histology were also studied. The first main result is that DPOAE intensities were significantly decreased 4 h post-soman and returned to baseline at 24 h. The amplitude changes were well related to the severity of symptoms, with the greatest change being recorded in the rats that survived long-lasting convulsions. The second main result is that baseline DPOAEs recorded 8 days before soman appear to predict the severity of symptoms produced by the intoxication. Indeed, the lowest baseline DPOAEs corresponded to the occurrence of long-lasting convulsions and brain damage and to the greatest inhibition in central ChE. These results thus suggest that DPOAEs represent a promising non-invasive tool to assess and predict the central consequences of nerve agent poisoning. Further investigations will be carried out to assess the potential applications and the limits of this non invasive method.

1.0 INTRODUCTION

The organophosphorus (OP) compound, Soman (O-1,2,2-trimethylpropylmethyl-phosphonofluoridate), a chemical warfare nerve agent, is a potent and irreversible inhibitor of both peripheral and central cholinesterases (ChEs). Acute exposure to soman can cause profound physiological debilitation, generalized convulsive seizures, coma and death [16]. In survivors, the development of long-lasting seizure activity is known to be related to brain damage [3, 16]. Neurochemically, acetylcholine (ACh) accumulation plays a key role in the initiation of seizure activity whereas the excitatory amino acid glutamate (Glu) is involved at a later stage in seizures as well as in brain cell damage [16].

Experimentally, there is a lot of available invasive methods to evaluate the acute impact of an OP intoxication on the brain or to follow the effect of an anti-OP medication in intoxicated animals (e.g.,

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histology and related techniques, neurochemistry, etc.). On the contrary, there is a lack of non invasive easy-to-use diagnostic method.

Among the numerous transmitters involved in the auditory function, ACh and Glu are thought to play crucial roles. For instance, cochlear outer hair cell (OHC) function is regulated from brain to cochlea (top-down regulation) by an important cholinergic system, the efferent medial olivocochlear system, and the afferent transmission from the cochlear inner hair cells (IHC) is under Glu control [22]. Recent publications [18,19] indicate that effects on the auditory system (decreased OHC function and decreased sound localization discrimination) appear during pathophysiological conditions in which the cholinergic and/or glutamatergic neurotransmissions are involved.

A promising audiometric method consisting in the measurement of the distortion product otoacoustic emissions (DPOAEs) emerged these last 15 years [11]. This technique relies on the contractile properties of the OHC which, in response to tonal sound stimuli, can generate retrograde wave sounds, the intensity of which being captured and recorded by a very sensitive probe microphone in the external auditory canal. DPOAEs are the results of the non linear properties of the cochlear basilar membrane: when two primary pure tones (*f1* and *f2)* are presented simultaneously with a ratio *f2/f1* comprised between 1.2 and 1.3, distortion products are generated with the largest occurring in the mammalian ear at the frequency 2*f1* – *f2*. The amplitude of DPOAEs, expressed in dB SPL (Sound Pressure Level), is mostly related to the integrity of the OHC function, and is regulated by the corticofugal efferent system [12]. DPOAEs have been proved useful to evaluate, objectively and non-invasively, the vulnerability of the cochlear function in clinical settings, [8-10] and are now routinely used in laboratory animals. They have been also used to evaluate the ototoxicity of various chemicals [14, 17, 21], among other applications.

Since, the same neurotransmitter systems (i.e., ACh and Glu) are involved in both hearing and the cerebral changes produced by OP poisoning, it can be postulated that OP-induced changes in the hearing function might reflect those in the brain. This hypothesis is supported by the fact that patients with *myasthenia gravis,* an autoimmune disease in which antibodies alter ACh receptors, show, in addition to evident muscular impairments, cognitive disturbances [20] and low DPOAE amplitudes [19]. Interestingly, their otoemissions can be improved by treatment with pyridostigmine bromide, a reversible ChE inhibitor [19]. Therefore, audiometric methods may become useful to non-invasively diagnose the severity of the central effects caused by OP poisoning. In order to assess these hypotheses, rats were exposed to sub-lethal doses of soman, and their DPOAEs were recorded before and after administration. The results were studied in relation to various indicators of intoxication severity (i.e., clinical symptomatology, measures of brain ChE inhibition, brain damage).

