ABSTRACT

Objective: Although recent advances in the treatment of congestive heart disease, mortality among patients' remains a questionable remark. Therefore, we evaluated the role of capsaicin on in vitro and ex vivo platelet aggregation induced by Adenosine Di-Phosphate (ADP) as well as in vivo thrombosis models and role of NO, KATP was also identified in the capsaicin-induced anti-platelet animal model as well as in vivo model of arterial thrombosis.

Methods: According to body weight wistar rats were divided into five groups. Group I and Group II was treated with saline and capsaicin (3 mg/kg, i.v), while animals from Group III were treated with N(ω)-nitro-L-arginine methyl ester (L-NAME) (30 mg/kg, i.v) 30 min before administration of capsaicin (3 mg/kg, i.v). Group IV animals were treated with glibenclamide (10 mg/kg, i.v) 30 min before administration of capsaicin (3 mg/kg, i.v). Group V was considered as a positive control and administered clopidogrel (30 mg/kg, p. o). Animals were subjected for in vitro, ex-vivo platelet aggregation assay. ADP (30µM) was utilized as an aggregating agent in these experiments. After these assays, animals of each group were subjected for subacute tail bleeding time in a rat model and FeCl3-induced arterial thrombosis model in rats.

Results: In ADP-induced in vitro platelet aggregation, a significant reduction in % platelet aggregation was observed at 50µM (64.35±4.641) and 100µM (52.72±4.192) concentration of capsaicin as compared to vehicle control (85.82±3.716). Capsaicin (3 mg/kg, i.v) also showed a significant reduction (49.53±4.075) in ex-vivo ADP-induced platelet aggregation as compared to vehicle control (69.36±2.057). In FeCl3-induced arterial thrombosis model, Capsaicin (3 mg/kg, i.v) exhibited an increase in time to occlusion in this rodent model and present of the L-NAME and glibenclamide inhibited the activity of capsaicin.

Conclusion: In our study, capsaicin (50 µM, 100µM) exhibited potent anti-platelet activity in ADP-induced platelet aggregation, similarly capsaicin exhibited significant anti-platelet activity in the ex-vivo study. Moreover, the presence of L-NAME and glibenclamide inhibited the anti-thrombotic and anti-platelet action of capsaicin. Therefore, it was concluded that NO and KATP may be involved in the anti-thrombotic action of capsaicin.

Keywords: Capsaicin, Anti-platelet activity, Glibenclamide, Nitric Oxide, TRPV1 channel

INTRODUCTION

Heart attacks and strokes are the most common causes of mortality and morbidity across the world and both represent clinical manifestations of acute arterial thrombosis [1]. Among them, atherosclerosis presents a greater risk to cardiovascular and peripheral vascular system causing mortality in cardiovascular disease patients [2]. Thrombus formation is a key mediator in the development of atherosclerosis [3]. The platelet is believed to play a pivotal role in pathogenesis and progression of atherosclerosis [4]. Previously it was believed that the platelet is having a minor role in this process; however, it is recognized that the platelet plays itself as a critical link between thrombus formation, inflammation, and atherosclerosis [5]. At the site of vascular injury, platelets come into contact with subendothelial components and form a plug-like structure to avoid future damage to the endothelium. However, if the injury continues to happen, it will activate the cascade of signalling molecules which will form thrombus at the site of injury. This will lead to life-threatening disease states such as myocardial infarction, atherosclerosis or ischemic stroke [6].

Upon platelet formation at the site of vascular injury a series of cascade initiates which mainly involves three phases. Phase I, Phase II and Phase III name as the initial phase, extension phase and stabilization phase respectively. The initial phase involves attachment of platelets to the exposed sub-endothelial layer following vascular injury and a monolayer of activated cells is formed. This activated monolayer further recruits more additional platelets to construct another strong layer during extension phase [7]. In order to restrict the recently formed thrombus to the initial injury site, regulation of platelet aggregation is the process to modulate a balance between activation and inhibition of signalling pathway of platelet [8]. Any defect in regulation of platelet activation or aggregation can cause arterial thrombosis, the major manifestation of atherosclerosis which triggers myocardial infarction and stroke. Nitric oxide (NO) is an endogenous gas present in endothelial cell of vascular endothelium, which is believed to play a predominant role in the regulation of platelet aggregation [9]. Endothelial NO responsible for maintenance of basal vascular tone and blood flow, and thereby regulation of blood pressure due to vasodilatory action [10].

