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Protection by sustained release of physostigmine and procyclidine of soman poisoning in rats

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Abstract

The efficacy of a combinational prophylactic regimen on the lethality, convulsions, and loss of morphological and functional integrities of the brain induced by an organophosphate soman was investigated in rats. The rats were implanted subcutaneously with osmotic minipumps containing the combinational prophylactic regimen composed of physostigmine, a reversible cholinesterase inhibitor, and procyclidine, an *N*-methyl-D-aspartate antagonist possessing anticholinergic action, for 3 days, and intoxicated subcutaneously with soman (160 μg/kg, 1.3 LD_{50}). The doses of combinational regimen in minipumps were optimized to achieve 30–35% inhibition of blood cholinesterase activity by physostigmine and 50–100 ng/ml of blood concentrations of procyclidine as clinically available doses, respectively. In comparison, 1-[([4-(aminocarbonyl)pyridinio]methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium (HI-6, 125 mg/kg) was administered intraperitoneally 30 min prior to the soman challenge in control groups to reduce mortality of rats without affecting convulsions. Soman induced profound limbic convulsions and 30% mortality, leading to increased blood–brain barrier permeability, neural injuries, learning and memory impairments, and physical incapacitation of survived rats pretreated with HI-6. The combinational regimen, at optimal doses without adverse effects on passive avoidance performances (72 μg/kg/h of physostigmine plus 432 μg/kg/h of procyclidine), exerted full protective effects against lethality, convulsions, blood–brain barrier opening, brain injuries, learning and memory impairments, and physical incapacitation induced by soman. Taken together, it is suggested that the combination of physostigmine and procyclidine, at adequate doses, could be a choice to provide the victims of organophosphate poisoning with chance of intensive care for survival and neuroprotection.

Keywords: Soman; Brain injury; Memory impairment; Prophylactic; Physostigmine; Procyclidine

1. Introduction

Organophosphates have been used worldwide as pesticides and are still one of the most threatening warfare agents (Somani, 1992). Recently, organophosphates also emerged as a major threat of terrorism, since the Tokyo subway system had been subjected to attack with sarin (Suzuki et al., 1995; Nagao et al., 1997). Exposure to organophosphates causes tremors, hypersecretion, respiratory distress, coma, and death (Somani, 1992; Taylor, 1985). The acute toxicity of organophosphates is exerted by accumulation of acetylcholine in synapses, neuromuscular, and neuroglandular junctions following irreversible inhibition of acetylcolinesterases in cholinergic nervous system.

In addition to acute cholinergic toxicities, organophosphate poisoning induces epileptiform seizures (McDonough and Shih, 1993; Shih et al., 1991), leading to increase

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in blood–brain barrier permeability (Carpentier et al., 1990), brain injuries (Kim et al., 1999; McDonough et al., 1989; Tryphonas and Clement, 1995), and cardiac failure (McDonough et al., 1989; Tryphonas et al., 1996). It has been demonstrated that such excitotoxic brain injuries were induced in the limbic system, resulting in abnormal behaviors, disturbances in concentration, schizophrenia, depression, insomnia, nervousness, visual abnormalities, and loss of learning and memory functions (McDonough et al., 1986; Metcalf and Holmes, 1969; Raffaele et al., 1987; Sidell, 1997; Tilson et al., 1990).

Traditionally, atropine and an oxime have been used as the standard treatment of organophosphate poisoning for their synergistic antidotal efficacy (Dunn and Sidell, 1989). However, coadministration of 2-pralidoxime or obidoxime with atropine did not exert synergistic protection against the lethality of soman which undergoes rapid 'aging' following inhibition of acetylcholinesterases (Berman and Decker, 1986; Fleisher and Harris, 1965; Talbot et al., 1988). In addition, rapid administration, within the first 5 min, with a high dose of anticholinergics is required for attenuation of convulsions (Lallement et al., 1998). Furthermore, diazepam, an agonist of γ-aminobutyric acid receptors, was not found to exert sufficient neuroprotective activity (McDonough and Shih, 1997) because a long duration after injection of diazepam is necessary for seizure termination, in contrast to a rapid induction, probably 20–30 min, of brain injuries (Lallement et al., 1994; McDonough et al., 1995; Myhrer et al., 2004b).