2.0 MATERIELS & METHODS

2.1 Animals

Adult male Wistar rats (300 g; Janvier, France) served as subjects. This model has been used extensively in our laboratory to study soman intoxication, and has been shown useful to assess auditory function, especially otoacoustic emissions [23].

Rats were housed in a controlled environment (21 ± 2 °C; 12 h dark/light cycle with light provided between 7 a.m. and 7 p.m.). Food and water were given *ad libitum*. Procedures were designed in accordance with the regulations regarding the “protection of animals used for experimental and other scientific purposes” from the relevant Directives of the European Community (86/609/CEE). The study protocols reported herein were approved by the ethical committee of our institute.

2.2 Intoxication

Soman (> 97 % pure as assessed by gas chromatography) was supplied by the Centre d’Etude du Bouchet (Vert Le Petit, France). Soman was administered subcutaneously at the dose of 45 µg/kg (0.5 LD_{50}; 500 µl

in saline). This dose was chosen after preliminary experiments showing that it is essentially sub lethal and produced various levels of symptom severity, from almost no clinical sign to convulsions and death (see Table 2 for details). All the intoxications were performed between 8 a.m. and 11 a.m. to reduce possible circadian variations of cholinergic parameters [6].

The clinical state was continuously observed for at least 8 h after the intoxication and on the following morning for at least 30 min. The presence and frequency of hypercholinergic signs (i.e., chewing, hypersalivation, fasciculations, tremors, and convulsions) were noted. The loss of body weight in survivors was also recorded as an indicator of general health status.

2.3 General experimental design

Control observations were provided by sham-operated animals in which soman was replaced by saline (500 µl; *s.c.*). As summarized in Table 1, three sets of experiments were performed.

In the first experiment, the rats were measured for DPOAEs either 4 h or 24 h after soman intoxication and then immediately sacrificed for brain histology.

The second experiment was undertaken to search for a possible correlation between the DPOAE and ChE responses to soman intoxication. Hence, no sham-operated rats were included. DPOAE and ChE measurements were successively performed in the same animals 24 h post-soman.

In a third additional experiment, the level of brain ChE inhibition produced by soman 4 h and 24 h post-challenge was verified in the absence of previous anesthesia and DPOAE measurement.

The study time-points were choosen as they correspond to two different stages in the progression of brain damage produced by convulsant doses of soman in rodents [1, 2, 4]: At 4 h post-challenge, developing cerebral changes are observed including edema and eosinophilic suffering cells; at 24 h post-treatment, brain damage is well settled, including numerous cell death.

2.4 DPOAE measurements

DPOAEs were measured in a thermoregulated and silent room using the DPOAE GSI 60 system (Grason-Stadler, Milford, NH USA). Immobility being required during DPOAE measurements, rats were anesthetized with sodium pentobarbital as in Rasmussen's study [23] (70 mg/kg; *i.p.*; Sanofi, France). The probe was then carefully inserted into the right auditory canal via a neonatal tip. The neonatal tip allowed tight adaptation of the probe into the external auditory canal. Any looseness of the probe could be detected by the system and, if any, measurements were aborted.

DPOAEs were investigated in a range covering about one octave, from 2375 Hz to 4812 Hz (*f2*). The range of the frequencies measured was voluntarily restricted to the low frequencies in order to be in the best possible accordance with the potential clinical application in humans. Indeed, it is well known that in humans high frequencies are especially vulnerable to a variety of factors, like age, noise or various hearing pathologies (i.e. otitis media) [7, 24]. As in a war context exposure to nerve agents may be associated to impulse noise due to firearms, the high-frequency range has to be avoided in order to use DPOAEs as a specific diagnostic tool for OP poisoning. Despite the fact that rats have rather poor hearing in the 2-4 kHz range, their DPOAEs were stable, and reliably measured. Nevertheless, further investigations will be performed to test the rat's whole hearing range in order to assess if other, more sensitive, frequency bands can be used in a diagnostic context.

The intensities of the pure tones *f1* (the lowest frequency) and *f2* (the highest frequency) were chosen from the literature in rats [13, 17] and from our previous pilot studies. In these, two stimulus protocols were tested (1) $f2 = f1 + 10$ dB = 70 dB SPL, (2) $f2 = f1 = 70$ dB SPL with the ratio $f2/f1$ set at 1.2. The stimulus protocol 70/70 dB SPL was finally chosen because it showed more reproducible responses and a better separation from the noise floor.

