Title page

Transdermal patch containing physostigmine and procyclidine protects rhesus monkeys against VX intoxication

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Abbreviations: LD₅₀: median lethal dose, 2-PAM: 2-pralidoxime, AchE: acetylcholinesterase, PYS: pyridostigmine, PHS: physostigmine, PC: procyclidine, BBB: blood-brain barrier, C_{max}: maximum concentration of drug in the serum, AUC_∞: area under the concentration-time curve from zero time extrapolated to infinity, T_{max}: mean time to maximum concentration

ABSTRACT

VX is an organophosphate cholinesterase inhibitor known as a chemical warfare agent. This study was designed 1) to determine the acute toxicity of VX in male rhesus monkeys by subcutaneous administration, 2) to evaluate the efficacy of a transdermal patch containing physostigmine and procyclidine. The median lethal dose (LD₅₀) of the subcutaneous injection of VX was 15.409 ug/kg, which was calculated using the up-and-down dose selection procedure based on deaths occurring within 48 h. To test the efficacy of the transdermal patch, rhesus monkeys were treated with a patch (5×5 cm²) alone or in combination with post-exposure therapy comprising atropine plus 2-pralidoxime (2-PAM), and then administered subcutaneous injection of VX at various doses. The rhesus monkeys pretreated with the patch alone were 100% protected against 1.5×LD₅₀ of VX, while the rhesus monkeys treated with the patch, atropine, and 2-PAM were 100% protected against 50×LD₅₀ of VX. This study demonstrated that patch pretreatment in conjunction with atropine and 2-PAM treatment is an effective regimen against high doses of VX.

Keywords: Nerve agent, Rhesus monkey, Transdermal patch, VX

51 **Introduction**

52 VX, an organophosphorus compound and nerve agent known for its use as a chemical warfare
 53 agent, is an extremely lethal chemical (Zurer, 1998). It is a potent and irreversible acetylcholinesterase
 54 (AChE) inhibitor. The high toxicity of VX contributes to its specific reaction with AChE. Unlike G
 55 agents, VX is more stable, more resistant to detoxification, less volatile, and penetrates skin more
 56 efficiently (U.S. Department of the Army, 1974). VX are attractive chemical weapons to terrorists
 57 because they are fast-acting even in minute quantities and can cause death by multiple routes,
 58 including inhalation (Munro, 1994). In the past, VX was used by the Aum Shinrikyo cult in Japan to
 59 kill people (Zurer, 1998). Recently, VX was used for an assassination in Malaysia in 2017 (Nakagawa
 60 and Tu, 2018).

61 Current antidotes for VX intoxication are composed of a combination of pretreatment with
 62 the reversible AChE inhibitor pyridostigmine (PYS) and post-exposure therapy with a three-drug
 63 regimen consisting of atropine sulfate, 2-pralidoxime (2-PAM), and diazepam (Dunn and Sidell,
 64 1989; Shih et al., 2003; Vrdoljak et al., 2006). Although PYS effectively inhibits the enzyme AChE in
 65 a reversible manner, PYS can hardly penetrate the blood-brain barrier (BBB). Therefore, PYS only
 66 acts on the peripheral nervous system (Rickett et al., 1987). VX easily passes into the BBB because
 67 of its high lipophilicity, and both the central and peripheral nerves are severely damaged (U.S.
 68 Department of the Army, 1974). Recently, physostigmine (PHS) has been proposed as an alternative
 69 to PYS (Solana et al., 1990). Previous studies have reported that PHS is very effective against sarin
 70 or soman poisoning (Cho et al., 2012; Philippens et al., 2007). However, PHS has a short half-life in
 71 plasma and a narrow therapeutic efficacy, thus requiring administration as a sustained release
 72 formulation (Jenner et al., 1994). Nevertheless, the antidotal efficacies of PHS augmented
 73 synergistically when combined with procyclidine (PC) or scopolamine (Myhrer et al., 2010; Myhrer
 74 et al., 2004; Choi et al., 2004). Procyclidine is an antagonist of cholinergic and NMDA receptors
 75 (McDonough Jr. and Shih, 1995). It functions as an anti-convulsant and neuroprotective (Kim et al.,
 76 1997).

Our group has studied a transdermal patch system on a long-term basis to find better alternatives to nerve agents. In a previous study, we developed a transdermal patch containing PHS and PC and demonstrated that a prototype of this transdermal patch system fully protected rats, beagle dogs, and rhesus monkeys against soman poisoning (Cho et al., 2012; Choi et al., 2004; Kim et al., 2005). In addition, the patch system showed attenuation of clinical signs and seizures induced by soman toxicity. These effects led to the minimization of brain injury.

