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Kajian Keberkesanan Anti-Hipertensi ke Atas Polipeptida Terpencil Dari Tapak Sulaiman (Crown of Thorns Starfish)

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Abstract

Acanthaster planci is one type of starfish distributed worldwide and its body is fully covered by long and sharp spines and is believed to contain poisonous venom which can cause severe sharp pain in the chest, nausea, vomiting, inflammation surrounding the injured site and may lead to paralysis and death if condition is severe.

The toxicity effects of the COTS has been focussed in the past five years and according to Johgalingam *et al.* (1995), the COTS crude extract contains at least three distinct fractions when partially purified by sephadex G-75, in which fraction 13-18 caused hypotension in normal albino rats (*Sprague Dawley*). Thus, this study is extended to elucidate the possibility of this fraction as a potential anti-hypertension agent including the mechanism of action, these will involve the *in-vitro* and *in-vivo* experiments in animals to evaluate the anti-hypertension activity of this fraction.

In *in-vivo* studies on WKY and SHR animals, fraction 10 to 25 are most frequently showed hypotensive effects. The COTS pooled solution (COTSPS) also showed strong anti-hypertensive effects, possibly via a calcium channel receptor antagonist effect on the cardiac muscle, an increased in GIT activity without any changes on the heart rate. These effects were substantiated by the *in-vitro* experiments on the isolated perfused guinea-pig heart and isolated perfused rabbit ileum preparations. However, the COTSPS was easily desensitized and associated with rebound hypertension. This rebound hypertension effect was clearly explained when the COTSPS was able to cause vasoconstriction on the isolated perfused rabbit ear artery via the agonist effect on a-adrenoceptor and calcium channel receptor.

In the toxicity study, 77.5 mg/kg and 57.4 mg/kg of COTSPS given by intraperitoneum into albino mice (ICR Strains), exhibited peripheral and central responses including gasping, piloerection, straub tail, hind limb placing, convulsion, blindness, paralysis and eventually death occurred approximately 1 hour. The toxicity curve showed the LD_{50} of COTSPS was 9.307 mg/kg.

Introduction

Crown of Thorns Starfish (COTS) or ***Acanthaster planci***, is not a medicinal marine life seems its' body fully covered by long sharp spines that contains poisonous venom which can causes severe pain in the chest, vomiting, nausea, and intense inflammation and numbness at site of injury. However, it is more popular with an uncommon outbreak that it has unique behavior to eat up and destroyed the coral reef community, gives a negative impact to the tourism industry as well as the ecology of the costal region. In the past five years, Kim KH *et al.*, has focused on toxicity effects caused by the COTS crude extract, the mechanism of action of the toxic effects and partially purified the active fractions which responsible for the toxic effects. In addition, Johgalingam *et al.* 1999, reported that the COTS crude extract contain at least 3 distinct active fractions when partially purified by Sephadex G-75: fraction 13-18 caused hypotension; fraction 31-39 caused increased in blood pressure and GIT activity; while fraction 47-52 caused increased in blood pressure without affecting the GIT activity.

Based on the findings, our aim of this research will elucidate the possibility of fraction 13-18 as a potential anti-hypertensive agent in the treatment of the hypertensive patients. Basically, it involved (1) the study of the toxicity effects of the fraction 13-18 in the experimental animals, including the determination of LD_{50} and the symptoms of the toxic effects, and (2) to identify the pharmacological action of fraction 13-18 especially focused on the mechanism of action in blood pressure regulation in in-vivo experimental normotensive (WKY) and spontaneously hypertensive rat (SHR) models, and lowering the blood pressure by using in-vitro experimental modules.

MATERIALS AND METHODS

All experiments were carried out in the neurophysiology laboratory of physiology department, UM.

