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沒食子酸抑制血小板的活化與血小板-白血球凝集：經由
Akt 與 GSK3β 訊息傳遞的作用

**Gallic Acid Attenuates Platelet Activation and Platelet-leukocyte Aggregation:
Involving Pathways of Akt and GSK3β**

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中文摘要

　　血小板活化及血小板與白血球共軛是動脈粥狀硬化的重要機轉。心血管疾病多與粥狀動脈硬化有關，其中心肌梗塞更是嚴重的致命性疾病。沒食子酸是紅酒與茶葉當中的主要成分，已被報導具有心血管保護效果。目前認為沒食子酸的心血管保護能力，可能與其抗氧化能力有關。直到最近，沒食子酸對血小板的影響的資料並不多，而且並不一致。因此我們此次的研究目的在於探討沒食子酸對血小板活化的影響及其機轉。我們以健康成年人的血小板作實驗，發現沒食子酸能夠抑制被致效劑刺激的血小板凝集、P-selectin的表現與血小板-白血球共軛，且有劑量效應。胞內鈣離子的增加，是血小板活化的重要步驟，我們的研究發現，沒食子酸能夠抑制血小板胞內鈣離子的釋放。另外，PKCα、MAPK 與 Akt 等訊息傳導，近來也被報導在血小板功能調節上，扮演重要的角色；而 GSK3β 也被證實在血小板的活化抑制上，當作 Akt 的受質 (substrate)。而我們的實驗也證明了。沒食子酸也能夠減少PKCα、p38 MAPK、Akt 與 GSK3β 的磷酸化。另外，在我們的血小板實驗當中，沒石子酸對於氧化壓力的抑制效果並不明顯。透過這樣的觀察，我們推測沒食子酸經由抑制 PKCα/p38 MAPK 與 Akt/GSK3β 等路徑，同時調節胞內鈣離子的釋放，而達到抑制血小板的功能。這樣的結果是第一次沒食子酸對於血小板功能抑制的機轉探討。另外，我們的研究也提供了動脈硬化性疾病的一個可能治療方向。

Abstract

Platelet activation and its interaction with leukocytes play an important role in atherothrombosis. Cardiovascular diseases resulted from atherothrombosis, e.g., acute myocardial infarction, remain the major causes of death worldwide. Gallic acid, a major constituent of red wine and tea, had been shown to have properties of cardiovascular protection, which maybe associated with its anti-oxidant effects. Till now, there were scanty data regarding the effects of gallic acid on platelet function. Therefore, we designed this *in vitro* study to determine whether gallic acid can inhibit platelet activation, aggregation and platelet-leukocyte aggregation and to elucidate the possible mechanisms. From our results, gallic acid can dose-dependently inhibit platelet aggregation, P-selectin expression and platelet-leukocyte aggregation. Increase of intracellular Ca^{2+} is important in platelet activation. We showed that gallic acid prevents intra-cellular Ca^{2+} release in stimulated platelets. It had been widely studied the activity of PKCα, MAPK and Akt had significant roles in platelet activation. Recently, GSK3β was recognized as an effector of Akt in regulating platelet function. Our data further disclosed that gallic acid inhibit the phosphorylation of PKCα, MAPK, Akt and GSK3β on platelets stimulated with the agonists (ADP and U46619). Taken together, gallic acid might exert the inhibitory effects on stimulated platelets through attenuating the activity of PKCα/p38MAPK and Akt/GSK3β and preventing the release of intracellular Ca^{2+}. This is the first mechanistic explanation of the platelet inhibitory effects from gallic acid. Our work also provide a possible therapeutic implication in cardiovascular diseases.

序文與致謝

這一次論文的完成，真的是經歷了許多的波折，除了原先嘗試的藥物以及條件有問題外，實驗總是沒進度，後來流式細胞儀還突然故障了一陣子，不過，最後還是做出了一些結果，終於可堪撰寫。過程中最困難的還是要兼顧臨床工作與實驗的進行，加上一些臨時的醫院任務，常常都會耽誤到實驗的進度。經過了兩年的學習，不敢說很充實，但是，對於醫學研究以及疾病的分子機轉，的確有了不一樣的觀點，我想這是我覺得最大的收穫。

兩年來，最要感謝的還是李繼源老師，老師帶著我一點一滴的累積實驗的觀念，修正研究設計的邏輯，還要容忍我常常忙於醫院的工作，耽擱實驗進度。當然還要謝謝文玲、玉珍、佳華還有李思沅博士，沒有她們的幫忙，這個實驗是很難完成的。另外還要感謝台大的陳玉怜老師的大力協助，以及慷慨的幫忙。

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CHAPTER 1 INTRODUCTION

1.1 Background

Cardiovascular disease, in particular, coronary heart disease (CHD) and associated myocardial infarction, remains a major cause of death in the developed counties. Platelets are essential for primary hemostasis and repair of the endothelium, but they also play a key role in the development of acute coronary syndromes and contribute to cerebrovascular events. The activation of platelets by inflammatory triggers may also be a critical component of atherothrombosis[1]. In addition, platelets participate in the process of forming and extending atherosclerotic plaques[2,3]. When activated, platelets co-aggregate with circulating leukocytes ia PSGL-1 and P-selectin interactions. These interactions trigger autocrine and paracrine activation processes that lead to leukocyte recruitment into the vascular wall, which results in development of atherosclerotic lesions[4]. In addition, in a large-scale prospective human study, the risk of future cardiovascular events increased with increasing plasma soluble P-selectin level[5].