Recently, we demonstrated that a prophylactic regimen composed of physostigmine and procyclidine fully prevented lethality, convulsions, and brain injuries induced by soman (Kim et al., 2002). Procyclidine antagonizes both muscarinic and nicotinic cholinergic receptors as well as the glutamatergic *N*-methyl-D-aspartate receptor (Gao et al., 1998; McDonough and Shih, 1995; Waelbroeck et al., 1990), and thereby stops convulsions rapidly, which might be a key element for the effective neuroprotection (Kim et al., 1997; Myhrer et al., 2003). In addition, it was demonstrated that physostigmine enhanced the antidotal, anticonvulsant, and neuroprotective effects of procyclidine (Kim et al., 1998, 2002). The effect of centrally active physostigmine, as a prophylactic candidate for the enhancement of the efficacy of antidotes, is believed to be superior to that of centrally inactive pyridostigmine (Harris et al., 1984; Kim et al., 1998).

In spite of high antidotal efficacies, the cognitive side effects of prophylactics are in controversy. In fact, dose-related adverse effects of scopolamine and procyclidine were exhibited from diverse behavioral and cognitive function tests (Myhrer et al., 2004a). In comparison with profound side effects at low doses (0.05–0.15 mg/kg) of scopolamine, a remarkable adverse impact was obtained with only a high dose (6 mg/kg) of procyclidine. Therefore, the doses of prophylactics must be optimized for both the effective protection against organophosphate poisoning and the avoidance of adverse effects on behavioral and cognitive functions.

Such a concept led us to investigate the effectiveness of controlled release of physostigmine and procyclidine, at optimal doses devoid of influence on memory functions because the prophylactic regimen is to be developed as a patch for the prophylaxis of organophosphate poisoning. In the present study, rats were implanted subcutaneously with osmotic minipumps containing optimal doses of physostigmine and procyclidine for 3 days prior to soman poisoning. In addition to convulsions and mortality of rats, blood–brain barrier permeability, microscopic findings, and memory impairments following soman challenge were examined as morphological and functional parameters of brain injuries.

2. Materials and methods

2.1. Materials

Physostigmine salicylate, procyclidine hydrochloride and Evan's blue were from Sigma (St. Louis, USA). Formamide was procured from Merck (St. Whitehouse, USA). ALZET osmotic minipumps (model 2001; release rate 1.0 μl/h for 7 days) were obtained from ALZA (St. Charleston, USA). Soman and 1-[([4-(amino-carbonyl)-pyridinio]methoxy)methyl]-2-[(hydroxyimino)methyl]pyridinium (HI-6) were synthesized in Single Small Scale Facility of Agency for Defense Development, Korea.

For injection, a stock solution of soman (10 mg/ml of 10% isopropyl alcohol) and HI-6 were dissolved in saline immediately before use, and administered in a volume of 1 ml/kg. For continued infusion using osmotic minipumps, physostigmine and procyclidine were dissolved in a solvent including 20% propylene glycol, 15% ethanol, and 65% water containing 0.05% glacial acetic acid.

2.2. Animals

Male specific pathogen-free Sprague–Dawley rats (body weights 240–260 g) were housed in an environmentally controlled room with temperature of 23±2 °C, relative humidity of 55±5%, a 12-h light/dark cycle, and feed and water available ad libitum. The experiments performed here were conducted according to the 'Guide Principles in the Use of Animals in Toxicology' which had been adopted by the Society of Toxicology in 1989.

2.3. Dose-range determination

To obtain profiles of blood concentrations and enzyme inhibition rates of physostigmine and procyclidine,

osmotic minipumps containing various doses of single prophylactic, physostigmine (18–72 μg/kg/h) or procyclidine (114–1152 μg/kg/h), were implanted subcutaneously to the back side of rats according to aseptic procedures of surgery provided by the manufacturer's instructions. After 3-day implantation of minipumps, blood (8 ml) was collected for the assay of cholinesterase activity and the measurement of blood concentrations of physostigmine and procyclidine. In brief, an aliquot (50 μl) of blood was collected into heparinized capillary tubes, and plasma, after centrifugation, was used for the enzyme assay by a slight modification of the method of Ellman et al. (1961) using butyrylthiocholine as a substrate (Kim et al., 1998). Separately, diisopropylfluorophosphate (final 50 μM) was added to the remaining blood samples, and serum was collected by centrifugation. Physostigmine was extracted by serial treatment with 0.7% ammonium hydroxide, *tert*-butyl ether and 0.01 M hydrochloric acid, and then analyzed with a high-pressure liquid chromatograph (Waters 2695 XC-D, Milford, USA) using an internal standard *N*,*N*-dimethylcarbamate (Brodie et al., 1987). Procyclidine was extracted with ethyl acetate-hexane (1:3), 1 M hydrochloric acid and 10 M sodium hydroxide plus ethyl acetate-hexane, and then analyzed with a gas chromatograph (Hewlett Packard 6890, Oxford, USA) using trihexyphenidyl as internal standard (Owen et al., 1989).