(A) COTS Crude Extract Preparation

Procedure: About 2 Kg of COTS thorns stored in the biofreezer was defrosted and then was blended by a machine with added 600 ml of distilled water, over 20 minutes. Then, the mixture was centrifuged for 10 minutes at 3,000 rpm (1x), the supernatant was then recentrifuged for 20 minutes at 3,000 rpm (2x), to remove large particles. The subsequent supernatant was then recentrifuged at 20,000 rpm for 20 minutes. The resulting supernatant is deemed the COTS crude extract. All extraction procedures were done at 4 ^{o}C or in the cold room.

(B) Gel Filtration

This technique was performed in order to separate out the 3 distinct active fractions and collected by using a fraction collector.

Procedure: 20 gram Sephadex G-75 powder was soaked with 250 ml distilled water, overnight. Then the gel was poured into a 50 cm long glass separation column, allowing precipitation occurred without forming any bubbles. Keep pouring the gel until the gel in the column precipitated and reach ~ 45 cm in length. The column was washed with saline continuously over a period of 1-2 hours before the COTS crude extract was added. After that, ~ 15 ml of COTS crude extract was added into the column gently without disrupting the gel layer. Start collection 14 minutes after added the COTS crude extract. The fraction collector should be adjusted 4 minutes collection time per each test tube. Keeping pouring saline again after the COTS crude extract went below the gel level during the course of collection. The final product is the individual fractions or the COTS Aqueous Extract (COTSAE). The whole process should be carry out in the cold room or at 4 ^{o}C and each tube that filled with COTSAE should be keep in the fridge immediately to prevent denaturation of the substance of interest.

(C) Guppy Fish Bioassay

This is a preliminary assay to identify the biologically active fractions from the COTSAE, which have the lethal effect on the Guppy fishes. By using a micropipette, 0.5ml of each fraction that had been collected before was mixed with 5 ml tape water in each correctly labeled well, followed by putting each well a healthy, approximate size 1-2 cm in length Guppy fish. The wells were then covered with plastic bags. The fishes were observed overnight to obtain the lethal effect of each fraction. Later, the identified active fractions that have the lethal effect were tested for the hypotensive effect by the in-vivo animals experiments, and were pooled together, so called the COTS Pooled Solution (COTSPS) and tested for its biological actions by the in-vitro and in-vivo animals experimental modules again.

(D) Pharmacological Studies: In-vivo studies

(1) **Direct blood pressure measurement on normotensive Wistar-Kyoto (WKY) rat and Spontaneous ly Hypertensive rat (SHR)** obtained from the faculty's animal house. Rats of both sexes, weighing approximately 190-250 gram body weight were used.

Procedures: WKY or SHR were anaesthesized by i.v injection of pentobarbitone sodium (25-30 mg/kg or 0.08ml/100g from stock solution). The trachea was cannulated to facilitate respiration. The venous cannula was filled with heparinized saline and inserted into the right external jugular vein for intravenous injection of COTSAE or COTSPS. Then, the left common carotid artery was cannulated for direct recording of the blood pressure via a pressure transducer. The gastro-intestinal tract (GIT) mactivity was measured via a balloon inserted into the ileum. Heart rate was measured via placing 3 electrodes in the right-length position. Both the blood pressure and intestinal pressure and also the heart rate were recorded on the MacLab data acquisition system. When the blood pressure displayed on the screen was came back to normal level, certain doses of COTSAE/COTSPS of each fraction were observed for at least 10-15 minutes before the next doses were introduced.

(2) **Toxicity Study** is a preliminary toxicity study to determine the optimum dose that is to be used in studying the sequential physiological responses of the mic e (ICR strains obtained from IMR) to COTSPS, and to determine the lethal dose of 50% (LD_{50}).

Procedures: 1.0 ml and 0.5 ml of COTSPS was separately injected i.p into 2 mice weighting 40 g and 27 g respectively, and both mice were observed for 2-3 hours. The length of time to death was recorded and the activity in each mouse was graded as follows: +1(moderate increase in activity);+2(strong increase in activity);-1(moderate decrease in activity);-2(strong decrease in activity); -ve(no reaction). For the LD_{50}, 6 groups of mice each group with 10 mice, were injected i.p with 0.025ml, 0.05ml, 0.75ml, 0.1ml, 0.2ml and 0.4ml of COTSPS, respectively. The number of mice surviving 24 and 48 hours after injection were determined for each dose and were converted into percentage. The graph of percentage verses concentration of COTSPS was plotted. A percentage value of 50% is taken as the LD_{50}.

