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Prediction of soman-induced cerebral damage by distortion product otoacoustic emissions

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ABSTRACT

The organophosphorus nerve agent soman is an irreversible cholinesterase (ChE) inhibitor that can produce long-lasting seizures and seizure-related brain damage (SRBD) in which acetylcholine and glutamate are involved. Since these neurotransmitters play a key-role in the auditory function, it was hypothesized that a hearing test may be an efficient way for detecting the central effects of soman intoxication. In the present study, distortion product otoacoustic emissions (DPOAEs), a non-invasive audiometric method, were used in rats administered with soman (70 μg/kg). Four hours post-soman, DPOAE intensities were significantly decreased. They returned to baseline one day later. The amplitude of the temporary drop of the DPOAEs was well related to the severity of the intoxication. The greatest change was recorded in the rats that survived long-lasting convulsions, i.e. those that showed the highest ChE inhibition in brain and severe encephalopathy. Furthermore, the administration, immediately after soman, of a three-drug therapy composed of atropine sulfate, HI-6 and avizafone abolished the convulsions, the transient drop of DPOAEs at 4 h and the occurrence of SRBD at 28 h without modifying brain ChE inhibition. This showed that DPOAE change was not directly related to soman-induced inhibition of cerebral ChE but rather to its neuropathological consequences. The present findings strongly suggest that DPOAEs represent a promising non-invasive tool to predict SRBD occurrence in nerve agent poisoning and to control the efficacy of a neuroprotective treatment.

1. Introduction

The organophosphorus (OP) compound, soman (*O*-1,2,2,-trimethylpropylmethyl-phosphonofluoridate), is a potent and irreversible inhibitor of both peripheral and central cholinesterases (ChEs). This "nerve agent" can cause generalized convulsive seizures, respiratory distress, cardiovascular disorders and death (McDonough and Shih, 1997). In survivors, the development of long-lasting seizure activity is related to dramatic brain damage (e.g. McDonough and Shih, 1997; Carpentier et al., 2000). While the initiation of seizures is due to accumulation of acetylcholine (ACh), their maintenance, as well as the build up of seizure-related brain damage (SRBD), is consensually attributed to glutamate (Glu) excitotoxicity (McDonough and Shih, 1997).

Recent history proves that the potential for exposure to OPs must still be considered as a major threat in the battlefield (e.g. the Gulf War in 1991) as well as following terrorist attacks (e.g. the Tokyo subway attack in 1995). In case of poisoning, a standard emergency treatment, often packaged in auto-injectors, is available. It is composed of an anticholinergic drug (atropine sulfate: AS), which antagonizes the effect of excess ACh at muscarinic receptors, and an oxime, which acts as a ChE re-activator at the peripheral level. In some countries, an anticonvulsant benzodiazepine, currently mostly diazepam, is also issued. In France, a new auto-injector has recently been licensed for use. The cartridge contains a freeze-dried combination of three drugs, which are made soluble just before i.m. injection: AS, pralidoxime methylsulfate and a pro-drug of diazepam (pro-diazepam or avizafone: AVZ). In contrast to diazepam, AVZ is water soluble and thus can be freeze-dried and stored in the same cartridge compartment as AS and pralidoxime. *In vivo*, AVZ undergoes rapid hydrolysis to give diazepam (Maidment and Upshall, 1990; Upshall et al., 1990). Early administration of the AS/pralidoxime/AVZ combination provides excellent

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protection against the mortality, the convulsions and the SRBD produced by even high doses of soman or sarin in rodents and primates (Taysse et al., 2003, 2006; Lallement et al., 2000, 2004). A similar cocktail in which pralidoxime was replaced by another oxime, HI-6, proved to be equally successful (Lallement et al., 1997; Myhrer et al., 2006).