Epidemiological studies have suggested that consumption of light to moderate alcohol, particularly red wine is associated with a reduction in overall mortality[1,6]. Although the exact nature of the protective effect of red wine is unclear, it might be attributable, in part, to its ability to reduce the progression of atherosclerotic lesions[7]. The protective effect of red wine seems to be related to its polyphenols which might prevent oxidation of LDL[8,9], activation of platelets[10], and expression of tissue factor and monocyte chemo-attractant protein-1[11]. Green tea had also been reported to have protective effects from cardiovascular diseases[12]. Data from clinical trials suggest that green tea use consistently leads to a significant increase in the antioxidant capacity of the blood[13]. Our attention was therefore drawn to gallic acid, a major constituent in red wine and green tea. Gallic acid itself had been shown to protect the myocardium against isoproterenol-induced oxidative stress in rates[14]. Previous reports on the favorable effects of gallic acid mainly are related to its antioxidant and anti-inflammatory properties[15,16]. Nonetheless, recent outcomes of randomized trials failed to confirm a protective effect of the antioxidant vitamin E on CHD[17]. Gallic acid may thus be atheroprotective at least in part through non-antioxidant mechanisms, e.g., through inhibiting platelets activation. Till now, there were

only scanty but inconsistent data reporting the effects of gallic acid on platelet function.

1.2 Study Objectives

Therefore, the purpose of our study was to determine whether gallic acid can inhibit platelet function in vitro and, if so, to elucidate the underlying mechanisms.

CHAPTER 2   METHODS

2.1 Materials and Reagents

The following reagents were used: anti-CD42a-PE antibody (Becton Dickinson, San Jose, CA, USA), a platelet-specific monoclonal antibody conjugated with phycoerythrin (PE) which recognizes platelet GPIX complex independent of activation; anti-CD62P-PE antibody (Becton Dickinson), a monoclonal antibody conjugated with PE that is directed against P-selectin expressed on the platelet surface; and anti-CD14-allophycocyanin (APC) (Becton Dickinson), a monoclonal antibody recognized a myelo-monocytic differentiation antigen expressed by monocytes. Adenosine 5'-diphosphate (ADP), gallic acid and paraformaldehyde were obtained from Sigma Chemicals (St. Louis, MO, USA). U46619, a thromboxane mimetic, was purchased from Cayman Chemical (Michigan, USA). Gallic acid was dissolved in dimethylsulfoxide (DMSO). Steps were taken to ensure that the concentration of DMSO was always the same (0.1%). Monoclonal antibodies against p38 MAPK (Mitogen-activated protein kinase) and PKCα (proten kinase C alpha) were obtained from Cell Signaling (Boston, USA). Monoclonal antibodies against Akt and glycogen synthase kinase (GSK) were obtained from R&D Systems (Minneapolis, USA).

2.2 Preparation of Platelet Suspension

Whole blood for this in vitro study was sampled from six healthy volunteers with age ranging from 27 to 53 years, who had not taken any medication for at least 15 days. Blood was collected from an antecubital vein with an 18-gauge needle. All samples were anti-coagulated with 1:7 volume of acid-citratedextrose (ACD, 2.5% trisodium citrate, 2.0% D-glucose, 1.5% citric acid) solution. After centrifugation, isolated platelets were washed twice with CGS buffer (0.123 M NaCl, 0.033 M D-glucose, 0.013 M trisodium citrate, pH 6.5) and resuspended in modified Tyrode's buffer (2.5 mM Hepes, 150 mM NaCl, 2.5 mM KCl, 12 mM NaHCO$_3$, 1 mM CaCl$_2$, 1 mM MgCl$_2$, 5.5 mM D-glucose, pH 7.4) to a final concentration of 3×10^8 /ml. In platelet-rich plasma (PRP) experiments, fresh blood was anti-coagulated with 1/9 volume of 3.8% trisodium citrate, and centrifuged at $150 \times g$ for 12 minutes (min) at room temperature (RT) to get PRP. Then, washed platelets and PRP were incubated at RT for 1 hour (h) to recover to resting state.