A substantial amount of research has been carried out to elucidate the role of NO in platelet aggregation and found that NO can inhibit platelet activation or aggregation in vitro and in vivo [11]. The mechanism by which inhibition of platelet aggregation turns out by NO is caused by the cGMP-dependent pathway. Soluble guanylylcy clase (sGC) is an enzyme responsible for the production of cyclic guanosine monophosphate (cGMP), and NO is involved in activation of sGC which further lead to activation of cGMP-dependent protein kinase (PKG) via a cGMP-dependent pathway. This activated PKG caused inhibition of platelet activation via phosphorylating T.tATPase receptors and thus inhibits its aggregating action on platelet. Further details include inhibition of influx of Ca2+ and other cations via activating sarcoplasmic reticulum ATPase (SERCA) [12]. PKG is also involved in blockage of the release of Ca2+ cation from the sarcoplasmic reticulum via inhibition of
Animals

Albino Wistar rats (150–200 g) were housed in a climate-controlled room (temperature 22±1 °C; relative humidity 55±5%) on a 12 h (light–dark) cycle. Animals had access to a standard pellet diet (Certified Amrut brand rodent feed; Pranav Agro Industries, Pune, India) and filtered tap water ad libitum. All experiments were carried out with strict adherence to ethical guidelines and were conducted according to the protocol (ICMP/Cology/14/10) approved by the Institutional Animal Ethics Committee (IAEC), and according to Indian norms set by the Committee for the Purpose of Control and Supervision of Experiments on Animals (PCPSEA, New Delhi, India). In addition, the animals were cared for in accordance with the Guide to the Care and Use of Experimental Animals (Vol. 1, 2nd ed., 1993, and Vol. 2, 1984) throughout the study. Throughout the entire study period, the animals were monitored for growth and health status to be certain that they were healthy.

Chemicals

Capsaicin, ADP (Adenosine Diphosphate) and Urethane were also purchased from Sigma Chemical, USA and ADP were prepared in phosphate buffer (pH 7.4). FeCl₃ was purchased from Hi-Media, India. Clopidogrel bisulfate was the generous gift from Zydus Cadila, India. In all experiments, water for injection was utilized for preparation of L-NAME and clopidogrel whereas capsaicin, glibenclamide was prepared in DMSO (0.5%)+saline.

Preparation of platelet-rich plasma (PRP) and platelet poor plasma (PPP)

Blood was collected into the tubes containing 3.8% trisodium citrate via retro-orbital route under light ether anesthesia from rats. All blood samples were centrifuged at 2000Xg for 20 min and supernatant platelet rich plasma (PRP) was collected carefully. The remaining amount of blood was centrifuged at 800Xg for 20 min and supernatant platelet poor plasma (PPP) was collected with utmost care.

Measurement of platelet aggregation using ADP as an aggregating agent

Platelet aggregation studies were performed on microplate reader in 96-well, flat-bottomed, microtiter plates. A 180-µl volume of PRP was placed in each well, followed by addition of 20 µl of ADP (30µM). For in vitro studies, PRP/PPP was incubated with various concentrations of capsaicin (25, 50 and 100 µM) for 2 min at 37 °C before addition of ADP. Readings were taken every 1 min over a 5-min period at 405-nm wavelength. During the runtime, the plate was incubated at 37 °C and was shaken vigorously in a shaking mode at the maximal speed available. All platelet aggregation studies were performed in triplicate. Change in optical density (OD) was measured by taking OD of buffer as blank.