From the enzyme inhibition profile, the optimal dose (72 μg/kg/h) of physostigmine was selected and combined with various doses (432–1152 μg/kg/h) of procyclidine. From 1 day after the implantation of minipumps containing physostigmine plus procyclidine, the rats were subjected for the passive avoidance trials for 3 days to obtain the procyclidine doses without influence on the learning and memory functions. Based on the previous (Kim et al., 2002) and preliminary efficacy studies of physostigmine plus procyclidine injection, the optimal doses of physostigmine and procyclidine in minipumps were selected, which achieve 30–35% inhibition of enzyme activity by physostigmine and 50–100 ng/ml of blood concentration of procyclidine.

2.4. Convulsions and mortality

Osmotic minipumps containing 72 μg/kg/h of physostigmine and 432 μg/kg/h of procyclidine, the optimal doses obtained from the dose determination study, were implanted subcutaneously to rats. After 3-day monitoring of enzyme activity and blood concentrations of the drugs, the rats were challenged subcutaneously with a high dose (160 μg/kg, $1.3\times LD_{50}$) of soman. In comparison, HI-6 (125 mg/kg) was administered intraperitoneally 30 min prior to the soman challenge in control groups to reduce mortality of rats without affecting seizures (McDonough and Shih, 1993). After the challenge with soman, the time course of convulsions and mortality of rats were monitored for 24 h (Kim et al., 2002).

2.5. Blood–brain barrier permeability

Rats implanted with minipumps containing physostigmine plus procyclidine for 3 days were injected intravenously with Evan's blue solution (2% in saline, 3 ml/kg), and then challenged with soman. In comparison, HI-6 (125 mg/kg) was administered intraperitoneally 30 min prior to the soman challenge in control groups, while normal control rats were administered with Evan's blue dye alone without soman challenge. After 1-h monitoring of convulsions following soman challenge, whole brain of rats survived was removed after intracardial perfusion with cold saline (100 ml/kg) under ether anesthesia (Carpentier et al., 1990; Petrali et al., 1991). The brain was sliced transversely to pass median eminence for the examination of dye penetration into brain tissues. After the examination of regions of dye leakage, the whole brain was immersed in formamide (4 ml) for dye extraction at 60° for 72 h (Harada et al., 1985). The dye penetrated into the brain was quantified by measuring at 620 nm with a standard curve.

2.6. Memory impairment

For the assessment of memory loss due to brain injuries, the rats that survived soman challenge were subjected to a passive avoidance apparatus (SD Gemini, San Diego, USA). The passive avoidance trials were performed twice a day from 1 day after soman poisoning for the acquisition of memory functions. In a separate set of performances, rats were subjected to the trials from 8 days after recovery from physical incapacitation induced by soman intoxication. The apparatus consists of two compartments equipped with a lamp and a steel-grid floor for electric shock (1 mA for 2 s). On the trials, electric shock was delivered when rats entered the dark compartment from the light room through guillotine door. The latency time of stay in the light room from light-on was recorded. The end-point was set at 300 s, denoting full acquisition of memory.

2.7. Brain injuries

Next day after 4.5-day avoidance performances, whole brain of rats was removed and fixed in 10% neutral-buffered formalin solution. For the evaluation of neural injuries, paraffin-embedded brain sections (4 μm in thickness) were stained with hematoxylin and eosin. Neuronal death and integrity of neuropils in hippocampus, piriform/entorhinal cortices, amygdala, and thalamus were examined under a light microscope (Kim et al., 2002).

2.8. Statistical analysis

The results were expressed as the mean±S.D. Tests of significance were performed using Duncan's multiple-range test after one-way analysis of variance (ANOVA), with $P<0.05$ as a criterion of difference.

3. Results

3.1. Dose–responses

Dose-related blood concentrations and enzyme inhibition rate were obtained by continuous infusion with physostigmine; 3-day implantation of minipumps containing 18, 36, and 72 μg/kg/h of physostigmine led to 2.6, 3.5, and 5.9 ng/ml of blood concentrations and 20.0%, 25.3%, and 33.6% inhibition of enzyme activity, respectively (Table 1). In addition, a similar pattern of blood concentrations, ranging from 28.0 to 202.3 ng/ml, of procyclidine was achieved after implantation of minipumps containing 144–1152 μg/kg/h.

In passive avoidance performances, high doses (≥864 μg/kg/h) of procyclidine with a fixed dose (72 μg/kg/h) of physostigmine significantly delayed the acquisition of memory ($P<0.05$; Fig. 1). At these high doses, however, a full acquisition was obtained by the 4th trial, requiring one more trial than in normal control. In comparison, procyclidine up to 576 μg/kg/h did not affect the learning and memory functions.