(E) Pharmacological Studies: In-vitro studies

(1) **Isolated Perfused Guinea-pig heart preparation (Langendorff's preparation)** is carried out to determine the effect of COTSPS on the heart.

Procedures: Adult Guinea-pig (450-550 g) were sacrified by a blow on the head. The heart was quickly removed and placed in a petri dish containing aerated Kreb's solution at 37 ^{o}C. The heart was gently squeezed several times so as to washed out as much blood as possible. The aorta was located and dissected free of all other vessels connected to the heart. The aorta was then tied to a glass cannula in the perfusion apparatus and prevent all bubbles from entering the aorta. Oxygenated Kreb's solution was thermostatically warmed to 37 ^{o}C and superfused at 8 ml/min at constant pressure from a reservoir. The coronary perfusion pressure was measured by connecting to a pressure transducer to a side arm in the connecting glass tube just before it entered the aorta. Ventricle contractility and heart rate were measured by connecting the ventricle via a hook and thread assembly to a force-displacement transducer. The COTSPS at different doses were injected into the aorta through a rubber tube attached to the cannula above the aorta. Coronary perfusion pressure, ventricle contractility and heart rate were recorded using the MacLab recording system.

(2) **Isolated Perfused Rabbit Ear Artery Preparation.** Adult rabbits (~ 1-1 ½ Kg) were restrained and the hair overlying the marginal vein of the ear was clipped and moistened with Kreb's solution to make the vein more visible. Pentobarbitone sodium 30 mg/kg was injected slowly through the marginal ear vein. The rabbit was transferred to a dissection board when the righting reflex was lost, and pentobarbitone sodium was further given slowly until there is no response to pain when the ear was pinched with a pair of forceps. Heparin at 100 IU/Kg was then injected through the same vein. The artery was separated from the vein and nerve and cleared of all connective tissue. The vessel was kept moist with Kreb's solution. The proximal end was tied off and the artery cannulated. About 1 cm of the artery from the tip of the cannula was cut, isolated and placed in a water jacketed organ bath containing Kreb's solution at 37 ^{o}C. The artery was perfused at 8 ml/min with Kreb's solution pumped with a peristaltic pump. The Kreb's solution was bubbled with carbogen. The organ bath and perfusate were maintained at 37 ^{o}C. COTSPS at different doses were administered as a bolus into the perfusing solution just before it entered the artery. Changes in perfusion pressure due to artery constriction were measured using a pressure transducer attached to a side arm in the cannula, and recorded using MacLab recording system. The solution in the bath was changed as soon as the peak response was recorded after each administration.

(3) **Isolated Perfused Rabbit Ileum Preparation.** Adult rabbit of either sex was killed by cervical dislocation. The abdomen was opened to expose the intestine. A length of 3-4cm ileum was removed and placed in a petri dish containing aerated Tyrode's solution. One end of the ileum was tied to a force displacement transducer connected to a MacLab recording system to monitor the contraction of ileum, while the other end was tied to a tissue holder and the preparation was positioned in a jacketed organ bath. The temperature of Tyrode's solution and surrounding jacket was kept constant at 37 ^{o}C. A four minutes cycle and two minutes contact time were used to test the effect of different volume of COTSPS on the rabbit ileum preparation.

(F) Determination of Mechanism of Action of the COTSAE/COTSPS.
In order to determine the mechanism of action of the anti-hypertensive effect in whole animals, several types of receptor blockers (?-adrenoceptor and ?-adrenoceptor blockers, calcium channel antagonist, potassium channel antagonist, atropine and yohimbine) were used simultaneously with the injection of COTSAE/COTSPS into the anasthesized rats. Later, the specific target organ of COTSPS was determined by in-vitro experiments include isolated perfused Guinea-pig's heart preparation, isolated perfused rabbit ear artery preparation, and isolated perfused rabbit ileum preparation.

