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PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 8 (1): 78-84, 2005
ISSN 1028-8880

The Effect of Coelomic Fluid *Stichopus hermanii* on Isolated Perfused Rat Hearts and the Involvement of Prostaglandin in its Mechanism of Action

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Abstract: The objective of this study was to investigate the effect of the coelomic fluid (freeze-dried) on the contractility, heart rate and coronary perfusion pressure of the isolated perfused rat hearts by using the Langendorff system. The second objective of the study was to investigate the involvement of prostaglandin in the mechanism action of the fluid. Isoprenaline (ISO) and sodium nitroprusside (SNP) were used as positive controls. The dose-response curve for ISO was determined in which 1.0 nmol ISO produced maximum contractility and heart rate. Then the effect of the coelomic fluid was determined followed by its dose-response curve. The fluid showed vasorelaxation effect on coronary arteries but not on the heart rate and contractility. The dose 1.0 mg fluid was chosen in the mechanistic study since it produced maximum vasorelaxation and this effect was similar to 70% effect of SNP ($1x10^{-6}$ mol). The mechanistic studies were divided into 3 parts. First, the time-matched control study. The result showed two identical doses (1.0 mg) of the fluid injected repeatedly into the heart gave similar effect. This study being used as a standard for further mechanism studies. The second and third parts, followed similar protocol as in the time-matched control. The first dose of the fluid was injected into the heart perfused with Kreb-Henseleit solution without adding Indomethacin (cyclooxygenase inhibitor). The second dose was injected into the heart perfused with the solution added Indomethacin (0.01 mM). The result showed Indomethacin reduced the vasorelaxation effect of the fluid by 52%. In conclusion, the studies indicated showed the coelomic fluid of *S. hermanii* caused vasorelaxation effect on coronary arteries and the mechanism involved prostaglandin.

Key words: Sea cucumber, *Stichopus hermanii*, role on isolated rat heart, contractility, heart rate, coronary perfusion pressure, mechanism, coelomic fluid, prostaglandin

INTRODUCTION

Sea cucumbers beside being a source of protein[1] are also widely used in traditional medicine. The genus Stichopus of these marine invertebrates, locally referred to as gamat is known to relief gastric ulcer, arthritis, pain, reduce hypertension and improve wound healing. Scientific studies supported some of these claims[2,3]. Other studies using sea cucumber extracts showed their ability to inhibit the growth of certain microorganisms[4] and to contain omega acids[5]. Although gamat reduce hypertension, its effect on the heart is unknown. This study, therefore, was aimed to determine the potential effect of one known species of sea cucumbers, *Stichopus hermanii* on isolated rat heart and the mechanism involved.

MATERIALS AND METHODS

Samples of sea cucumber *Stichopus hermanii* were collected from coastal areas of the Peninsular Malaysia. Meanwhile, the hearts for perfusion were isolated from male Sprague-Dawley rats averaging of 275 g b.w. obtained from the Animal Unit, Institute Medical Research, Kuala Lumpur.

Preparation of powdered form of coelomic fluid: The coelomic fluid was collected immediately by making a longitudinal incision 3-5 cm on the ventral side of the sea cucumber without damaging the internal organs using a scarpel. It was then filtered using filter paper Whatman No. 1. The filtrate (50 mL) was kept in a deep freezer (-20°C) overnight and then freeze-dried (freeze dryer

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Heto Drywinner 3) at -50°C under a vacuum pump for 24 h converted into a white powder.

Preparation of a modified Krebs-Henseleit solution: A modified Krebs-Henseleit solution was prepared with chemicals composition (mM); NaCl (118), $C_6H_{12}O_6$ (11.6), $NaHCO_3$ (25.0), KCl (4.7), KH_2PO_4 (4.7), KH_2PO_4 (1.2), $MgSO_4.7H_2O$ (1.2) and $CaC_{12}.6H_2O$ (1.23). These substances were dissolved in distilled water to obtain 2 L. Calcium chloride, $CaCl_2.6H_20$, was added to prevent the solution from cloudiness.