From preliminary studies on antidotal effect of physostigmine plus procyclidine injection, 72 μg/kg/h of physostigmine and 432 μg/kg/h of procyclidine, as effective doses which achieve 30–35% inhibition of enzyme activity and 50–100 ng/ml of blood concentration of procyclidine, were selected for the subsequent efficacy studies.

3.2. Effect on convulsions and mortality

A lethal dose (160 μg/kg, 1.3 LD_{50}) of soman induced cholinergic signs and profound convulsions in all rats pretreated with HI-6 (125 mg/kg), leading to tonic–clonic convulsions during 8–90 min after poisoning (Table 2). Three out of ten animals pretreated with HI-6 died within 1 h following soman poisoning. In contrast, the rats implanted with minipumps containing physostigmine (72 μg/kg/h) plus procyclidine (432 μg/kg/h) 3 days before soman poisoning did not show toxic signs other than lowered activity, indicative of a transient immobility following intoxication (Myhrer et al., 2004b). All the animals pretreated with physostigmine and procyclidine survived the soman challenge.

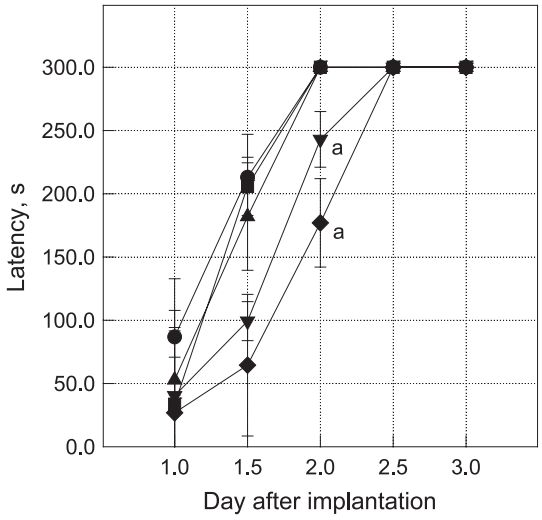

Fig. 1. Passive avoidance performances from 1 day after implantation of osmotic minipumps containing various concentrations of procyclidine in combination with a fixed concentration of physostigmine (72 μg/kg/h). Test of significance was performed when the latency time in control rats reached end-point of 300 s. [a]Significantly different from normal control ($P<0.05$). ●, Normal control; ■, procyclidine 432 μg/kg/h; ▲, procyclidine 576 μg/kg/h; ▼, procyclidine 864 μg/kg/h; ◆, procyclidine 1152 μg/kg/h.

3.3. Effect on blood–brain barrier permeability

During 1 h of convulsions, a marked penetration of Evan's blue dye was observed mainly in ventral thalamus, and less frequently in hypothalamus, amygdala, and ventricles surrounding hippocampus of rats pretreated with HI-6 (Fig. 2). In these animals that underwent convulsions, significant increase in the penetration of dye was confirmed by extracting and quantifying the dye from the brain tissues ($P<0.05$; Table 1). In comparison,

Table 1
Mean blood concentrations and enzyme inhibition following 3-day implantation of osmotic minipumps containing physostigmine or procyclidine

Treatment	Dose (μg/kg/h)	Blood concentration (ng/ml)	Enzyme inhibition (%)
Physostigmine	18	2.6±1.3	20.0±5.8
	36	3.5±2.1	25.3±7.4
	72	5.9±2.7	33.6±6.1
Procyclidine	144	28.0±7.5	–
	288	44.8±17.8	–
	432	73.7±21.4	–
	576	105.4±30.7	–
	864	173.6±46.6	–
	1152	202.3±30.3	–

Table 2
Effect of HI-6 (125 mg/kg) or physostigmine (72 μg/kg/h) plus procyclidine (432 μg/kg/h) on convulsions, lethality, and blood–brain barrier permeability induced by soman (160 μg/kg, 1.3 LD_{50})

Treatment (dose) ratio	Convulsion response ratio	Mortality response ratio	Evan's blue penetration (μg/brain)
Normal control	–	–	0.21±0.17
HI-6 injection (125 mg/kg)	10/10	3/10	13.42±4.82[a]
Physostigmine +procyclidine pump (72+432 μg/kg/h)	0/10	0/10	0.41±0.23

While HI-6 was pretreated intraperitoneally 30 min prior to poisoning with soman, osmotic minipumps containing physostigmine plus procyclidine were implanted subcutaneously 3 days before soman challenge.

[a] Significantly different from normal control ($P<0.05$).