% Aggregation was calculated using formula:

\[ \% \text{Aggregation} = \left( \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \right) \times 100 \]

Experimental design for ex vivo platelet aggregation study in rats

On the basis of body weight wistar rats were randomly divided into following groups. Experimental design was given in fig. 1.

![Fig. 1: Experimental design for ex vivo platelet aggregation study](image)

Via the retro-orbital route blood samples were collected in tubes containing 3.8% trisodium citrate under anesthesia. PRP/PPP was prepared as described above and samples were subjected to ADP-induced platelet aggregation assay. % Aggregation was calculated in order to estimate the influence of capsaicin in platelet aggregation.

Subaqueous tail bleeding time in rat

Anaesthetized rats were fixed in supine position on a temperature-controlled (37 °C) heating table. After a defined latency period, the tail of the rat was transected with a razor-blade mounted on a self-constructed device at a distance of 4 mm from the tip of the tail. Immediately after transection, the tail was immersed into a bath filled with isotonic saline solution (37 °C). The time until continuous blood flow ceased for >30 s was measured, with a maximum observation time of 30 min (longer bleeding times were assigned a value of 30 min). Clopidogrel (30 mg/kg, p. o., 120 min pretreatment time) was used as positive control.

FeCl₃-induced arterial thrombosis model in rats

Rats (n=6) were treated as per given protocol and then subjected to FeCl₃-induced arterial thrombosis. The fecl₃-induced chemical injury was used as a model of arterial thrombosis. A midline cervical incision was made on the ventral side of the neck and left carotid artery was isolated. Cannulation of the carotid artery was performed and connected to blood pressure measurement instrument (Biopac Systems, Inc, California, USA for B. P. measurement). A2×3 mm strip of Whatman filter paper No. #1 saturated with 35% (w/v) FeCl₃ was placed in each well, followed by addition of 20 µl of ADP (30µM). For in vitro studies, PRP/PPP was incubated with various concentrations of capsaicin (25, 50 and 100 µM) for 2 min at 37 °C before addition of ADP. Readings were taken every 1 min over a 5-min period at 405-nm wavelength. During the runtime, the plate was incubated at 37 °C and was shaken vigorously in a shaking mode at the maximal speed available. All platelet aggregation studies were performed in triplicate. Change in optical density (OD) was measured by taking OD of buffer as blank.

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% Aggregation was calculated using formula:

\[ \% \text{Aggregation} = \left( \frac{\text{Initial OD} - \text{Final OD}}{\text{Initial OD}} \right) \times 100 \]
RESULTS

Effects of capsaicin on ADP-induced in vitro and ex vivo platelet aggregation and mechanisms

In ADP-induced in vitro platelet aggregation, capsaicin exhibited a significant reduction in % platelet aggregation at 50µM (64.35±4.641) and 100µM (52.72±1.992) concentration as compared to vehicle control (85.82±3.716) (fig. 2). On another hand, capsaicin (3 mg/kg, i.v) also showed a significant reduction (49.53±4.075) in ex-vivo ADP-induced platelet aggregation as compared to vehicle control (89.38±2.057) (fig. 3). We also explored the mechanism of antiplatelet action of capsaicin by using L-NAME and glibenclamide to identify the role of NO and K+ in capsaicin mediated antiplatelet action. Capsaicin at 3 mg/kg, i.v exhibited a significant reduction in % platelet aggregation (49.53±4.075) as compared to vehicle control (89.38±2.057) (fig. 3). Pretreatment of L-NAME significantly blocked the antiplatelet effect of capsaicin, while on another hand a significant rise in bleeding time was observed with the addition of glibenclamide to capsaicin as compared to vehicle control (fig. 4). However, bleeding time for glibenclamide pretreated group was significantly lower than positive control. Hence, it can be concluded that NO may be involved in the antiplatelet activity of capsaicin.