Preparation of isoprenaline control stock: The isoprenaline (ISO) stock was prepared using saline and 1 mM ascorbic acid to produce 10 mM ISO, stored at -18°C. The solution was diluted accordingly before used as positive control.

Preparation of sodium nitroprusside: Fresh stock of 10 mM sodium nitroprusside (SNP) was prepared by diluting with 0.9% w/v sodium chloride to the required concentration $1x10^{-6}$ mol. This dose was chosen as a positive vasorelaxation control since it gave a consistent effect[6].

Preparation of coelomic stock solution *Stichopus hermanii*: The coelomic powder was diluted with saline as a stock solution of 100 mg mL^{-1}. This stock solution was further diluted to doses of 0.0001, 0.001, 0.01, 0.1, 1.0 and 10.0 mg in 0.1 mL saline.

Preparation of a modified Langendorff isolated perfused heart: Male Sprague-Dawley rats were anaesthetised with sodium pentobarbitone (40 mg mL^{-1} i.p.) then killed by cervical dislocation. The rat heart was immediately isolated by cutting its aorta and placed in a Petri dish, which contained cold Krebs-Henseleit solution before being fixed with a cannula by tying its aorta to allow the perfusion of Krebs-Henseleit solution, rate 10 mL min^{-1} and temperature 37°C. The solution was then gassed with a mixture of 95% of oxygen and 5% of carbon dioxide until it reached to the pH of 7.4. The cannula was connected to a pressure transducer (GRASS Model Pressure Transducer 300) that measured the coronary perfusion pressure. The hook was placed at the apex of the heart, which was also connected to the tension isometric transducer (GRASS Force-displacement Transducer, FT03C). This isometric transducer measured the contractility and the heart rate by a fixed pulley at the rest tension of 2.0 g. Then the drainage needle was placed in the left ventricle. The signals from both transducers were recorded using a GRASS Model 7D Polygraph machine. The heart was stabilized for approximately 20 min before the experiment started. ISO, SNP, coelomic fluid or saline was injected via the rubber tube (injection port) nearest to the cannula.

Determination of isoprenaline dose-response curve: A dose of ISO 0.01 nmol was injected into the heart soon after its heart rate, contractility and coronary perfusion pressure stabilized. The effect of ISO was allowed to disappear (>5 min) and then repeatedly administered with increasing doses of ISO until the dose 1000.0 nmol followed by saline as a negative control at the end of the experiment. A dose-response curve was plotted to obtain a suitable control dose.

Determination the effect of coelomic fluid on an isolated rat heart: Six isolated rat hearts were used, each treated with the respective doses of coelomic fluid; 0.0001, 0.001, 0.01, 0.1, 1.0 and 10.0 mg in 0.1 mL saline via an injection port when all the three parameters; heart rate, contractility and coronary perfusion pressure stabilized. The effect of the coelomic powder on the three parameters was observed and then allowed to disappear (>5 min) and then repeated with higher doses until 10.0 mg. Saline was then administered followed by SNP ($1x10^{-6}$ mol) and ISO (1.0 nmol), respectively as positive controls. A suitable dose of the coelomic fluid was selected from the dose-response curve to be used in time match control study as well as determination of mechanism of action.

Studies on mechanism of action of the coelomic fluid

Time-matched control group: The heart was stabilized for 20 min, then injected with coelomic fluid (1.0 mg) via an injection port. Its effect was allowed to disappear and the heart to stabilize for 20 min before a second dose (1.0 mg). SNP $1x10^{-6}$ mol SNP was administered soon after the coelomic effect disappeared. The effect of these two doses of coelomic fluid on the coronary perfusion pressure was used as a standard for studying vasorelaxation mechanism. A Krebs-Henseleit solution low in potassium (3.2 mM) was used to increase the coronary artery tonus, thus augmented the vasorelaxative effect of the coelomic fluid.