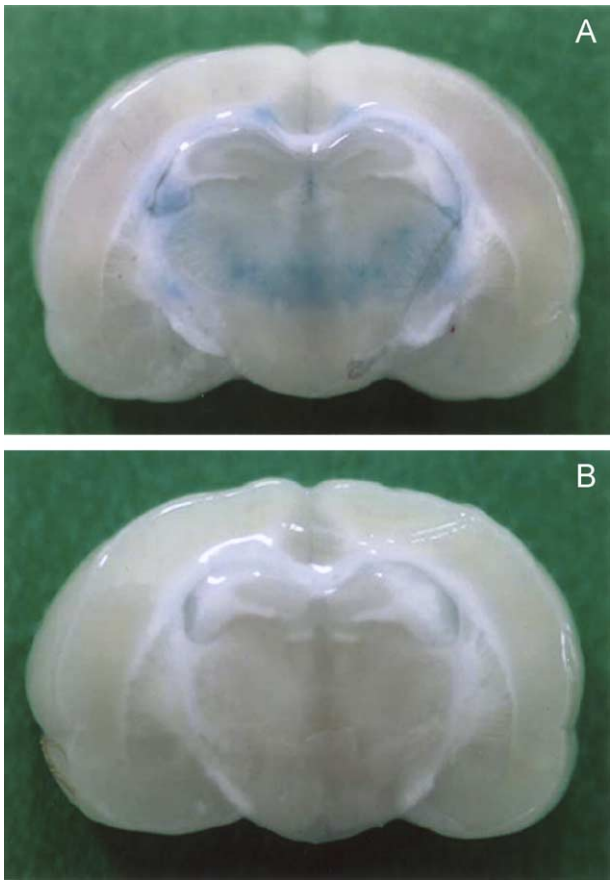

Fig. 2. Effect of HI-6 (125 mg/kg) or physostigmine (72 μg/kg/h) plus procyclidine (432 μg/kg/h) on blood–brain barrier permeability induced by soman (160 μg/kg, 1.3 LD_{50}). Marked penetration of Evan's blue is observed in ventral thalamus of animals treated with HI-6 (A), in comparison with full prevention in rats implanted with physostigmine plus procyclidine (B).

the dye penetration was prevented by pretreatment with physostigmine plus procyclidine to the control level of normal rats that was administered with only Evan's blue, without exposure to soman, and washed by intracardial perfusion with saline.

3.4. Effect on memory impairment

The rats that survived soman challenge were subjected to the passive avoidance performances from 1 day after poisoning to test the integrity of learning and memory functions. In contrast to a rapid acquisition of memory at the 3rd trial in normal rats administered with vehicle, a complete loss of learning and memory functions was observed in soman-intoxicated animals pretreated with HI-6 ($P<0.05$), in which no memory acquisition was obtained during 8 trials for 4.5 days (Fig. 3). Furthermore, the animals showed severe physical incapacitation, followed by ataxia, emaciation, and body weight loss. In contrast, the rats pretreated with physostigmine plus procyclidine were found to preserve the learning and memory functions,

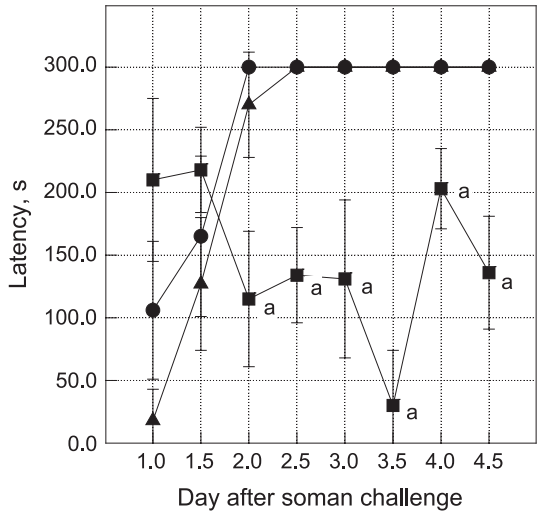

Fig. 3. Effect of HI-6 (125 mg/kg) or physostigmine (72 μg/kg/h) plus procyclidine (432 μg/kg/h) on passive avoidance performances of rats assessed from 1 day after challenge with soman (160 μg/kg, 1.3 LD_{50}). Test of significance was performed when the latency time in control rats reached end-point of 300 s. [a]Significantly different from normal control ($P<0.05$). ●, normal control; ■, HI-6; ▲, physostigmine plus procyclidine.

although the latency did not reach the end-point of 300 s on the 3rd trial.