DP-grams were obtained by sweeping *f2* from 2375 Hz to 4812 Hz over 20 frequencies. For each rat, 5 successive DP-grams were collected without removing the probe. Because DPOAE responses were relatively flat (amplitude between 15 and 20 dB SPL) in this narrow frequency range the average response for the 20 frequencies was calculated for each time point. Baseline DPOAEs were measured 8 days before saline (sham-operated rats) or soman treatment. Post-soman (or post-saline in sham-operated rats) DPOAEs were performed in different rats either 4 h (between 2.00 p.m. and 5 p.m.) or 24 h after the treatment (between 9.00 a.m. and 12.00 p.m.). The impact of intoxication was evaluated using the difference in DPOAE intensity (in dB) between pre- and post-challenge values.

2.5 Brain ChE assays

For brain ChE assay, the rats were sacrificed by decapitation either 4 h or 24 h after the intoxication. The brains (without the cerebellum) were immediately homogenized in Tris buffer (pH 7.4) / saccharose 0.32 M and centrifuged (15 min, 1000 g). Assay was performed on brain supernatant according to the method of Ellman [5] using an automated procedure (Hitachi 704; Kit Reagent MPR2 124117, Roche Molecular Biochemical) with the following modifications : acetylthiocholine concentration set at 0.95 mM final and absorbance measured at 412 nm.

Control brain ChE activity was obtained from sham-operated animals sacrificed either 4 h or 24 h after saline administration and from untreated rats (same age and weight as in the present study) of a pilot study undertaken to verify the reproducibility of the enzymatic method (see Table 1). Brain ChE activity was expressed in $nmol.min^{-1}.mg^{-1}$ protein and post-soman inhibition was expressed in percent of the average activity in control animals.

Experiments	4 h post-challenge			24 h post-challenge		
	Sham	Intoxicated rats		Sham	Intoxicated rats	
		survivors	death		survivors	death
DPOAE + histology	12	14	0	14	17	3
DPOAE and brain ChE	-	-	-	-	29	1
Subtotal DPOAE	(12)	(14)	(0)	(14)	(46)	(4)
Brain ChE	7	11	0	7	11	0
Total	19	25	0	21	57	4

Table 1: Distribution of animals in the different experiments

2.6 Histopathology

Following post-soman measurement of DPOAEs, the anesthetized rats were transcardially perfused with heparinized saline followed by a fixative solution (formaldehyde 4 %; acetic acid 3 %). The brain was immediately removed, postfixed (formaldehyde 4 %) and processed for embedding in paraffin. Coronal sections were stained with Hemalun-Phloxin for the detection of eosinophilic cells that demonstrated cell damages. The brain structures examined were among those known to be especially sensitive to convulsive doses of soman [2] i.e., the hippocampus, the thalamus, the cortices and the amygdala. In addition, a special attention have also been given to structures of the auditory pathways, namely the ventral and dorsal cochlear nuclei, the inferior colliculus, the medial geniculate body and the auditory cortex.

2.7 Statistics

Data are presented as mean ± SEM. Statistical analyses were performed using SPSS v.10.0 software (Chicago, IL). The level of significance was set at $p = 0.05$ for all the tests.

Because of a non gaussian distribution and a small samples size, the Mann and Whitney test (M&W test) was used to compare two groups of different animals (e.g.: brain ChE activity in rats sacrificed at 24 h *vs* rats sacrificed at 4 hr). The non-parametric Kruskall-Wallis test (K-W test) and post-hoc tests taking into account Bonferroni correction were used to compare data in more than two groups (e.g., brain ChE inhibition in the different symptom groups).

ANOVA with repeated measures, taking into account the five stimulations, was used to establish if DPOAE responses differed in the various symptom groups. This was followed either by a Bonferroni post-hoc test to determine which groups were different from the others (multiple comparisons) or by a Dunnett (2-sided) post-hoc test to determine which soman groups were different from the sham-operated group (paired comparison to one reference). Correlation between two variables were evaluated using Pearson's test (e.g.: ChE inhibition *vs* symptom severity; ChE inhibition *vs* DPOAE response).