The involvement of prostaglandin in vasorelaxation mechanism: Initially the heart was perfused with Krebs-Henseleit solution low in potassium ions. After the heart stabilized for 20 min, a dose of coelomic fluid (1.0 mg) was injected and its effect was allowed to disappear. Then the heart was perfused with Krebs-Henseleit solution low in potassium containing $10x10^{-6}$ M indomethacin. After 20 min, a second dose was

injected. Soon after its effect disappeared, a dose of SNP ($1x10^{-6}$ mol) was given as a positive control. Indomethacin is an enzyme cyclooxygenase inhibitor. Its usage was to inhibit prostaglandin synthesis. This determined whether prostaglandin was involved in the mechanism of vasorelaxation due to the coelomic powder.

Data analysis: All results were stated in mean±SEM. Value n = number of isolated hearts used. The statistical analyses were paired t test and ANOVA test. Paired t test was used to evaluate the effect of the coelomic fluid, the negative control and the positive control for the same group of isolated hearts. ANOVA test compared the three populations. The probability value, $p<0.05$ (*) indicated a significant effect.

RESULTS

Dose-response curve for isoprenaline: Five isolated rat hearts were used to study the effect of ISO on the heart rate, contractility and coronary perfusion pressure. Doses of ISO used were 0.01, 0.1, 1.0, 10.0, 100.0 and 1000.0 nmol. All doses of ISO increased the heart rate with maximum increased 109.0±16.4 beat per min at the dose 1.0 nmol (Fig. 1). The basal heart rate was 286.0±6.0 beat per min.

Isoprenaline increased developed tension (contractility) of the hearts. There were two phase changes in the developed tension (Fig. 2). In phase I, ISO dose 0.01 nmol increased developed tension 86.7±23.2%. This increased with increasing dose of ISO 1.0 nmol to 216.5±56.4%. In phase II, developed tension increased, but lower than in phase I was observed. The tension increased to 60.4±18.8% with the dose 1.0 nmol ISO. Although increased developed tension was low, its duration, however, was longer than in phase I. The basal developed tension was 4.7±0.5 g.

Generally there were two phases change in the coronary perfusion pressure after the injection of ISO (Fig. 3). In phase I, the coronary perfusion pressure decreased from 12.5±4.0 mm Hg (0.01 nmol ISO) to 26.8±14.5 mm Hg (1.0 nmol ISO). Its duration was short. In phase II, the coronary perfusion pressure decreased from 2.0±0.9 mm Hg (0.01 nmol ISO) to 12.0±3.4 mm Hg (10 nmol ISO) with a longer duration. The results showed changes in coronary perfusion pressure were prominent in phase I than in phase II. The basal coronary perfusion pressure was 66.8±6.1 mm Hg.

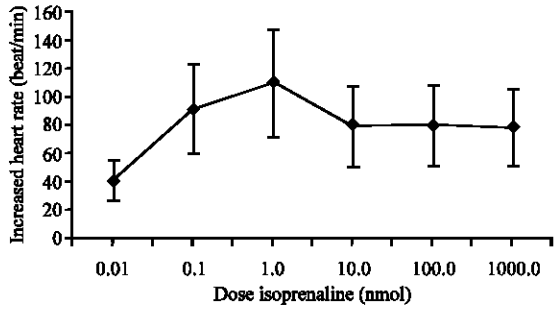

Fig. 1: Heart rate of isolated rat hearts by different doses of isoprenaline

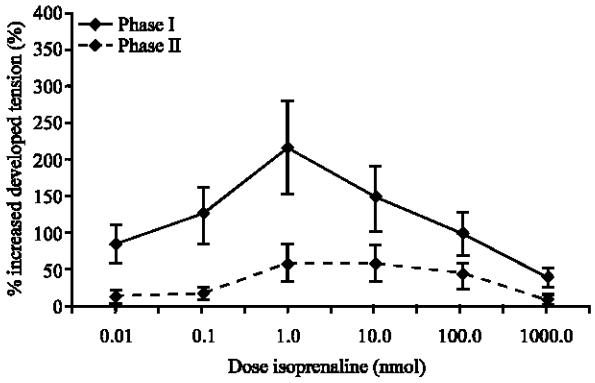

Fig. 2: Developed tension in phase I and phase II by different doses of isoprenaline on isolated rat hearts