In this study, we first analyzed the drug concentration in the blood after applying patches on rhesus monkeys. Next, we evaluated the median lethal dose (LD₅₀) of VX by subcutaneous injection in rhesus monkeys. To the best of our knowledge, this experiment has not been performed on rhesus monkeys before. Third, the effectiveness of the patch, alone or in combination with an antidote consisting of atropine plus 2-PAM were evaluated based on survival rate and clinical signs against VX intoxication in the rhesus monkeys.

MATERIALS AND METHODS

Chemicals

Atropine sulfate and 2-pralidoxime chloride were purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, MO). For animal injection, atropine (0.5mg/kg) and 2-pralidoxime (15mg/kg), based on the field formulas (Field Manual, FM 8-285) were dissolved in 0.9% saline and administered in a volume of 0.1ml/kg. VX of 98% purity were synthesized in single small scale facility of Agency for Defense Development, Republic of Korea. Stock solutions (2.0 mg/ml) of VX were prepared in iso-propanol and subsequent dilutions were performed in 0.9% saline for injection immediately before administration to animals.

Transdermal patch

Matrix-type transdermal patch was made as previously described (10). In briefly, for the preparation of patch, desalted procyclidine (8.7mg) and physostigmine (34.8mg) in 7×7 cm² were

103 dissolved in ethyl acetate-ethanol and mixed with an acrylic adhesive DURO-TAK 387-2287/87-287
104 (National Starch and Chemical Co., USA).

105

106 **Animals**

107 The fifty six male rhesus monkeys (*Macaca mulata*) were obtained from China by Woo Jeong Bio
108 (Woo Jeong Bio, Suwon, South Korea). These monkeys were quarantined on arrival for one month and
109 monitored for health condition. The animals were 3-4 years old and their body weights ranged
110 between 3.0 and 4.5 kg. The animals were individually housed in stainless cages and maintained in a
111 temperature (23± 2°C) and relative humidity (55±3%) controlled vivarium under a 12h light-dark
112 cycle. Water was available ad libitum and all monkeys are fed commercial primate pellets and fresh
113 fruit. The Animal Care and Use Committee at the Agency for Defense Development approved the
114 rhesus monkey experiments (ADD-IACUC-17, 18, 19).

115

116 **Drug concentration and Measurement of AchE activity in blood**

117 To obtain profiles of blood concentration of PHS, PC and AchE inhibition rates, various size (3×4cm²,
118 5×5cm², 7×7cm²) of patch were attached to the dorsal area of rhesus monkey after removal of the
119 hair with clipper and a depilation cream (Table 2). Blood was collected with heparinized tube at 0, 2,
120 4, 6, 8, 12, 24, 48, 72, 96 and 144 hours. AchE activity in blood was determined using modified
121 Ellman method (21).

122

123 **LD₅₀ of VX**

124 Five monkeys were given doses of VX ranging from 20 to 12.6 µg/kg by subcutaneous (SC) challenge
125 (Table 1). The experiments were performed using a sequential up-and-down method for small scales
126 (19) Using this approach, a first animal was injected at specific dose and the outcome obtained before
127 the next animal was challenged. The starting dose was 20 µg/kg, which is approximately halfway
128 between the reported VX intravenous administration LD₅₀ for rhesus monkeys (20). The dose of VX

was usually increased if an animal lived and decreased if an animal died within 48hour. An estimate of the LD₅₀ and confidence intervals were calculated by up-and-down method.

Efficacy of transdermal patch and combination therapy against VX toxicity.

5×5 cm² patch was attached on the dorsal area of the respective animal after removal of hair with a clipper and a depilation cream. The subjects were challenged with 2.0 ~ 1.5×LD₅₀ VX by SC (Table 3). The challenge dose was calculated from the LD₅₀ calculation experiment described above. For analyzing synergistic effect of combination therapy composed of transdermal patch and antidote, the monkeys, attached patch for 24 hours, were intramuscularly challenged with 10.0 ~ 100×LD₅₀ of VX. In addition, the traditional antidote composed of atropine plus 2-PAM were treated intramuscularly within 1 minute after VX exposure (Table 4). The animals were monitored for cholinergic symptoms and survival.