3.0 RESULTS

3.1 Clinical observations

The survival rate in animals intoxicated by 45 µg/kg of soman was 95 % (82/86). The 4 subjects that did not survive the soman administration died within the first few hours following soman administration, after displaying convulsions and dramatic episodes of labored breathing followed by profound respiratory distress. Survivors (n = 82) generally showed less impaired respiration. They presented a variety of symptoms (Table 2): (**i**) rats with almost no sign (Grade 1); (**ii**) rats with mild symptoms, the most common (Grade 2); (**iii**) rats with rare (1-3) and brief (1-5 seconds) episodes of convulsions observable within the first hour post-soman (Grade 3); (**iiii**) rats with convulsions appearing within minutes after the intoxication and lasting for at least the following 4 h (Grade 4). In survivors, the loss of the body weight recorded 24 h post-soman paralleled symptom severity: While no weight loss was observed in the Grade 1 group (+ 0.1 ± 5.1 g), a drop of 2 % (- 6.5 ± 7.7 g), 2.6 % (- 7.9 ± 6.9 g) and 16.5 % (- 49.7 ± 4.8 g) was recorded in Grade 2, Grade 3 and Grade 4 groups, respectively.

Symptom Groups	n %	Main Symptoms
Grade 1	14 17 %	rare chewing
Grade 2	39 48 %	frequent chewing; prostration
Grade 3	14 17 %	constant chewing; hypersalivation; tremors; fasciculations; brief convulsions
Grade 4	15 18 %	long-lasting convulsions

Table 2: Survivors to soman intoxication (45 µg/kg): clinical effects

3.2 Effect of soman on brain ChE activities

Brain ChE activity in sham-operated animals sacrificed either 4 h (n = 7) or 24 h (n = 7) post-saline did not differ from that detected in non-injected normal rats (n = 12) used in a preliminary study (K-W test). Therefore, the control brain ChE activity presented in Table 3 is the mean value obtained from all of these animals (n = 26). Compared to controls, brain ChE activity in the intoxicated animals was significantly decreased 4 h and 24 h after the administration of soman (M&W test, $p<0.001$).

Brain ChE activity seemed to recover by about 9 % between 4 h (inhibition ≈ 49 %) and 24 h post-soman (inhibition ≈ 40 %), however, the difference did not reach statistical significance (M&W test).

Figure 1 show the relationship between ChE inhibition in brain, and symptom severity grades for animals studied 4 h or 24 h post-challenge. The brain inhibition appears to be well correlated with symptom severity (4 h post-soman: $r = 0.887$, $p < 0.01$, $n = 11$; 24 h post-soman: $r = 0.645$, $p < 0.01$, $n = 40$). In the 24 h survivors, the mean percentages of brain ChE inhibition in the Grade 1 (22 ± 5 %), Grades 2-3 (40 ± 5 %), and Grade 4 (70 ± 5 %), groups differed significantly from each others (K-W test, $p<0.001$).

Sites of ChE activity measurement	Treatments	n	Time post-treatment	Control ChE activity	Post-treatment ChE activity	Post-treatment ChE inhibition(%)
Brain ($nmol.min^{-1}.mg^{-1}$ proteins)	None or saline	26	§	206 ± 19	-	-
	Soman	11	4 h	-	106 ± 52 £££	49 ± 25£££
		40	24 h	-	123 ± 41 £££	40 ± 20 £££

§ : this group was composed of 14 sham-operated rats sacrificed either 4 or 24 h after saline administration and 12 controls receiving no saline.

Comparison of brain ChE activity in rats sacrificed either 4 h or 24 h post-soman *vs* activity in control rats (M&W test): **£££**: $p<0.001$.

Comparison of brain ChE activity in rats sacrificed 24 h post-soman *vs* activity in rats sacrificed at 4h (M&W test): **NS**

Table 3: Brain ChE activity in rats intoxicated by soman (45 µg/kg)