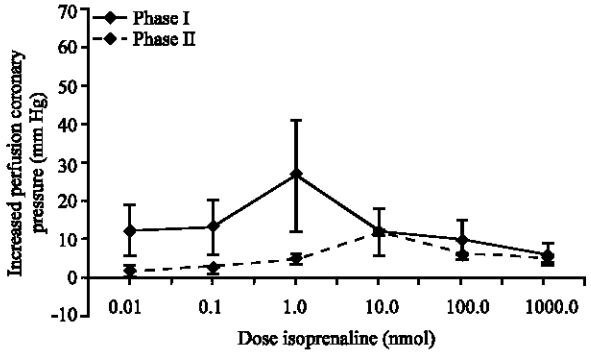

Fig. 3: Coronary perfusion pressure in phase I by different doses of isoprenaline on isolated rat hearts

Dose-response curve for the coelomic fluid of *S. hermanii*: For this study, six isolated rat hearts were used to determine the coelomic effect on the three parameters; contractility, heart rate and coronary perfusion pressure. The doses used were 0.0001, 0.001, 0.01, 0.1, 1.0 and 10.0 mg. Bolus dose of the coelomic fluid extract decreased developed tension from 0.9±0.9% (0.0001 mg) to 31.2±4.6% (10.0 mg). The basal developed tension was 6.0±0.5 mg. Saline had no effect on the developed tension (Fig. 4). For the dose 10.0 mg, two phases change in developed tension occurred. In phase I, developed tension increased by 30% for a short

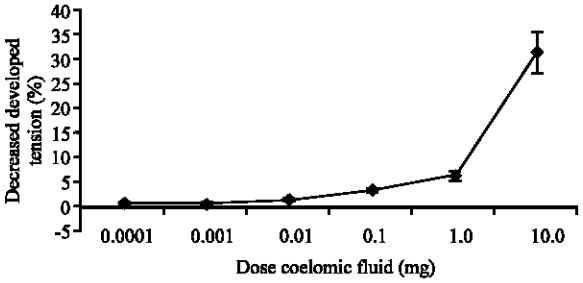

Fig. 4: Decreased developed tension by coelomic fluid *Stichopus hermanii* and saline (the carrier) on isolated rat hearts

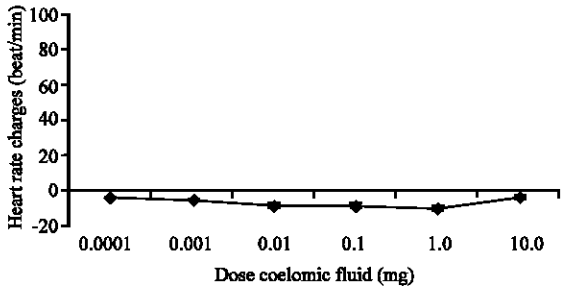

Fig. 5: Heart rate changes by coelomic fluid *Stichopus hermanii*, saline (the carrier) and isoprenaline (1.0 nmol) on isolated rat hearts

duration. In phase II, the tension decreased without recovery.

Compared to saline, the coelomic effect on the heart rate was non-significant (Fig. 5). However, the coronary perfusion pressure (Fig. 6 and 7)) decreased from lower dose to higher dose, 0.6±0.4 mm Hg (0.0001 mg) to 28.3±4.7 mm Hg (1.0 mg). The basal coronary perfusion pressure was 136.3±9.8 mm Hg. Saline had no effect on the perfusion pressure whereas SNP ($1x10^{-6}$ mol) decreased the coronary perfusion pressure 39.2±4.9 mm Hg.