2.3 Platelet Aggregation

Platelet aggregation was performed using a turbidometric platelet aggregometer (Payton, Scaborough, Canada). Briefly, PRP were pre-incubated with different concentration of gallic acid (100 μM, 500 μM, 1 mM) or control vehicles at 37 °C for 15 min, and then aggregation was induced by addition of ADP and U46619 at 37 °C with a stirring speed of 1000 rpm. Aggregation was measured turbidimetrically at 37°C with constant stirring at 1000 rpm. The absorbance of Tyrode's solution was assigned as 100% aggregation and the absorbance of platelet suspension as 0% aggregation.

2.4 Assess Platelet-leukocyte Aggregation and P-selectin Expression by Flow Cytometry

Anti-coagulated whole blood or PRP was preincubated with the desired concentration of gallic acid (50 μM to 500 μM) for 15 minutes at 37°C. Platelet agonist, ADP and U46619 were used at a final concentration of 2 μM in whole blood and 5 μM in PRP for 15 minutes' stimulation at room temperature. To determine platelet P-selectin expression, PRP samples were mixed with saturated concentrations of anti-CD62p-PE monoclonal antibody and anti-CD42a-PE monoclonal antibody. To determine platelet-leukocyte aggregate (PLA), whole blood was mixed with saturated concentrations of anti-CD42a-PE monoclonal antibody and anti-CD14-APC monoclonal antibody. After staining with antibodies, both the whole blood and PRP samples were incubated for 20 minutes in the dark. Both samples were then fixed with 1% paraformaldehyde and maintained at 4°C. After fixation, blood samples were immediately processed for flow cytometric analysis in a FACS Calibur flow cytometer (Becton Dickinson), having recourse to CELLQuest cell analysis software (Becton Dickinson).

To determine platelet CD62P expression in PRP, individual platelets were identified by size (forward scatter) and anti-CD42a-PE immunofluorescence using a logarithmic scaled dot plot. P-selectin expression on the surface of platelets was defined as positive for anti-CD62P-PE. Results are expressed as mean fluorescence intensity (MFI) and percentage of positive CD62P cells. The anti-CD62P MFI reflects the number of P-selectin epitopes expressed on the platelet surface membrane. Granulocytes were recognized by size (forward scatter) and granularity (side scatter). Anti-CD14-APC fluorescence was

used to further differentiate monocytes. The two-color analysis enabled discrimination of platelet-coupled and platelet-free leukocytes, and calculation of the percentage of platelet-coupled leukocytes in the leukocyte population. Similarly, the percentages of platelet-conjugated monocytes, and granulocytes could be measured by analysis of the individual leukocytes populations.

2.5 Measurements of Intracellular Ca^{2+} Concentration

Intracellular Ca^{2+} levels were determined with the Ca^{2+}-sensitive fluorochrome fluo-3 AM using flow cytometry. Briefly, washed human platelets (3×10^8 platelets/ml) were loaded with 8 μM fluo-3 AM for 30 min at 37°C in the dark. After being washed once, platelets were re-suspended at a concentration of 5×10^7 platelets/ml. The external Ca^{2+} was adjusted to 1 mM and then the dyed platelets were incubated with ADP (10 μM) or U46619 (2 μM), different concentrations of gallic acid (100 μM, 500 μM), or control vehicles at 37 °C for 30 min in the dark, and analyzed by flow cytometry.

2.6 Determination of Reactive Oxygen Species Formation

PRP were preloaded with 10 μM 2′7′ dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) for 30 min at 37°C. DCFH-DA is a non-fluorescent lipid soluble probe which diffuses across the plasma membrane and is converted intra-cellularly to a cell impermeant (DCFH) by intracellular esterases. The probe can then be oxidized by H$_2$0$_2$ to the fluorescent species. Oxidation was quantified by measuring the increase in mean fluorescence with a flow cytometer.

2.7 Western Blot Analysis

Total proteins (46.4 μg) were subjected to SDS PAGE (12%) and blotted on PVDF membrane. Immunodetection was carried out using antibodies directed against phosphorylated and origin forms of P38 MAPK, PKCα, Akt and GSK3β. The immunoreactive band was detected by enhanced chemiluminescence.

2.8 Statistical Analysis

All data are expressed as the   mean ± standard   error of the mean. Each sample was

compared with the corresponding control sample. Analysis of statistical significance was performed using one-way analysis of variance (ANOVA) combined with the Tukey test. For comparison between two groups Student's t test was used. $p < 0.05$ was considered to be significant for a difference.