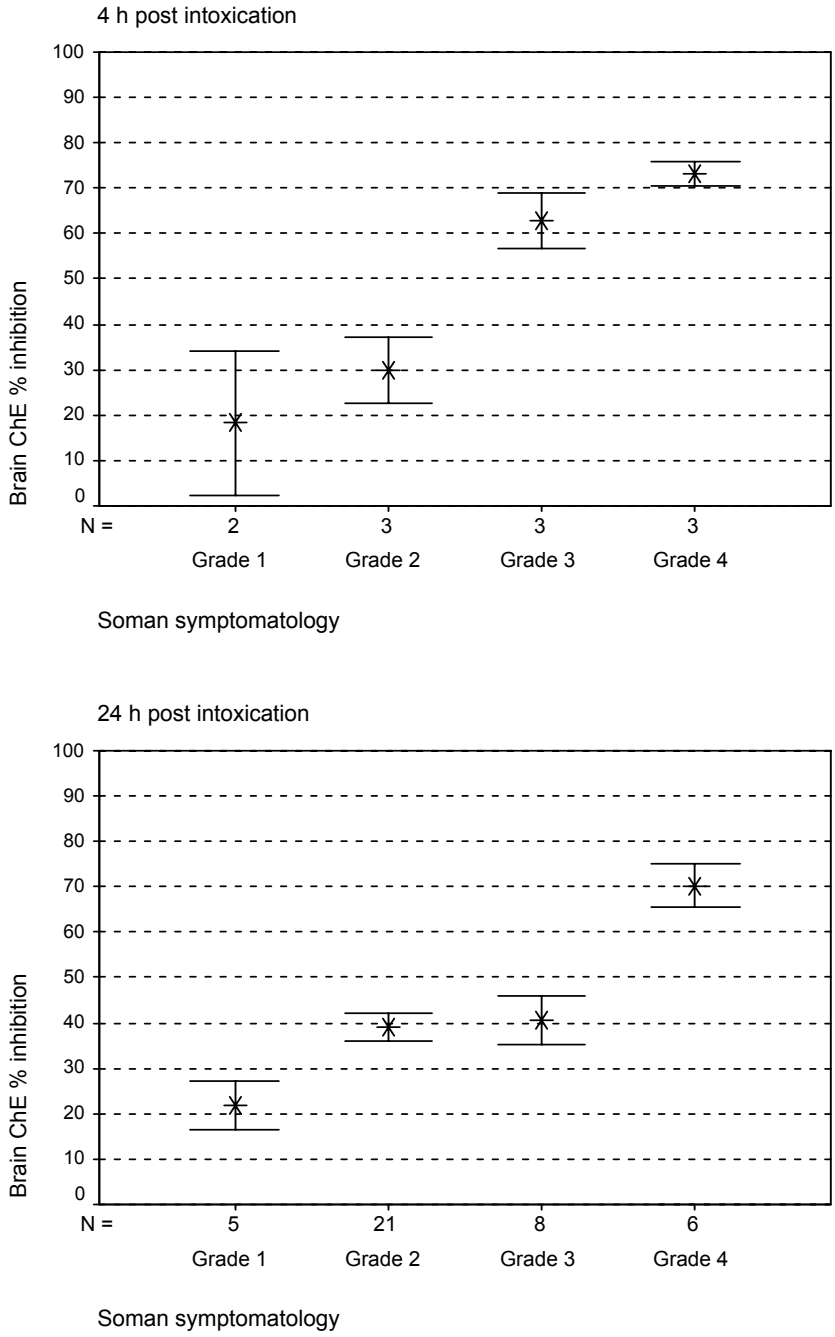

Figure 1: Brain ChE inhibition produced by soman as a function of symptom severity.

3.3 Effect of soman on brain histology (Figure 2)

No damage was ever detected in any brain structures of the sham-operated animals or of the intoxicated rats that did not experience convulsions (Grades 1 and 2) or experienced convulsions of short duration (Grade 3). By contrast, all rats which showed long-lasting convulsions (Grade 4) displayed edema and eosinophilic cells. In these animals, structural changes, the severity of which increased from 4 h to 24 h post soman, could be seen in the hippocampus, the thalamus, the cortex and the amygdala as well as in some structures of the auditory pathway, such as the auditory cortex and the medial geniculate body. In the

latter, eosinophilic cells and edema remained confined to the periphery of the structure 4 h post-soman but invaded the entire area 24 h later. Other anatomical components of the auditory system such as the inferior colliculus, the ventral and dorsal cochlear nuclei did not appear damaged.

3.4 DPOAE changes in intoxicated rats

Four hours after the intoxication (n = 14), no Grade 3 animals were obtained. In other groups, DPOAE responses were shown to decrease compared to the pre-challenge values. This change depended on the severity of symptoms, with the greatest decrease observed in the Grade 4 rats (Figure 3). Accordingly, ANOVA with repeated measures (5 stimulations) indicated that DPOAE changes were significantly different between the various symptom groups ($F = 6.03$, $df = 3$, $p = 0.004$). Post-hoc Dunnett test (2-sided) showed that Grade 4 rats differed significantly from sham-operated animals ($p = 0.003$). The difference between Grade 2 rats and sham-operated animals was close to the significant limit ($p = 0.07$).