RESULTS

Pharmacokinetic analysis of PHS and PC by application of transdermal patch

The drug pharmacokinetic parameters in the blood were summarized in the supplementary material. Pharmacokinetic parameters of PHS and PC in rhesus monkeys following application were evaluated at 0, 2, 4, 6, 8, 12, 24, 48, 72, 96 and 144 hours. The maximum concentration of PHS in the serum (C_{max}) and the area under the concentration-time curve from zero time extrapolated to infinity (AUC_∞) increased almost proportionally after patch application. The mean time to maximum concentration (T_{max}) ranged from 5.2 to 12.0 h after patch application, then decreased gradually during the 3-day application. For the 3×4 and 5×5 patch, the drug levels showed similar kinetics in the blood, whereas the drug concentration of the 7×7 patch was higher than that of the patch during the 3-day application. Twenty-four hour application of the patch resulted in PHS and PC concentrations of 2.4–20.8 ng/ml and 45.3–115.0 ng/ml, respectively, in the blood. The PHS level returned to baseline three days after application, and the PC level returned to baseline three days after

155 application.

156

157 **AchE inhibition in blood**

158 Blood AchE inhibition reached its maximum level 6 h - 24h after patch application, which is
159 consistent with the profile of PHS concentration. However, AchE inhibition rate was maintained at
160 26% for six days and slightly decreased in a time-dependent manner (Fig. 1).

161

162 **LD₅₀ evaluation of VX**

163 For LD₅₀ evaluation of VX by subcutaneous administration in rhesus monkeys, a series of
164 administrations was carried out according to the rule of increasing the dose following a survival and
165 decreasing the dose following a death (Table 1). Within minutes of VX injection after a death,
166 monkeys showed severe chewing and facial automatisms. This was followed by strong and
167 continuous tremors throughout the body. This phase was followed by seizure, prostration, and
168 unconsciousness. At the starting dose, death occurred within 24 h. At the second dose, the monkeys
169 received 15.8 ug/kg of VX and exhibited delayed onset of seizures. After termination of the seizures,
170 the animal slowly restored consciousness, although clinical signs persisted for 24 h. The surviving
171 monkeys were able to sit in their cage 24 h after administration. The third dose had similar results as
172 the first. At the fourth administration, animals dosed with 15.8 ug/kg of VX elicited severe signs of
173 VX intoxication, and died within 24 h. At the fifth administration, animals survived at a dose of 12.6
174 ug/kg; however, they showed tremor, fasciculation, and salivation for 12 h after which normal
175 behavior returned.

176

177 **Efficacy of transdermal patch**

178 All animals exposed to VX were carefully monitored for clinical signs and mortality. The monkeys
179 treated with patch for 24 h were exposed to various doses, and the results are summarized in Table 3.
180 The patch group exposed to a 1.5×LD₅₀ dose showed mild symptoms of intoxication, such as transient

tremors and seizures on the day of VX challenge. Weak locomotor activity was observed after 48 h, and then returned to normal three days after VX challenge. Monkeys with patches, exposed to $2 \times LD_{50}$ dose (30.8 ug/kg) of VX exhibited salivation, tremors, and defecation followed by seizures, and three monkeys out of five died within 24 h. For further experiments on the combinational effects of the prophylactic patch and the therapeutic atropine and 2-PAM, patch-treated monkeys were additionally administered atropine and 2-PAM one minute after injecting various doses of VX. The results are summarized in Table 4. Despite the intoxicative symptoms induced by VX challenge, monkeys treated with patch plus antidote had 100% survival against $30 \times LD_{50}$ of VX. However, monkeys treated with antidote alone died within 24 h. Remarkably, monkeys treated with patch plus antidote had 50% survival against $100 \times LD_{50}$ of VX.

DISCUSSION

Carbamates have demonstrated effective antidotal efficacy as anticholinergic drugs against nerve agents in guinea pigs and marmoset monkeys (Leadbeater et al., 1985; Philippens et al., 1998). Pretreatment with carbamates attenuates seizure and brain damage (Shin et al., 1992; McDonough Jr. and Shih, 1993). Therefore, our group has focused on developing a simple prophylactic regimen containing carbamates such as PHS. A previous study demonstrated that a combination of PHS and PC was effective in the prevention of lethality and seizure against organophosphorus intoxication (Kim et al., 2002). In addition, continuous infusion of PHS and PC by osmotic pump was found to be highly effective (Choi et al., 2004). More recently, a transdermal patch containing PHS and PC was developed, and its prophylactic efficacy against soman poisoning was demonstrated in rhesus monkeys and beagle dogs (Cho et al., 2012; Kim et al., 2005). In this study, we showed that a transdermal patch alone or in combination with traditional antidotes protects rhesus monkeys against VX. A transdermal patch alone containing PHS 34.8 mg and PC 8.7 mg protected rhesus monkey against $1.5 \times LD_{50}$ of VX. Furthermore, the treatment regimen composed of pretreatment with patch and post-exposure treatment with atropine plus 2-PAM exerted synergistic survival against $50 \times LD_{50}$