Moreover, FeCl3-induced arterial thrombosis model was also performed to elucidate the role and mechanism of capsaicin in platelet aggregation. In that, time to occlusion (TTO) was measured as endpoint parameter. Capsaicin (3 mg/kg, i.v) exhibited an increase in time to occlusion in this rodent model as shown in fig. 5. This action was completely reversed in presence of L-NAME, while the presence of glibenclamide partially inhibited the activity of capsaicin as compared to vehicle control. Reduction in thrombus weight was observed in capsaicin-treated group whereas L-NAME and glibenclamide exhibited inhibition of capsaicin activity partially depicted in the graph (fig. 6).

Effects of capsaicin in vivo models of subaqueous tail bleeding time and FeCl3-induced thrombosis in rats and mechanisms involved

Following ex-vivo and in vitro platelet aggregation assay we carried out in vivo model of thrombosis to explore the role of capsaicin in thrombosis and its underlying mechanism. Capsaicin (3 mg/kg, i.v) (446.3±38.75) showed a significant rise in bleeding time as compared to vehicle control (241.8±17.94). Further, bleeding time for capsaicin (3 mg/kg, i.v) is significantly lower than positive control clopidogrel (30 mg/kg, p. o.) (fig. 4). Bleeding time is a significant parameter of antiplatelet or anticoagulant activity; therefore it has a major impact on the identification of antiplatelet or anticoagulant effect of the substance. In our study, pretreatment of L-NAME significantly blocked the antiplatelet effect of capsaicin, while on another hand a significant rise in bleeding time was observed with the addition of glibenclamide to capsaicin as compared to vehicle control (fig. 4). However, bleeding time for glibenclamide pretreated group was significantly lower than positive control.
suggests NO and K partially blocked anti-platelet effect of capsaicin in similar models ex-vivo of capsaicin in the same model. Above observations suggest that capsaicin may have antithrombotic activity via NO-cGMP pathway. A drug characteristic belongs to antiplatelet or anticoagulant can be identified on the basis of bleeding profile of the drug, hence we studied the bleeding profile of capsaicin using tail vein bleeding time rodent model. We observed that bleeding time for the capsaicin-treated group was significantly decreased as compared to vehicle control but much more so than with the glibenclamide treatment. Therefore, it can be concluded that capsaicin has a little effect on blood coagulation but not significant. It has been demonstrated that thrombus composition is an indicator of coronary occlusion time. "Fresh" thrombi have the highest portion of platelets, while the proportion of fibrin fibers increases over time, as the level of thrombin increases, leading to "older" fibrin-rich thrombi [25]. In our study, thrombus formed by FeCl3 was less than within a day hence it is to be considered as fresh thrombus composed of high portion of platelet and it is also documented in previous studies. In a model of FeCl3-induced thrombus, capsaicin has significantly showed inhibitory action on thrombus formation. Therefore, it can be concluded that capsaicin may exert antithrombotic effect by inhibiting structural platelet inhibition via induction of NO from endothelium. In our result, effect of fibrin was not observed due to thrombin structure was confined to rich level of platelet than fibrin clot. Hence, our study supports the previous finding of antiplatelet activity of capsaicin [26]. Eguchi et al. demonstrated that endothelial cell mitochondria have decisive role on thrombus formation process. Upon injury to endothelium, reactive oxygen species (ROS) enhance the process of thrombus formation which is attenuated by KATP channel opener [27]. In our study of FeCl3-induced thrombus; thrombus was formed as a result of damage to the endothelium was caused by FeCl3. This thrombus formation time and its extent were attenuated by the treatment of capsaicin. However, addition of glibenclamide to capsaicin somehow shifted the antithrombotic activity of capsaicin towards KATP channel which was not significant. Our results are not consistent with observation of Mittelstadt who stated that Capsaicin-induced inhibition of platelet aggregation is not mediated by TRPV1 [28]. In our experiments results, partial involvement of KATP was seen in various parameters of arterial model of thrombosis hence the role of KATP channel in capsaicin offered antithrombotic action could not be neglected. Therefore, it would not be wrong to say that activation of TRPV1 channel by capsaicin may have opened KATP in the endothelium of artery of rat.

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