In a separate set of learning and memory tests, the rats were subjected to passive avoidance trials from 8 days after the soman challenge to avoid the effect of physical incapacitation on passive avoidance performances. As a result, the profile of latency of rats pretreated with HI-6 was markedly different from that of early trials performed 1 day after soman poisoning (Fig. 4). In this performance, the memory acquisition was obtained on the 4th

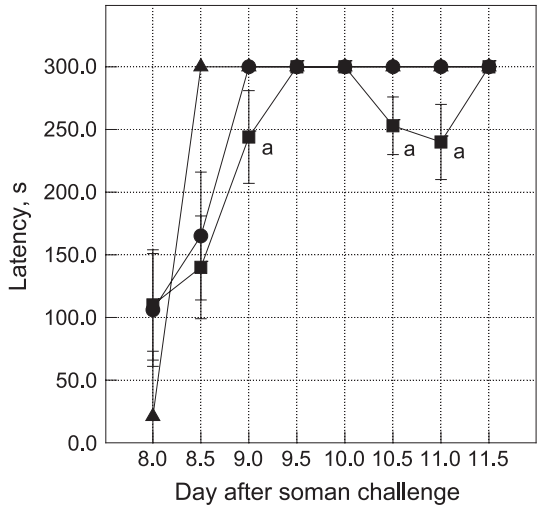

Fig. 4. Effect of HI-6 (125 mg/kg) or physostigmine (72 μg/kg/h) plus procyclidine (432 μg/kg/h) on passive avoidance performances of rats assessed from 8 days after challenge with soman (160 μg/kg, 1.3 LD_{50}). Test of significance was performed when the latency time in control rats reached end-point of 300 s. [a]Significantly different from normal control ($P<0.05$). ●, normal control; ■, HI-6; ▲, physostigmine plus procyclidine.

trial, although the retention of memory once acquired was not fully maintained thereafter ($P<0.05$). In addition, the animals pretreated with physostigmine plus procyclidine reached 300 s of latency on the 2nd trial, compared to full acquisition on the 4th trial when subjected to the performances from 1 day after exposure to soman.

3.5. Effect on brain injuries

Tonic–clonic convulsions in rats pretreated with HI-6 led to dark shrinkage degeneration and loss of neurons as examined on the 5th day following soman challenge. The neuronal death was observed predominantly in hippocampus (Fig. 5A) and piriform cortices. In severe cases, the neurodegeneration and malacia were found to spread to all hippocampal formation (CA1–CA4 and dentate gyrus), amygdala, thalamus, and entorhinal cortex. Such histopathological changes are not seen in the brain of rats implanted with minipumps containing the combinational regimen of physostigmine plus procyclidine in all brain regions observed (Fig. 5B).

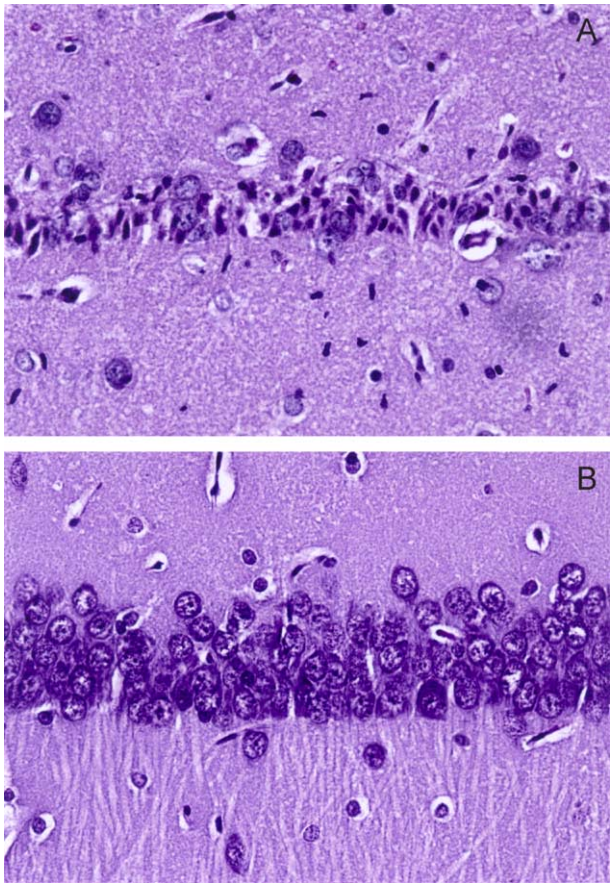

Fig. 5. Effect of HI-6 (125 mg/kg) or physostigmine (72 μg/kg/h) plus procyclidine (432 μg/kg/h) on hippocampal injury induced by soman (160 μg/kg, 1.3 LD_{50}). Extensive degeneration and loss of pyramidal neurons are observed in the brain of animals treated with HI-6 (A), in comparison with intact features of hippocampus of rats implanted with physostigmine plus procyclidine (B).