As illustrated in Figure 3, DPOAEs measured 24 h post-soman were shown to recover compared to the values recorded at the 4 h time-point. These values (n = 46) reached or even exceeded those recorded in the sham-operated animals (n = 14) from which they did not appear significantly different (ANOVA with repeated measures, $F = 2.13$, $df = 4$, $p = 0.089$). This DPOAE recovery was a function of symptom severity since the greatest shift was recorded in the Grade 4 rats, which displayed the lowest DPOAEs at 4 h and the highest at 24 h. DPOAE changes between the 4 symptom groups at the 24 h time-point were not significantly different but close to the significant limit (ANOVA with repeated measures, $F = 2.13$, $df = 3$, $p = 0.07$).

3.5 Baseline DPOAEs in relation to symptoms of poisoning

Interestingly, in the rats that survived soman administration and were studied for DPOAEs (n = 60), a relationship was found between their pre-soman baseline DPOAEs and the severity of symptoms produced later by the intoxication. As illustrated in Figure 4, the baseline DPOAEs were the lowest in the rats which ultimately displayed the most severe post-soman symptoms (Grade 4). Statistically, baseline DPOAEs were significantly different between groups (ANOVA with repeated measures, five stimulations; $df = 3$; $p = 0.025$). Specifically, Bonferroni post hoc test showed that Grade 4 rats baseline DPOAEs (amplitudes < 20 dB) were significantly lower than baseline of Grade 2 rats (amplitudes > 20 dB) ($p = 0.031$).

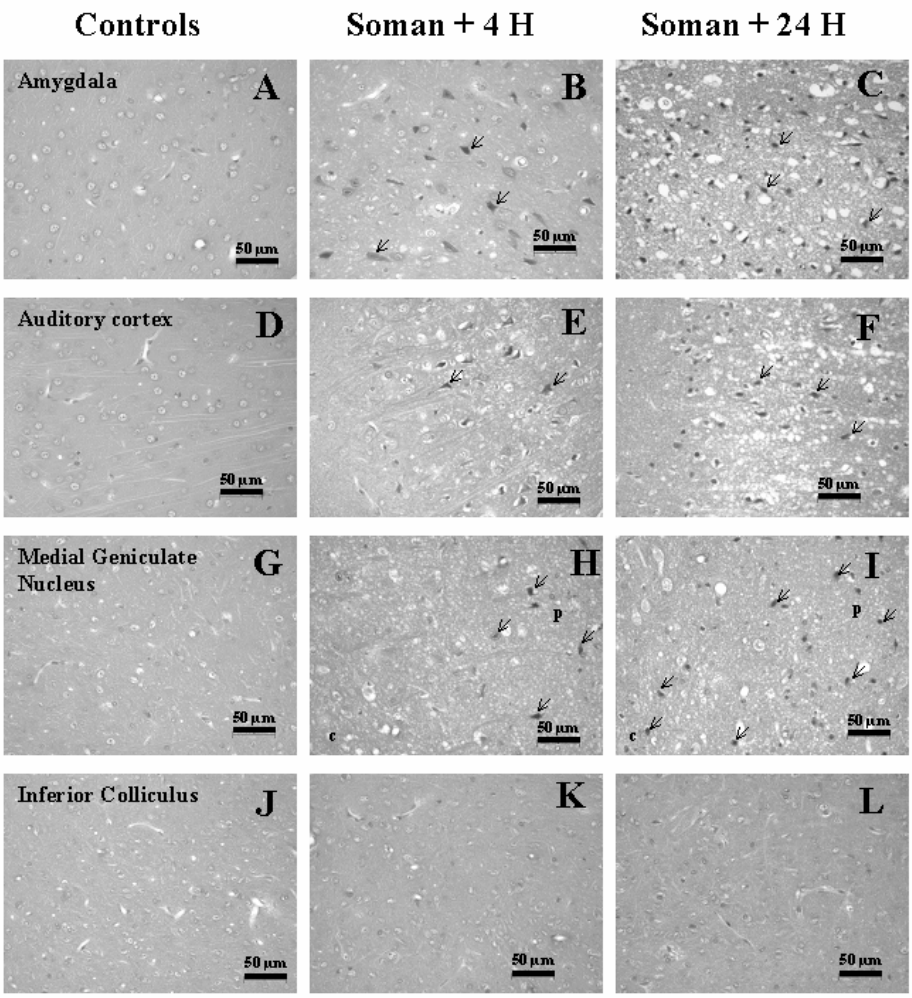

Figure 2: Histopathological consequences of soman in various brain tissues (H&P stain):