(G) Data Handling (Statistical Analysis)
The data from each experiment was expressed as the mean ± S.E.M for each group and *n* represents the number of experiments. In bar charts, S.E.M values are indicated by error bars (in some cases the error bars were so small they were obliterated by the line symbols). For the direct measurement of BP method, the differences of each mean values from both WKY and SHR were compared by using t-test: paired-two sample for means with probability of $p>0.05$ were considered statistically not significant (n=3; d.o.f =2). For the in-vitro studies, each mean values were compared between the drugs itself and with the present of blockers by using same kind of t-test as mentioned above. Negative notification indicates reduction.

RESULTS

(A) Guppy Fish Bioassay
6 sets of COTSAE fraction have been tested on the Guppy fishes. Table 1 summarized the number of active fractions that causing dead to the Guppy fishes within 24 hours.

(B) Pharmacological Studies: in-vivo studies
(1) **Direct blood pressure measurement on normotensive Wistar-Kyoto (WKY) and Spontaneously Hypertensive rats (SHR).** I.v infusion of 1.55 mg/kg of each COTSAE active fractions into anaesthetized WKY or SHR showed fraction number 10 to 25 are most frequently caused decreased in systemic BP with no significant effect on HR, but increased the GIT activity of both WKY and SHR. The hypotensive effect is very prominent and long lasting both in WKY and SHR (almost 6 minutes) before went back to normal level. However, the reductions of systemic BP are not statistically significant differences between WKY and SHR (except fraction 16, p=0.0012). Whereas, i.v infusion of 1.55 mg/kg COTSPS into anaesthetized WKY and SHR, similarly caused a strong decreased in systemic BP with mean value of -32.45±10.57% and -28.69±6.70% respectively and increased in GIT activity (14.60+0.50% and 12.74+4.25% respectively) without any changes on HR. They are not statistical significant differences either (p=0.3862, n=3) (Figure 1). The COTSPS hypotensive effect in both WKY and SHR are dose-dependent begin from the minimum dose of 0.620 mg/kg. At high dose (85.32 mg/kg), COTSPS induced a sharp decreased in systemic BP (<40mmHg) and HR with a strong increased in GIT activity, which then led to death of the rat (Figure 2). In addition, no blocking effect on COTSPS hypotensive effect being observed in both anaesthetized or vagotomised WKY and SHR when pretreated with i.v 0.1ml;10^{-5} of non-selective $?_1$ and $?_2$ blocker (phentolamine), ? blocker (propranolol), Ca^{2+} channel receptor blocker (diltiazem), K^+ channel receptor blocker (TEA), muscarinic receptor blocker (atropine), and $?_2$ blocker (yohimbine), as well as the GIT stimulation in both WKY and SHR. Desensitization of the

COTSPS hypotensive effect and rebound hypertension occurred when 0.05ml COTSPS in ascending concentration manner are continuously infused i.v into the same preparation.

(2) **Toxicity Study.** Table 2 summarized the toxic effects of COTSPS on 27 g and 40 g mice (ICR strain) respectively. From the toxicity curve of COTSPS on mice, the LD_{50} = 9.307 mg/kg.

(C) Pharmacological Studies: in-vitro studies

(1) **Isolated Perfused Guinea-pig heart preparation (Langendorff's preparation).** Injection of 0.31mg of COTSPS into isolated perfused Guinea-pig heart increased the coronary perfusion pressure (mean = 14.47$\pm$0.92%), decreased ventricle contractility (mean = -14.11$\pm$0.76%) and no significant changed being observed on the HR (Figure 3). These effects were only blocked when pretreated with 0.3ml;10^{-5} M Ca^{2+} channel blocker-diltiazem(p<0.005 & p=0.0136; n=3)(Figure 4), but not with non-selective ?$_1$ and ?$_2$ blocker (phentolamine), ? blocker (propranolol) and K^+ channel receptor blocker (TEA).