CHAPTER 3   RESULTS

3.1 Effects of Gallic Acid on Platelet Aggregation

We determined the effects of a range of concentrations of gallic acid in PRP induced by ADP and U46619. The PRP was incubated with the appropriate concentration of gallic acid for 15 minutes before the addition of ADP or U46619. Gallic acid at concentrations of 100μM, 500μM and 1000 μM inhibited 2.5 μM ADP- and 1.5 μM U46619-induced platelet aggregation in a dose dependent manner (n=5, $p<0.05$; Fig. 1A, 1B, 1C). One mM gallic acid inhibited platelet aggregation by 25% and 20% with the agonists of ADP and U46619, respectively. Moreover, the solvent control (0.5% DMSO) did not significantly affect platelet aggregation stimulated by agonists in either washed platelets or PRP (data not shown).
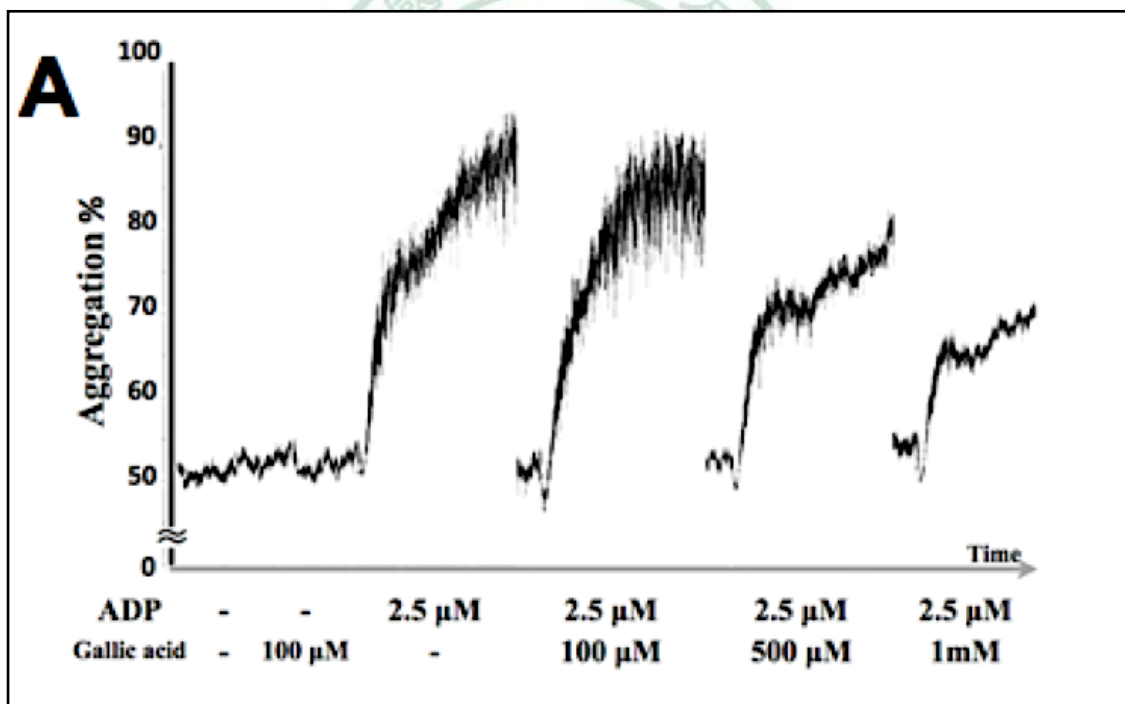

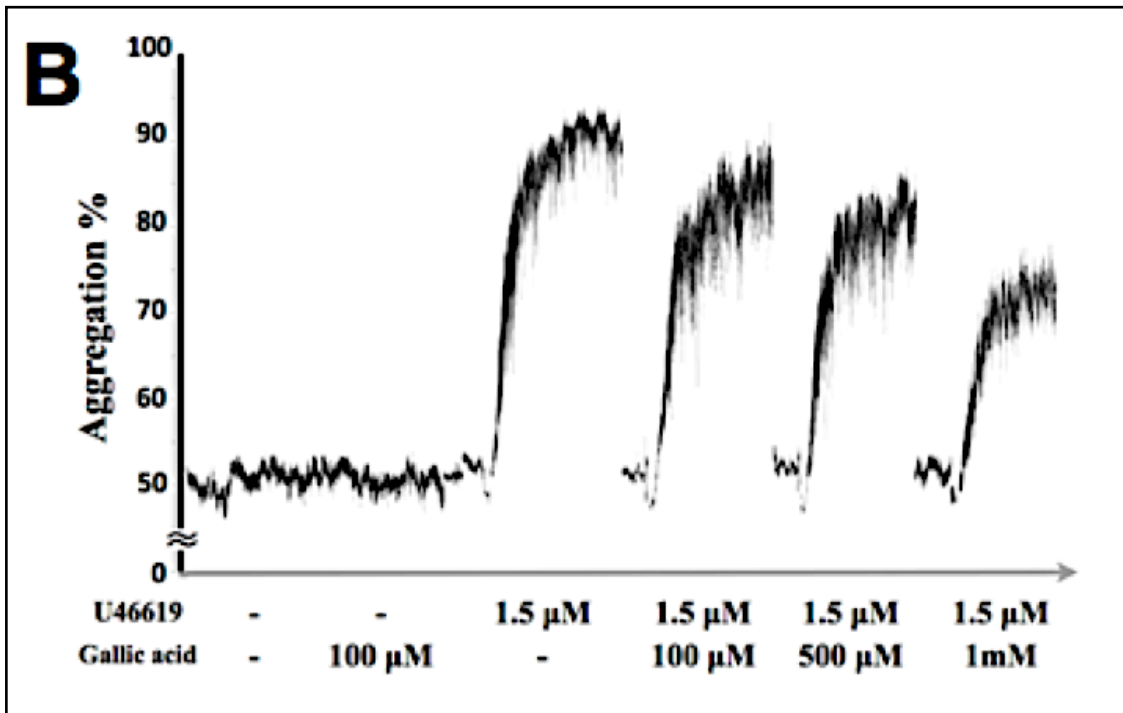Fig. 1A, gallic acid inhibited platelet aggregation induced by ADP