Study on action mechanism of coelomic fluid *Stichopus hermanii*: The analysis ANOVA showed there was non-significant vasorelaxation effect of the first dose of coelomic fluid for either the time control or indomethacin study. Therefore, a comparative study could be performed between mechanism study and time control study for the second dose extract together with the inhibitor.

Time control study: Six isolated rat hearts were used to investigate the effect of injecting twice the coelomic fluid extract with the second dose administered at an interval 29 min to the same heart (Fig. 8). The first dose decreased the coronary perfusion pressure by 36.7±6.5 mm Hg from the basal pressure 164.2±25.8 mm Hg while the second

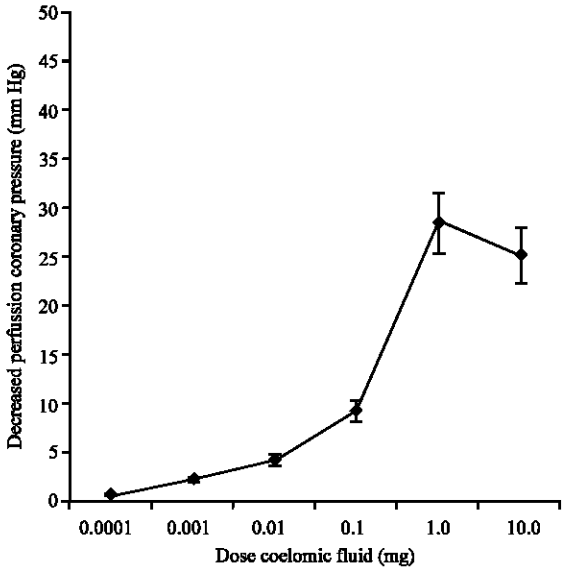

Fig. 6: Decreased perfusion coronary pressure by ceolomic fluid *Stichopus hermanii*, saline (carries) and sodium nitroprusside (1000 nmol) on isolated rat hearts

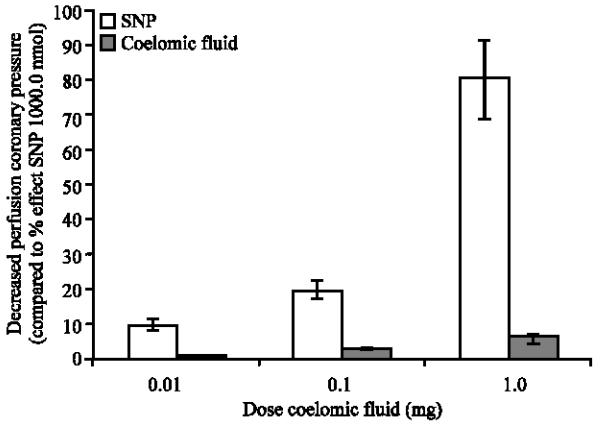

Fig. 7: Decreased perfusion coronary pressure by coelomic fluid *Stichopus hermanii* compared to the percentage of decreased perfusion coronary pressure by sodium nitroprusside (1000 nmol) on isolated rat hearts

dose by 40.8±7.8 mm Hg from the basal pressure 173.3±28.8 mm Hg. Both decreased values, however, were not significant.