Amygdala (A, B, C): Compared to controls (A), this region shows an emergent interstitial edema and a number of eosinophilic cells (arrows) 4 h post soman (B). At 24 h (C), the edema becomes dramatic, with larger vacuoles and almost all the cells appear highly condensed (arrows). Auditory cortex (D, E, F) and medial geniculate nucleus (G, H, I): these structures show damage similar to those seen in the amygdala. Note that in the medial geniculate nucleus, edema and cell changes were essentially confined to the periphery of the structure (p) at 4 h but invaded the central parts (c) at 24 h. Inferior colliculus (J, K, L): this structure remains intact throughout the intoxication.

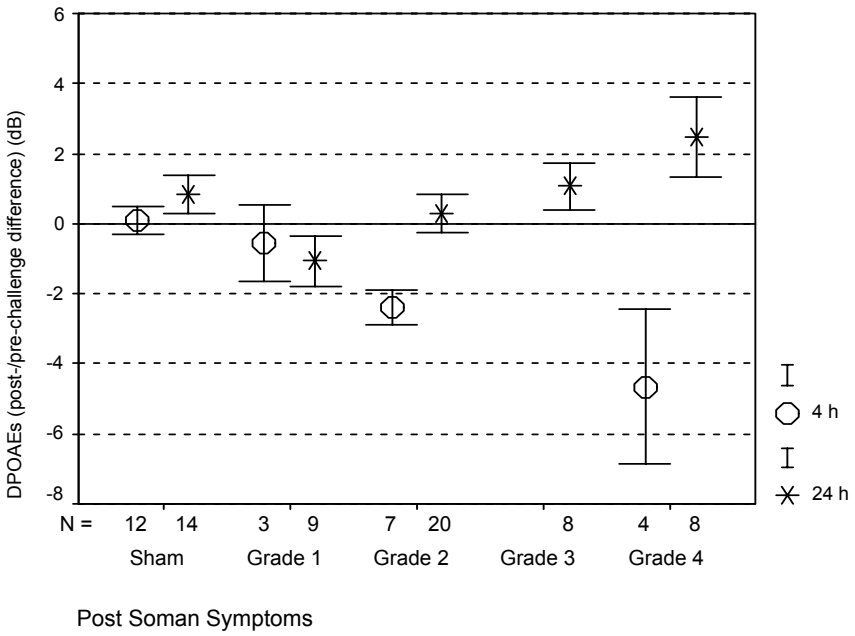

Figure 3: Mean DPOAE changes recorded 4 h and 24 h after soman intoxication: relation with the severity of symptom.

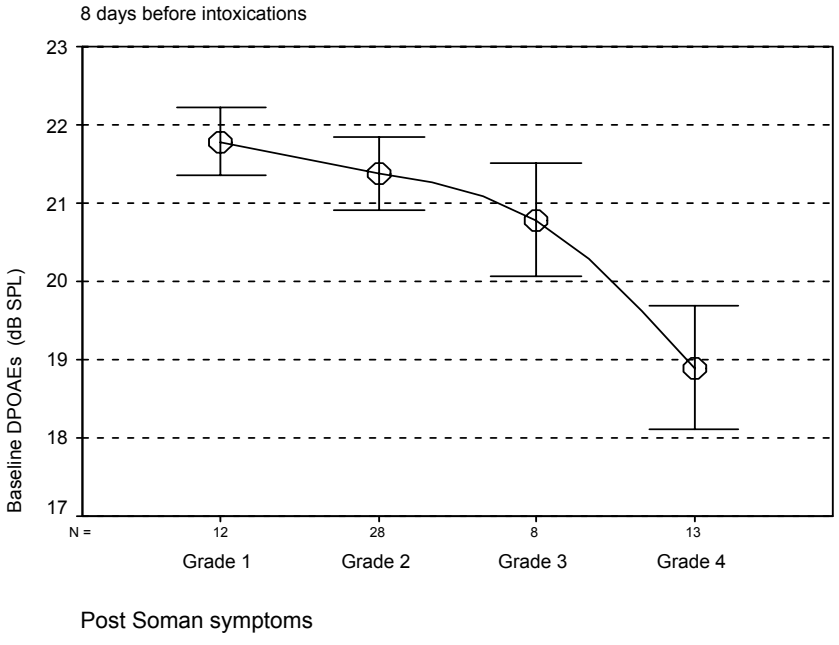

Figure 4: Baseline DPOAEs as a function of symptom severity after soman intoxication