4. Discussion

Because of low effectiveness of therapeutic antidotes in emergency situations, some simple prophylactic regimens containing physostigmine and anticholinergics, such as trihexyphenidyl and scopolamine, were proposed as one of the effective measures for survival and neuroprotection from chemical warfare and terrorism (Lim et al., 1988, 1991; Meshulam et al., 1995; Philippens et al., 1996, 2000a; Wetherell, 1994). In a previous study, we demonstrated that a combinational pretreatment with physostigmine and procyclidine exerted additive protection against soman poisoning, in which protection ratios of 1.92–5.07 (fold of control LD_{50}) were achieved with 0.3–6 mg/kg of procyclidine in combination with 0.1 mg/kg of physostigmine (Kim et al., 2002). Furthermore, a relatively low dose (1 mg/kg) of procyclidine combined with physostigmine (0.1 mg/kg) fully prevented rats from lethality, convulsions, and brain injuries induced by 1.3 LD_{50} of soman. Recently, it was confirmed that appropriate doses of the combinational prophylactics were found to exhibit complete protection up to 1.6 LD_{50} and partial effect against 2 LD_{50} of soman (Myhrer et al., 2004b).

In spite of beneficial effect, a high dose (6 mg/kg) of procyclidine was found to cause cognitive side effects (Myhrer et al., 2004a). Hence, we optimized the doses of physostigmine and procyclidine, based on the enzyme inhibition rate, blood concentrations, and the influence on passive avoidance performances. Because adequate enzyme inhibition (20–40%) by carbamates, in combination with anticholinergics, was found to be required for the effective protection against organophosphate poisoning (Cook and Kolka, 1992; Dunn and Sidell, 1989; Wetherell, 1994), 72 μg/kg/h of physostigmine was selected from 3-day implantation of osmotic minipumps. At the dose of physostigmine, 5.9 ng/ml of mean blood concentration and 33.6% inhibition of enzyme activity were obtained, wherein no influence on the learning and memory trials was observed (data not shown).

To match the adverse effects with blood concentrations of procyclidine, the rats implanted with minipumps containing various doses of procyclidine and a fixed dose (72 μg/kg/h) of physostigmine were subjected to passive avoidance performances. On the trials, 864 μg/kg/h of procyclidine, resulting in 173.6 ng/ml of blood concentration, delayed the acquisition of memory (Fig. 1). On the other hand, 576 μg/kg/h of procyclidine, leading to 105.4 ng/ml of blood concentration, did not affect the learning and memory functions. Such results are consistent with previous observations that 1–3 mg/kg of procyclidine led to 50–150 ng/ml of blood concentrations 30 min after subcutaneous injection (unpublished data), and did not affect preference for novelty (Myhrer et al., 2004a). Despite the effect of high doses (≥864 μg/kg/h) of procyclidine on the delay of memory acquisition, a full acquisition was obtained by the 4th trial, requiring only one more trial than in normal control, indicating that procyclidine is well tolerated in rats.

It was expected that the possible adverse effects of carbamates and anticholinergics used prophylactically might be offset by each other (Berry and Davies, 1970; Lim et al., 1991; Philippens et al., 1996, 2000a). In our preliminary study, the subcutaneous maximum sign-free dose (3 mg/kg) of procyclidine was increased to 10 mg/kg by coadministration of physostigmine (0.1 mg/kg) in step-through tests (unpublished data). Moreover, adverse effects and mortality were markedly attenuated and eliminated in dogs implanted with minipumps containing both physostigmine and procyclidine, although profound symptoms and death were induced by each extremely high dose of physostigmine (90 μg/kg/h) or procyclidine (900 μg/kg/h; Huang et al., 2003). However, it was found that combination of physostigmne and anticholinergics not always offset their adverse effects; that is, physostigmine rather potentiated the reduction of preference for novelty induced by scopolamine and procyclidine (Myhrer et al., 2004a). Therefore, it is believed that the combination of carbamates and anticholinergics may affect differently the behavioral, motor, and cognitive functions.