207 of VX. In contrast, monkeys that received only post-exposure therapy with atropine plus 2-PAM
 208 showed a 50% survival rate against 50×LD₅₀ of VX. The surviving monkeys exhibited profound
 209 symptoms of intoxication, such as tremors and seizures. PHS plays a role in inhibiting AchE
 210 temporarily, thus shielding AchE from permanent inhibition by inflow of the nerve agent (Bartolini
 211 et al., 1973). In addition, PHS can protect the central nervous system because it is an unquaternized
 212 carbamate that penetrates the BBB (Solana et al., 1990). Therefore, PHS has been studied as a
 213 promising prophylactic agent against organophosphorus intoxication. In particular, when PHS was
 214 used with PC, synergistic effects were observed in marmoset monkeys and guinea pigs (Philippens
 215 et al., 2007).

216 PC is an antagonist of both nicotinic and muscarinic receptors. Therefore, it has antagonistic
 217 activity on NMDA receptors. Involuntary seizure after nerve agent intoxication stimulates NMDA
 218 signaling. Thus, treatment regimens including PHS (carbamate) combined with PC (anti-NMDA)
 219 would be synergistic against organophosphorus toxicity and brain damage after nerve agent
 220 intoxication. Our group performed brain histopathology to confirm whether the patch attenuated brain
 221 injury in a previous study. Severe neuropathological changes, such as eosinophilic neuronal injury,
 222 neuronal vacuolation, and shrunken neurons as a result of seizure were observed in the atropine plus
 223 2-PAM-treated animal brains. In contrast, patch-treated animal brains showed normal features of
 224 histology (Cho et al., 2012). In this respect, the patch demonstrated brain protection against a nerve
 225 agent.

226 Rhesus monkeys have been used previously as a non-human primate research species of
 227 choice to evaluate nerve agent toxicity and efficacy of medical countermeasures. However, the supply
 228 of rhesus monkeys has been reduced recently, and the availability of animals has greatly decreased.
 229 The LD₅₀ of VX administered through the subcutaneous, intramuscular, and intravenous routes in
 230 rhesus monkeys has not been published. Previous studies performed small-scale experiments using 2
 231 or 3 monkeys and showed that rhesus monkeys treated with VX developed tremors, severe seizures,
 232 and death (Raveh et al., 1997). Therefore, researchers have predicted the LD₅₀ dose of VX on the

basis of the results of the following animals. The LD₅₀ of VX in mice, rats, rabbits, and guinea pigs was valued at 9 to 16 ug/kg (Wolfe et al., 1987). The LD₅₀ dose of VX in cats was reported at 5 ug/kg by intravenous route (Rickett et al., 1986). These differences were thought to be related to endogenous carboxylesterase in the body of animals (Maxwell et al., 1992). As a result, the LD₅₀ of VX administered intravenously was estimated to be < 7.5 ug/kg. The present study demonstrates the LD₅₀ of VX (15.4 ug/kg, sc) in rhesus monkeys for the first time. The subcutaneous LD₅₀ dose was two times higher than that of the predicted intravenous LD₅₀. In the soman agent, the intravenous LD₅₀ dose was 5.5 ug/kg and the subcutaneous LD₅₀ dose was 12.3–13.0 g/kg in rhesus monkeys. Therefore, the VX LD₅₀ dose is considered to be similar to that of soman LD₅₀ in rhesus monkeys. It should be noted that LD₅₀ evaluation in this study using the up-down method was performed with fewer number of animals than with other methods such as probit analysis in previous studies. Notably, this result is considered an important administration route in the efficacy test of medical countermeasures for nerve agents. The present study also showed that the signs of VX intoxication in rhesus monkeys were consistent with what has been described in previous experiments. Within 20 min of VX injection, chewing and facial spasm were observed, followed by continuous tremor and convulsion within 30 min.