4.0 DISCUSSION

In the present study, the 45 µg/kg subcutaneous dose of soman produced minimal mortality but a large panel of symptoms, from minimal (Grade 1) to severe (Grade 4), in surviving rats. Even in the absence of EEG recordings, the clinical evaluation of convulsions seemed correct as all animals of the Grade 4 group showed the highest brain inhibition and are the only ones that showed brain damage along with considerable loss in body weight, two reliable indicators of long-lasting seizure activity [3, 15]. The first important finding is that, compared to controls, the intoxicated rats displayed biphasic DPOAE responses, with a decrease at 4 h and a recovery/rebound at 24 h. Above all, soman-induced DPOAE changes were demonstrated to be well related to the severity of the intoxication. Strikingly, the amplitude of the decrease at 4 h and of the increase at 24 h was the lowest in the rats that showed the less severe symptoms (Grade 1) and the greatest in those that displayed the most severe symptoms, the greatest brain ChE inhibition and brain damage (Grade 4).

Our observation that DPOAEs diminished 4 h after intoxication and recovered/rebounded 24 h later suggests that the cochlear function was initially partially inhibited and later de-inhibited or stimulated, with responses that tended to reach or even exceed those observed in controls. Since the cochlear function recovered within 24 hr, the DPOAE changes could hardly be related to irreversible structural damage of the cochlear apparatus (this will be assessed in further experiments by performing cochlear histology). Possible explanations could stem from the changes produced by soman in the regulation of cholinergic, glutamatergic and GABAergic systems [16], all known to be involved in the auditory function, or from the changes produced by soman itself on ionic status of cochlear fluids. However, the greater DPOAE changes observed in the Grade 4 rats might be in part due to the structural damage detected in some areas of the central auditory pathway (the auditory cortex and the medial geniculate bodies).

Further experiments are necessary to understand the significance of the increase in DPOAE responses observed at 24 h in the Grade 4 rats. This result can be interpreted as a temporary physiological rebound of the cochlear function, but a permanent dysfunction cannot be excluded.

The second major finding of the present study is the observed relationship between the baseline DPOAEs and the clinical and neurochemical effects of soman. Indeed, 8 days before intoxication, animals that showed the highest baseline DPOAEs prior to the intoxication were those that experienced the less severe symptoms and the less important central effects after the intoxication (Grade 1 rats). Conversely, the lowest baseline DPOAEs prior to the intoxication were found in the animals that displayed, after the intoxication, the most severe symptoms, development of brain damage, and the greatest inhibition in brain ChE (Grade 4 rats). Therefore, there is a hope that DPOAE measures could be used not only as a marker of soman intoxication but also as a predictor of susceptibility to soman exposure. In other words, our results suggest (**i**) that, before the intoxication, the OHC status of the Grade 4 rats was functionally less good than that of the other animals, (**ii**), that DPOAEs might reflect the overall status of the central cholinergic functionality regarding its capacity to resist to an OP aggression, and (**iii**), that otoemissions might help detecting the most sensitive individuals to soman-induced inhibition of central ChE and the most susceptible to develop convulsions, and brain damage. Also correlation between DPOAE baseline and Brain ChE activity baseline will be further assessed.

In conclusion, the results reported above provide elements suggesting that DPOAEs may become a novel non-invasive tool to be used as (**i**), a biomarker, and (**ii**), a predictor of the central effects of soman. In the experimental, as well as in the clinical field, such a method may supplement the existing ones, in order to increase the accuracy and potency of the prognosis/diagnosis of intoxication. With regards to human use, the DPOAE technique provides interesting advantages: it requires no particular preparation of the measured subject, can be used outside the hospital environment, and the low weight and bulk of the device allows measurements to be performed anywhere it is needed.

5.0 REFERENCES

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