Furthermore, there were marked differences in the sensitivity to test methods for physiological, behavioral, motor, and cognitive functions. Among them, passive avoidance trial was one of the most sensitive parameters for procyclidine, showing maximum sign-free dose of 3 mg/kg in step-through test, in comparison with that of 10 mg/kg in Morris water-maze test (unpublished data). Therefore, we selected 432 μg/kg/h of procyclodine, a dose much lower than the sign-free dose because we previously showed enough protective effects with 1 mg/kg of procyclidine in combination with physostigmine (0.1 mg/kg) against 1.3 LD_{50} of soman (Kim et al., 2002). In the present study, the blood concentration of procyclidine was 73.7 ng/ml which was slightly higher than that of injection of 1 mg/kg (50 ng/ml), and was comparable to that for the treatment of Parkinsonism (Dean et al., 1980; Whiteman et al., 1985). In addition, the enzyme inhibition rate by 72 μg/kg/h of physostigmine infusion was 33.6%, comparable to 36% by 0.1 mg/kg of physostigmine injection in Sprague–Dawley rats (unpublished data), although it was reported that 67% of inhibition was obtained in Wistar rats with the same dose (Lennox et al., 1985).

We attempted to evaluate the efficacy of combinational prophylactic regimen based on the both morphological and functional integrities of the body, including convulsions, mortality, blood–brain barrier permeability, microscopic findings, and learning and memory performances. Convulsion-related dye leakage was fully prevented by pretreatment with physostigmine plus procyclidine, in parallel with the protection of brain injuries. Because the dye penetrated into brain tissues can be extracted easily with formamide (Harada et al., 1985), it is suggested that the dye quantification might be a good tool for the evaluation of the efficacy of anticonvulsants. Interestingly, it was demonstrated that blood–brain barrier opening was induced predominantly in thalamus during convulsions following soman intoxication (Carpentier et al., 1990; Petrali et al., 1991; the present study), although the most susceptible regions were somewhat different according to the convulsive agents (Nitsch and Klatzo, 1983). It is important to note that the topographical distribution of early vascular leakage following soman intoxication was similar to that of delayed apoptotic neural injury (Carpentier et al., 1990; Kim et al., 1999), implying a relationship between "vasogenic" edema and apoptotic change. In comparison, hippocampus and pyriform/entorhinal cortices, the "cytotoxic" regions that exerted poor permeability of blood–brain barrier during soman-induced convulsions (Carpentier et al., 1990; the present study), exhibited predominantly necrosis (Kim et al., 1999). Therefore, it is not excluded that the neural apoptosis may be related to vascular responses during central seizures.

Noteworthy, the convulsive rats pretreated with HI-6 exhibited a long-term physical incapacitation, showing incoordination, prolonged clonic epilepsy, emaciation, and loss of body weights. In addition, the stressed rats did not show efficiency in the acquisition of memory as subjected to passive avoidance performances from 1 day after soman poisoning. Because it was not excluded that such a full loss of learning and memory functions partially come from damaged physical integrity, we tested the rats after recovery of body functions for the avoidance trials. In comparison with the poor early performances, the rats recovered from the physical incapacitation responded to the trials. In spite of the response, however, the rats exhibited a delayed acquisition as well as deficits in the retention of memory obtained, suggestive of an irreversible impairment of memory functions following extensive loss of neurons. On the other hand, the rats implanted with combinational prophylactic regimen fully gained memory from the 2nd trial, although the rats showed a slight delay in memory acquisition when subjected to the trials from 1 day after poisoning.

It is of interest to note that the efficacy of carbamates in combination with anticholinergics was high in monkeys followed by guinea pigs, dogs, rabbits, mice, chickens, and rats (Berry and Davies, 1970; Dirnhuber et al., 1979; Gordon et al., 1978). In previous results (Kim et al., 2002), the efficacy of physostigmine (0.1 mg/kg) alone or in combination with low doses (0.3–3.0 mg/kg) of procyclidine in guinea pigs was much higher than that in rats which might be due to the species differences in soman-detoxifying enzymes such as carboxylesterase (Maxwell et al., 1988). In addition, it was proposed that continuous infusion of physostigmine via osmotic minipumps in primates was much more effective than a bolus injection or continuous infusion in rodents (Lim et al., 1988; Philippens et al., 2000b). In a separate experiment, we observed a high protective effect of a prototype of patch system containing physostigmine and procyclidine against soman in beagle dogs (manuscript in preparation). Thus, it is expected that a remarkable effect could be achieved with a long-lasting

patch containing physostigmine and procyclidine in primates including human.

In the present study, the efficacy of combinational prophylactic regimen was evaluated using osmotic minipumps as a substitute method for the sustained release of a patch system. The combinational regimen, at doses without adverse effects itself, fully protected against the lethality and both morphological and functional injuries induced by a lethal dose of soman. Altogether, from the previous and present studies, it is proposed that the combination of physostigmine and procyclidine, at adequate doses, could be a choice to provide the victims of organophosphate poisoning with chance of intensive care for survival and neuroprotection.

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