Fig. 1B, gallic acid inhibited platelet aggregation induced by U46619
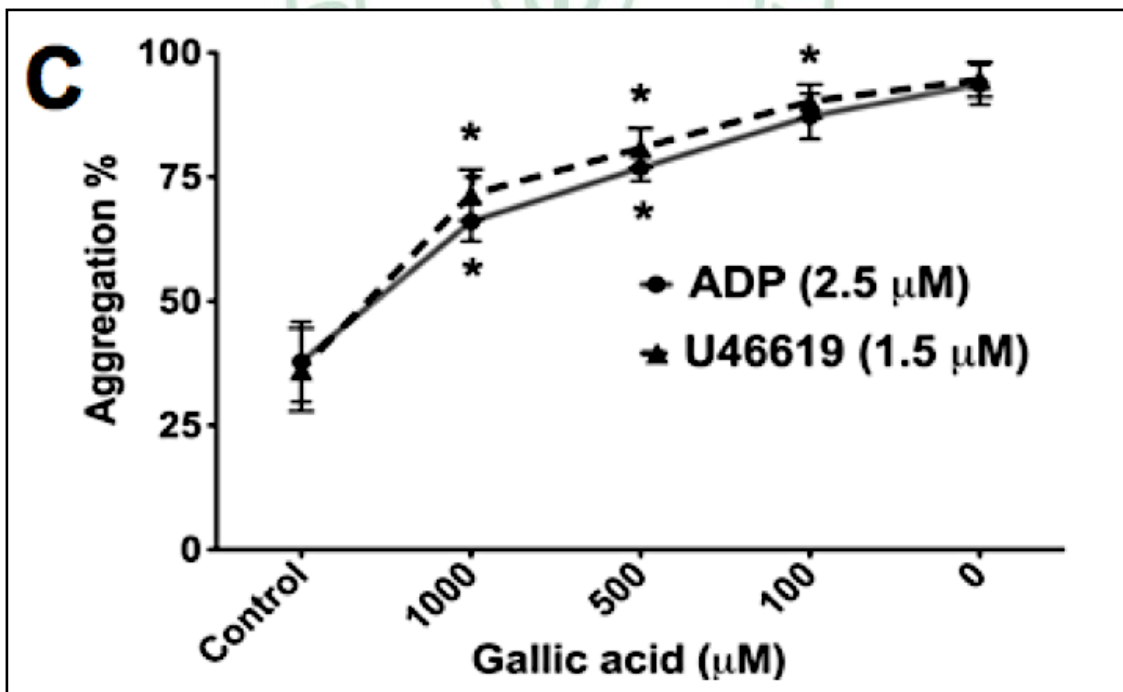

Fig. 1C, gallic acid dose-dependently inhibited platelet aggregation induced by ADP or U46619