(2) **Isolated Perfused Rabbit Ear Artery Preparation.** Injection of 0.31 mg of COTSPS caused vasoconstriction (mean=25.55$\pm$4.45 mmHg;n=3) (Figure 5) and this effects were blocked when pretreated with 0.3ml;10^{-5} M diltiazem (p=0.0053;n=3) and phentolamine (p=0.0079;n=3). Whereas, ? blocker (propranolol) and K^+ channel receptor blocker (TEA) showed no significant blocking effect.

(3) **Isolated Perfused Rabbit Ileum Preparation.** Injection of COTSPS increased the contraction of isolated rabbit ileum (figure 6) approximately 1.00 gram tension, but the contraction was not dose dependent.

DISCUSSION

The hypotensive effect of COTSPS in the whole animals studies is mainly due to the ability of COTSPS to decrease the contractility of the heart. This is clearly explained when 0.31 mg of COTSPS infused into the isolated Guinea-pig heart causing strong decreased in heart contractility. The mechanism of action is believed to be due to the antagonized action of COTSPS on the Ca^{2+} channel receptor of myocyte (indicated when COTSPS effect is blocked by diltiazem), that it prevents the Ca^{2+} entry into the cells and lowered down the intracellular concentration of Ca^{2+}, leads to decrease in heart contractility and decreased in cardiac output, thus causing fall in systemic BP. Besides that, COTSPS can acts as Ca^{2+} channel receptor agonist and ?$_1$ receptor agonist at the peripheral artery (indicated when COTSPS effect is blocked by diltiazem and phentolamine), that it increases the entry of Ca^{2+} into the cells and increases the intracellular concentration of Ca^{2+}, leads to vasoconstriction and thus causing increased in systemic BP. This action is clearly explained the rebound hypertension phenomenon, where its vasoconstriction effect is initially masked by the hypotensive effect, and become more obvious when the COTSPS desensitized after repeated exposure. There are 2 mechanisms are being proposed to explain the desensitization of COTSPS after repeated exposure: (1) the COTSPS permanently bound to the Ca^{2+} channel receptor of myocyte and thus preventing subsequent binding of COTSPS to the receptor; (2) the COTSPS after binds to the receptor, caused conformation changed to the receptor, thus preventing subsequent binding of COTSPS to the receptor.

CONCLUSION

The hypotensive effect of COTSPS is mainly due to the antagonized action on Ca^{2+} channel receptor of myocyte, and desensitization of COTSPS can occurred after repeated exposure. However, no significant reflex tachycardia being observed. In addition, the vasoconstriction effect of COTSPS contributes to rebound hypertension especially at the end of experiments and COTSPS can gives rise to a variety of toxic effects. In conclusion, based on above findings, relatively COTSPS is not an ideal antihypertensive agent due to its desensitization and rebound hypertension effect although it has a strong and long acting hypotensive effect on the heart with no significant reflex tachycardia.

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No.	Lethal Effect (No. of active fraction)
1	9,10,11,12,……..,23,24,25
2	7,8,9,10,……..,24,25,26
3	11,12,13,14,……..,24,25,26
4	8,9,10,11,……..,24,25,26
5	7,8,9,10,…….,23,24,25
6	11,12,13,14,…….,24,25,26

Table 1: Active fractions from Guppy fishes

Table 2: List of toxic effects of the COTSPS on 27g and 40g bioassay mice , respectively.

Toxic effect	Doses of COTSPS	
	57.4mg/kg	77.5 mg/kg
Gasping	after 7 mins (+2)	-ve
Spontaneous activity	-2	2
Piloerection	1	1
Aggressiveness	1	-ve
Tremor	2	2
Convulsion	2	2
Stretching activity	2	2
Tail pinch	1	1
Cyanosis	2	2
Straub tail	2	2
Visual placing	2	2
Corneal reflex	-ve	1
Righting reflex	Lost at 50 mins (+2)	Lost at 40 mins (+2)
Paralysis	After 40 mins (+2)	After 50 mins (+2)
Death	After 60-65 mins	After 1 hour