The involvement of prostaglandin in vasorelaxation mechanism: Bolus dose coelomic fluid extract decreased coronary perfusion pressure by 30.8±8.4 mm Hg (basal pressure 132.5±23.4 mm Hg) for the heart perfused with the Krebs solution only (Fig. 9). On adding Indomethacin dose 0.01 nM to the Krebs solution, the

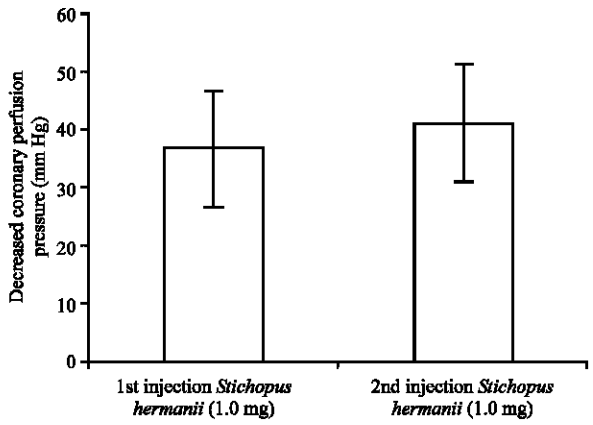

Fig. 8: Time control study; Comparison on the effect of vasorelaxation by injecting twice coelomic fluid *Stichopus hermanii* (1.0 mg) at 29 min interval on the same isolated rat heart

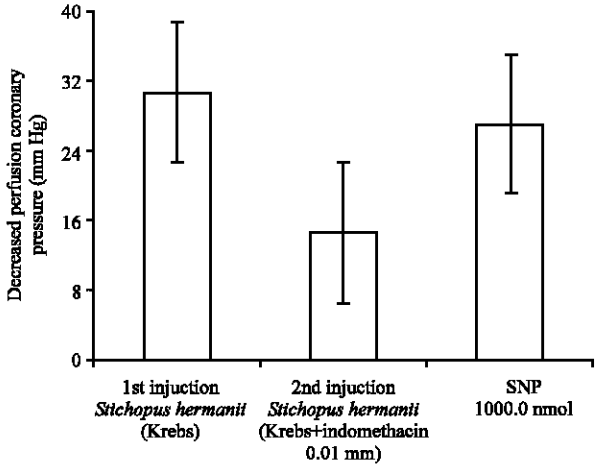

Fig. 9: Effect of vasorelaxation by coelomic fluid *Stichopus hermanii* (1.0 mg) on coronary perfusion pressure of isolated rat hearts before and after being exposed to Indomethacin

second dose of the extract reduced coronary perfusion pressure by 14.6±4.0 mm Hg (basal pressure 121.3±17.9 mm Hg 20 min after adding Indomethacin). These two values decreased in coronary perfusion pressure were different significantly ($p<0.05$, paired t-test). However, there was non-significant difference between the basal coronary perfusion pressure of the heart perfused only with Krebs solution and the same heart perfused with Krebs solution plus Indomethacin.

DISCUSSION

Dose-response curve for isoprenaline

Increased heart rate: Isoprenaline is a type of catecholamine ß-adrenergic agonist and has an effect on $ß_1$-and $ß_2$-adrenoceptors. Stimulation of ß -qdrenoceptor by Isoprenaline, will activate protein Gs in which the later activate adenyl cyclase. Activation of adenyl cyclase caused transformation of ATP to cyclic adenosine monophoshate which will activate Protein Kinase A (PKA). PKA will increased permeability towards calcium ion. Stimulation of β-adrenoceptor will also increased sodium ions permeability[7]. From these combined mechanisms, $ß_1$-adrenoceptor caused the prepotensial slope to become much steeper. The steeper is the slope the faster is the depolarization process and the number of impulses produced in SA nodes. Therefore, the heart rate increases.

Increased developed tension (contractility): In this study isoprenaline increased heart contractility by stimulating phosphorisation of calcium ion channel via PKA thus increased calcium ions permeability into the cell which further released of calcium (calcium-induced calcium release). Thus, more calcium bind with troponin-C to increase the heart contraction. In phase I, however, the developed tension increased briefly.

The effect on coronary perfusion pressure: The decreased coronary perfusion pressure is a measurement for coronary artery vasorelaxation of an isolated heart rat. Due to the constant perfusion of Krebs-Henseleit into the heart during the experiment, any change in coronary perfusion pressure therefore would reflect change in vascular resistance[8].