Due to the neuropathological effect of soman, there is a crucial need to monitor the evolution of SRBD over time and to assess the neuroprotection afforded by medications. Assessment of SRBD through histopathology is time-consuming, requires animal sacrifice and provides only snapshots of structural changes at a given time. Above all, this invasive technique is not possible in man. Besides various other tools (clinical observations, analysis of EEG tracings and spectrum, measurement of blood ChE activity, magnetic resonance imaging: review in Carpentier et al., 2008), we were recently interested in a non-invasive audiometric method (Kemp et al., 1990) based on the measurement of distortion product otoemissions (DPOAEs). Our interest stemmed from the hypothesis that soman might induce changes in hearing that might reflect those in brain since the same neurotransmitter systems (mainly ACh and Glu) are involved in both hearing and OP-induced seizures and SRBD (see references in Job et al., 2007). Otoacoustic emissions rely on the contractile properties of the cochlear outer hair cells (OHC) which can generate retrograde wave sounds whose intensity can be captured and recorded by a sensitive microphone inserted into the external auditory canal. DPOAEs were proved useful to evaluate the cochlear function in laboratory and clinical settings following noise and/or ototoxic injuries (Harris, 1990; Mills et al., 1999; Job and Nottet, 2002; Lataye et al., 2003; Job et al., 2004; Pouyatos et al., 2005).

In a first study performed in rats (Job et al., 2007), DPOAEs were measured in a low frequency range (2.3–4.8 kHz) and, immobility being required, under sodium pentobarbital anesthesia. To avoid the possible influence of repetitive anesthesia, measurements were made eight day before (baseline) and either 4 h or 24 h after exposure to a moderate dose of soman (45 μg/kg). This dose produced various symptoms, from almost none to long-lasting convulsions. Interestingly, the pre-soman DPOAE baseline was found to be the lowest in rats that, after the intoxication, displayed the highest brain ChE inhibition, long-lasting convulsions and SRBD. This first observation suggested that DPOAEs might be used as a predictor of susceptibility to soman-induced convulsions and SRBD. The second main finding was that the intoxicated rats showed a decrease of DPOAEs at 4 h post-challenge and the greatest drop was again recorded in the rats displaying the most severe symptoms (i.e. long-lasting convulsions), the highest inhibition of cerebral ChE and extensive SRBD. At 24 h DPOAEs tended to normalize in all the rats. The temporary change of DPOAEs in the acute hours of the intoxication suggested that this audiometric method might be a useful tool to non-invasively foresee the occurrence of SRBD.

The main purpose of the present study was to confirm that DPOAEs could actually be predictive in soman poisoning. For this, two approaches were used: First, we investigated if our previous results could be reproduced in different experimental conditions: (i) the rats were intoxicated with a higher dose of soman (70 μg/kg) to increase the percentage of animals with long-lasting convulsions; (ii), the upper limit of the studied frequency range was extended from 4.8 kHz to 12 kHz to reach frequency regions of best hearing in the rats; (iii), gaseous anesthesia with isoflurane was preferred to sodium pentobarbital as it produces slighter anesthesia followed by fast recovery thus allowing repetitive pre- and post-challenge measurements in the same animals. Second, we wished to demonstrate that the suppression of soman-induced seizures and SRBD could be accompanied by the disappearance of the DPOAE drop observed 4 h after the intoxication. With this aim, supplementary rats were intoxicated and immediately treated with an anticonvulsant and neuroprotective combination of HI-6, AS and AVZ. In these animals, the impact of the therapy on DPOAEs, brain histology and blood and brain ChE activities were assessed. In addition, the cochlear histology was investigated in some of the intoxicated rats.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (300 g; Janvier, France; $n = 126$) served as subjects. Animals were housed in a controlled environment (21 ± 2 °C; 12 h dark/light cycle with light provided between 7.00 a.m. and 7.00 p.m.). Food and water were available *ad libitum*. Procedures were designed in accordance with the regulations regarding the "protection of animals used in experimental and other scientific purposes" from the relevant Directives of the European Community (86/609/CEE). The protocols reported herein were approved by the ethical committee of our institute.