This study included a large-scale experiment using 60 monkeys and demonstrated that applying patches alone protected monkeys against 1.5×LD₅₀ of VX, while combined administration with antidotes protected monkeys against 50×LD₅₀ of VX. As a result, the preventive application of patches and the therapeutics of antidotes such as atropine and 2-PAM are very effective against VX. Recently, this transdermal patch cleared a nonclinical study and is currently under clinical trial.

Acknowledgements: This work was supported by grant from Agency for Defense Development, Republic of Korea (571665-271987001, 671665-271987001, 771665-271987001).

Funding: This work was supported by the Agency for Defense Development, Republic of Korea

259 (571665-271987001, 671665-271987001, 771665-271987001)

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Figure Legends

Fig. 1. Analysis of blood concentration of physostigmine (A), procyclidine (B) and AchE inhibition rate (C) during attachment with patch.

368 Table 1. Summary of animal responses and LD₅₀ calculation for the Up and Down method.

Dose (µg/kg)	Log ₁₀ Dose			Response (animal No.)		
20.0	1.3	X		X		
15.8	1.2		O		X	
12.6	1.1					O

369

<p>O = 48hr survival, X= Fatality</p> <p>$LD_{50} = X_f + (K \times d)$</p> <p>$LD_{50} = 1.1 + (0.878 \times 0.1)$</p> <p>$LD_{50} = 1.1 + 0.0878$</p> <p>$LD_{50} = 1.1878(\log_{10}) \pm (0.61 \times 0.1)$</p> <p>$LD_{50} = 15.40991 \mu\text{g/kg} \pm 1.1508$</p>	<p>X_f = last dose tested</p> <p>K = Dixon & Massey table 0.878</p> <p>d = interval between test doses = 0.1 ($\log_{10}$)</p> <p>N' = total number of subjects tested = 5</p> <p>N = sample size = 5</p> <p>$\sigma = d, SE = 0.61\sigma$</p>
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372 Table 2. Group of drug concentration and Ach E activity in the Rhesus monkey.

Patch size	Animal No.	Blood collection
3×4 cm ² patch	5	0, 2, 4, 8, 12, 24, 48, 72, 96, 144 hr (Total 10 times collection)
5×5 cm ² patch	5	
7×7 cm ² patch	5	

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379 Table 3. Effects of the patch against VX intoxication.

Treatments	VX challenge Dose (LD ₅₀)	Animal No.	Survival No/Total No. (%)
None	23.1 ug/kg (1.5LD ₅₀)	1	0/1 (0)
5×5 cm ² patch	23.1 ug/kg (1.5LD ₅₀)	5	5/5 (100)
5×5 cm ² patch	24.6 ug/kg (1.6 LD ₅₀)	5	4/5 (80)
5×5 cm ² patch	30.8 ug/kg (2.0 LD ₅₀)	5	3/5 (60)

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385 Table 4. Effects of the combination therapy against VX intoxication.

5×5 cm ² Patch	Atropine + 2PAM	VX challenge Dose (LD ₅₀)	Animal No.	Survival No /Total No
X	O	154.0 ug/kg (10.0LD ₅₀)	1	1/1
O	O		1	1/1
X	O	462.0 ug/kg (30.0LD ₅₀)	1	1/1
O	O		1	1/1
X	O	770.0 ug/kg (50.0LD ₅₀)	6	3/6
O	O		6	6/6
X	O	1540.0 ug/kg (100.0LD ₅₀)	2	0/2
O	O		2	1/2

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392 Supplementary Table 1. Physostigmine pharmacokinetic mean data.

Patch size	AUC (last)ng·h/ml	AUC (last)ng·h/ml	Cmax (ng/ml)	Tmax (hr)	T1/2 (hr)
3×4 cm ² Patch	218.5	231.8	16.9	5.2	11.30
5×5 cm ² Patch	348.6	355.5	9.8	12.0	20.6
7×7 cm ² Patch	994.4	1023.0	27.1	7.0	23.9

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395 Supplementary Table 2. Procyclidine pharmacokinetic mean data.

Patch size	AUC (last)ng·h/ml	AUC (last)ng·h/ml	Cmax (ng/ml)	Tmax (hr)	T1/2 (hr)
3×4 cm ² Patch	2833.5	3017.8	91.3	4.4	35.0
5×5 cm ² Patch	4839.3	5461.7	85.2	12.4	49.3
7×7 cm ² Patch	11081.7	12211.2	195.6	10.5	45.2

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