3.2 Effects of Gallic Acid on Platelet-Leukocyte Aggregation

The influence of gallic acid on PLA was determined by flow cytometry with measurement on resting platelets and compared to ADP and U46619 activated platelets. The percent positive platelets and the mean fluorescence intensity (MFI) of platelets expressing CD62P were measured. The effect of gallic acid on ADP- (Fig. 2A, 2B) or U46619- (Fig. 2C, 2D) induced PLA in whole blood revealed a modesty inhibition on total PLA at the concentration of 100 μM and 500 μM, while platelet−monocyte (upper panels of 2A and 2C) and platelet−granulocyte (lower panels of 2A and 2C) aggregates were both inhibited in a dose-dependent manner (n=5, $p < 0.05$).
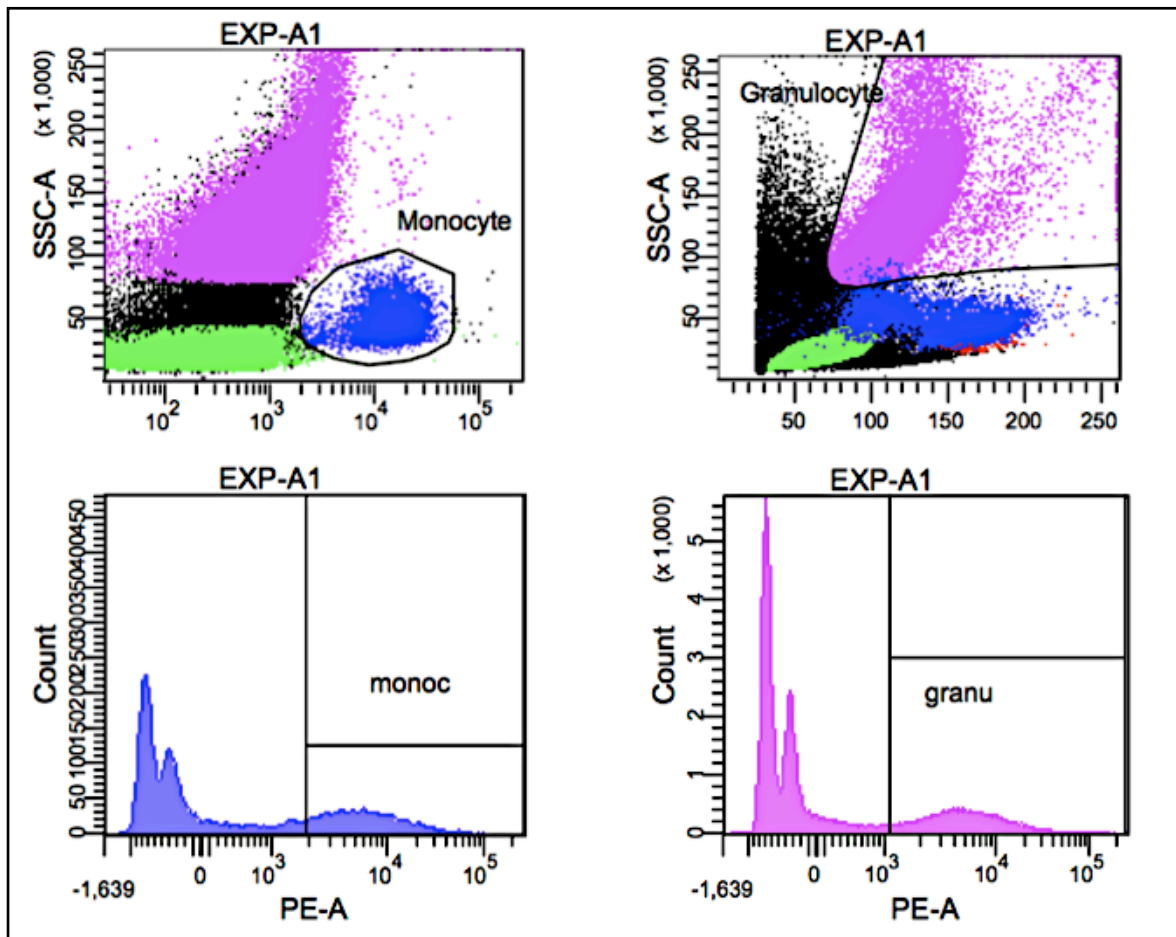