2.2. Drugs, doses and injection routes

Soman (>97% pure as assessed by gas chromatography) was supplied by the Centre d'étude du Bouchet (Vert-le-Petit, France). Solutions were freshly prepared by diluting the initial solution (2 mg/mL in isopropanol) in ice-cold 0.9% (w/v) saline. Soman was administered subcutaneously at 70 μg/kg (~0.8 LD_{50}; 500 μL in saline). All the intoxications were performed between 9.30 a.m. and 11.30 a.m. to reduce possible circadian variations of cholinergic parameters (Elsmore, 1981). HI-6 dichloride was generously provided by DRDC Suffield (Canada). Atropine sulfate (AS) and avizafone (AVZ) were purchased from Sigma–Aldrich (Saint Louis, MO, USA) and Neosystem (Strasbourg, France), respectively. The dose and i.m. route for HI-6 (42 mg/kg), AS (14 mg/kg) and AVZ (3 mg/kg) were chosen according to a previous study on rats by Myhrer et al. (2006). The three drugs were mixed in saline before administration (150 μL) in the hind leg. Isoflurane was from Belamont (France).

2.3. DPOAE

2.3.1. Experimental design

The first group (SOM) of rats ($n = 31$) was administered with soman only. The second group (SOMTREAT) of rats ($n = 22$) was intoxicated and, within 1 min received the treatment mixture composed of HI-6, AS and AVZ. Two control groups were constituted with sham-poisoned animals administered with saline (500 μL; s.c.) instead of soman. In the first control group (SAL; $n = 13$), no supplementary injection was performed after saline. In the second control group (SALTREAT; $n = 20$), the rats were administered, 1 min after saline, with the therapeutic cocktail used in the SOMTREAT group.

The clinical observation was continuous for at least 8 h after the intoxication and on the following morning for at least 30 min. In our previous study (Job et al., 2007), the most significant DPOAE changes were detected only in the animals that experienced long-lasting convulsions appearing within minutes after the intoxication and lasting for at least the following 4 h. Therefore, in the present study, only two symptom subgroups were considered in the SOM group and SOMTREAT group: rats without long-lasting convulsions (with either no signs at all, or mild symptoms, or rare and brief convulsive episodes within the first hour post-soman) noted C− and rats with long-lasting convulsions noted C+. All animals were weighed 1 h before and 24 h after the administration of soman or saline.

2.3.2. Anesthesia

Anesthesia was induced by placing the animals for approximately 3–5 min in a Plexiglas box receiving 5% isoflurane in air at 0.3 L min^{-1} and then maintained (via an adapted mask) with 1.5% isoflurane in air throughout the acquisition of DPOAEs. After the end of the anesthesia, the rats recovered apparently normal behavior within 10–25 min.

2.3.3. DPOAE measurements

DPOAEs were measured in a thermoregulated and silent room using the DPOAE GSI system (Grason-Stadler, Milford, NH, USA). During anesthesia, the probe was carefully inserted into the right auditory canal via a neonatal tip, which allowed tight adaptation into the external auditory canal.

To avoid any circadian variations, all DPOAE measurements were always performed between 13:30 h and 16:30 h. Baseline DPOAEs were measured 24 h before soman (or saline in controls). Post-soman (or post-saline in controls) DPOAEs were performed on each animal 4 h and 28 h post-challenge. DPOAEs were investigated in a frequency range covering from 2601 Hz to 9832 Hz for *f1* and from 3175 Hz to 11988 Hz for *f2* over 24 frequencies.

Conditions of measurements were selected from pilot experiments on control (saline-treated) rats. In these, DP-grams were obtained from each animal using three different protocols of stimulation (expressed in decibel sound pressure level: dB SPL): 70/70 dB SPL; 60/50 dB SPL; 55/35 dB SPL with either 5 runs ($n = 6$) or 3 runs ($n = 14$) applied in succession in the same animal at the three different time points. For each stimulation protocol, no significant change was ever detected in