β-adrenoceptors of large coronary channel are mainly β_1 while of smaller channel mainly β_2[8]. Therefore, in this study, phase I the vasoconstriction was caused by ISO which activated β_1 adrenoceptor in the large coronary channel while vasorelaxation in phase II was due to the activation of β_2-adrenoceptor in small coronary channel. Referring to Isoprenaline dose-response curve, the contractility and maximum heart rate occurred at the dose 1.0 nmol. Thus, this dose of ISO was chosen as a positive control.

Dose-response curve for coelomic fluid: In this observation, the coelomic powder 1.0 mg caused maximum vasorelaxation, i.e. 70% of the vasorelaxation by SNP at dose 1.0 nmol. The carrier (saline) had no effect on the coronary perfusion pressure indicating that vasorelaxation effect was caused by the coelomic powder.

Although the coelomic fluid decreased the developed tension, only dose 10.0 mg was effective. This dose initially increased and later decreased the developed

tension without recovery. As for the heart rate, there was no significant effect by the coelomic fluid.

Krebs solution low in potassium: Vasorelaxation effect could be observed clearly when the basal coronary perfusion pressure is high[9]. This allowed vasorelaxation to be measured easily[10]. But, for the rat isolated heart the basal coronary perfusion pressure was low. Thus Krebs solution low in potassium being used for the heart perfusion. According to Norton and Dexter[11], basal coronary perfusion pressure is influenced by the level of extracellular potassium. Potassium level lower than physiologic value caused vasoconstriction and increased muscle tone[12].

Studies on the mechanism of action

Time control study: Coelomic fluid dose 1.0 mg was used since this dose gave maximum effect on vasoconstriction. This dose gave similar effect on the same heart 29 min after its injection. In this time control study, the effect of the fluid was not influenced by the inhibitor.

Involvement of prostaglandin in vasorelaxation mechanism: Indomethacin inhibited prostaglandin synthesis[13,14] via cyclooxygenase inhibitor. The study showed indomethacin 0.01 mM could inhibit vasorelaxation effect of the coelomic fluid. The inhibitor did not change the basal coronary perfusion pressure significantly before and after the heart being perfused with Krebs-Henseleit solution added Indomethacin. This indicated the vasorelaxation effect of the extract on coronary vessel was not being influenced by changes in the basal coronary perfusion pressure. After the heart being perfused with Krebs solution added Indomethacin 0.01 mM, vasorelaxation effect of the coelomic fluid 1.0 mg was reduced by 52% compared to the heart perfused with Krebs solution only. This proved that prostaglandin was involved in vasorelaxation mechanism of the fluid.

Prostaglandin such as PGI_2 and PGE_2 have vasorelaxation effect. Coelomic fluid might produce an effect at coronary vessel via an increased in prostaglandin production. These two types of prostaglandin caused vasorelaxation by stimulating adenyl cyclase for conversion ATP to cAMP which inhibited enzyme myosin light chain kinase (MLCK). MLCK inhibition caused smooth muscle blood vessel to relax. PGI_2 is the main product of coronary vessel[15] because endothelium has high concentration of PGI_2 syntase[16]. PGI_2 has a potent vasorelaxation activity on rat isolated heart. Normally endothelium produced PGE_2 lower than PGI_2[17]. Further investigation therefore needed to identify whether PGE_2 is the main component in vasorelaxation caused by the sea cucumber extract.

From the results it could be concluded that the coelomic fluid caused vasorelaxation of coronary vessels but, had no effect on the contractility and the heart rate This vasorelaxation effect mechanism involved prostaglandin as proven by using Indomethacin.

ACKNOWLEDGMENTS

This study was supported by IRPA grant Ministry of Science and Technology Malaysia (MOSTE) and thanks to Miss Hayati Idris and Nor Azira Johari for typing the manuscript.

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