Fig. 2, Determine the impacts of platelet-monocyte and platelet-granulocyte interaction from gallic acid at different dose.
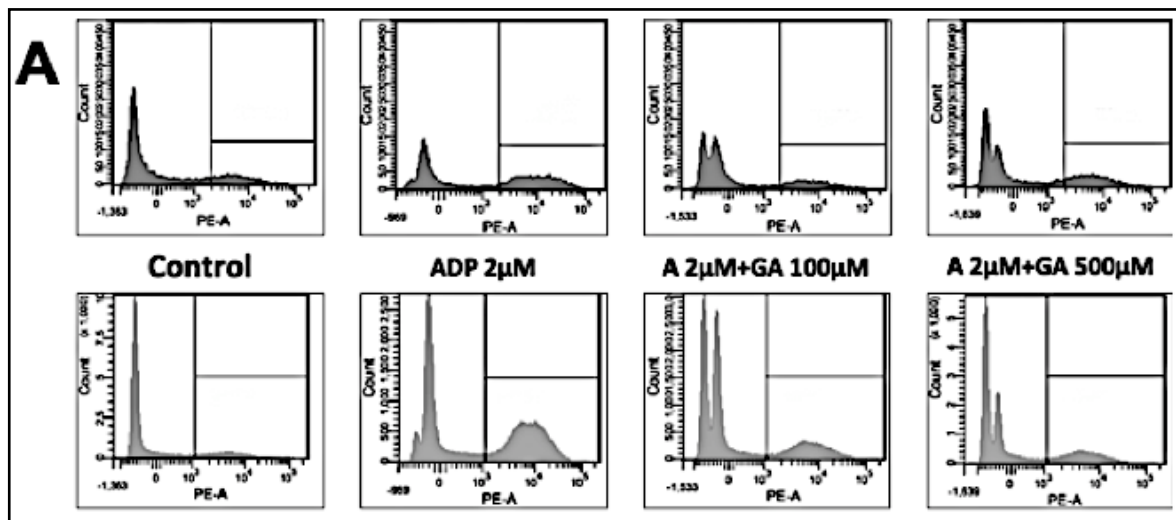
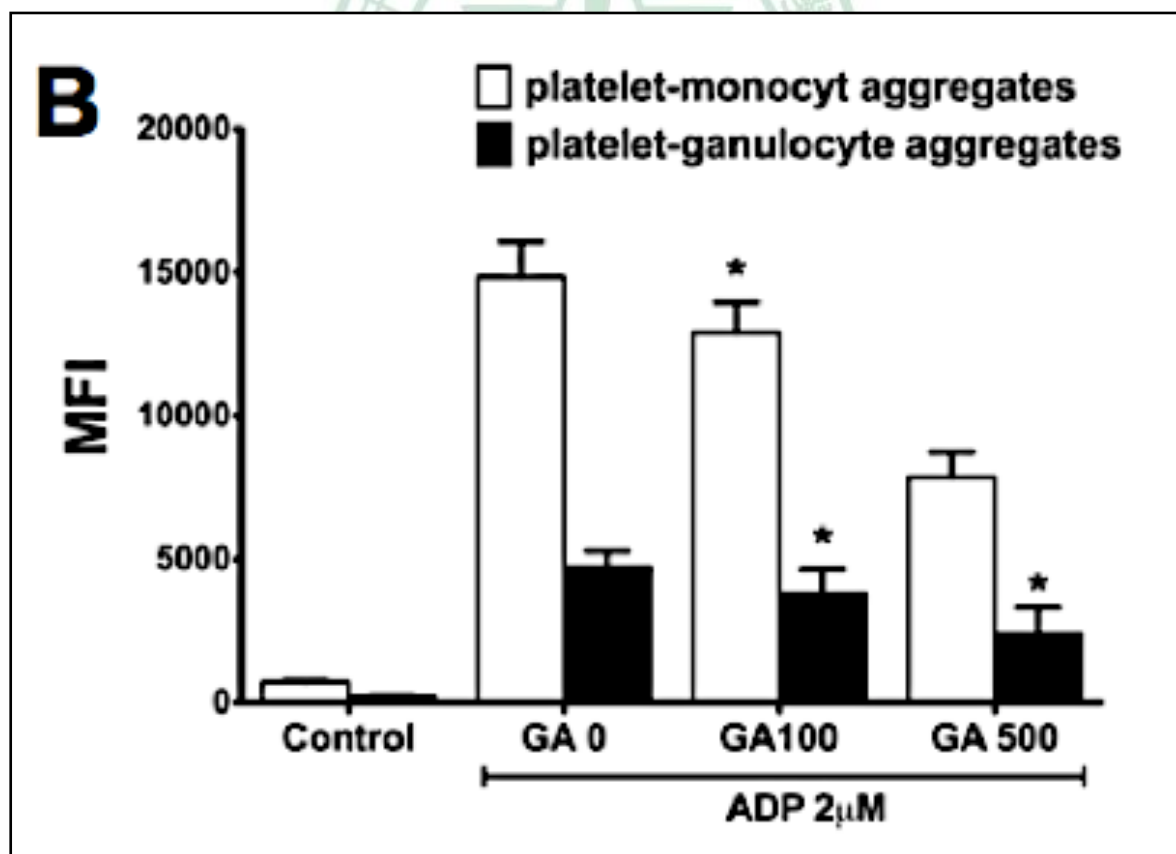Fig. 2A, Gallic acid inhibited ADP-induced PLA in whole blood at different dose.

Fig. 2B, Gallic acid inhibited ADP-induced PLA in whole blood at different dose.
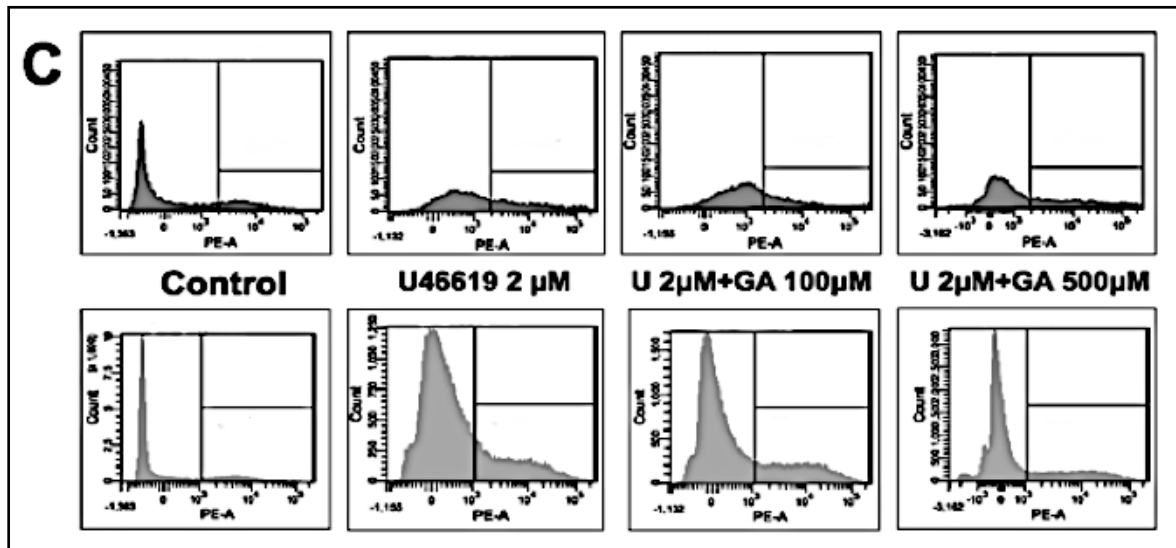
Fig. 2C, Gallic acid inhibited U46619-induced PLA in whole blood at different dose.
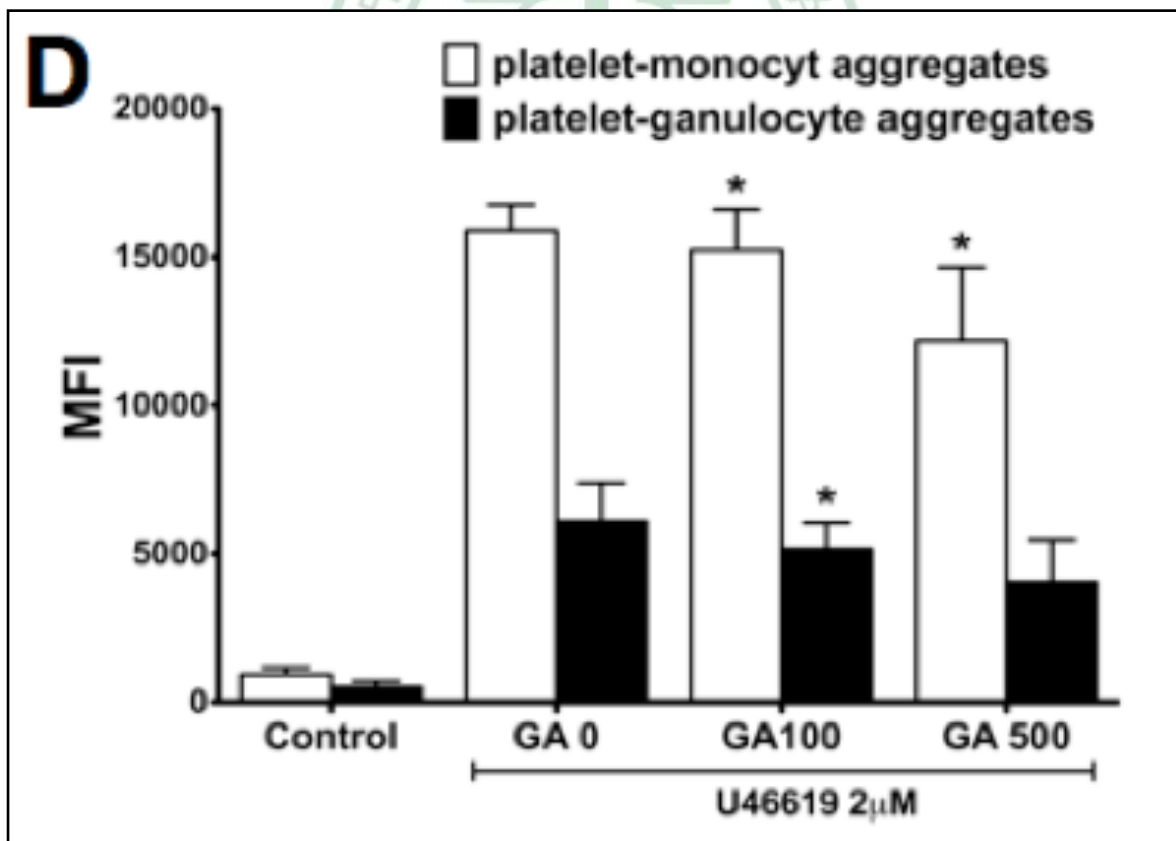

Fig. 2D, Gallic acid inhibited U46619- or ADP- induced PLA in whole blood at different dose.

3.3 Gallic Acid Interfere P-selectin expression of Platelets

The influence of gallic acid on CD62P platelet surface expression using flow cytometry was measured on resting platelets and compared to ADP and U46619 activated platelets. The platelets were exposed to 5 μM ADP and 5 μM U46619 in the presence of 50-500 μM gallic acid. Upon addition of 5 μM ADP and U46619, CD62P percent positive platelets and MFI representing CD62P cell surface density increased dramatically compared to that observed with no agonist treatment. After preincubation with increasing gallic acid concentrations had a small effect on the percentage of platelets expressing CD62P in response to ADP or U46619 (n=5, $p<0.05$, Fig. 3A, 3B). And the cell surface density of CD62P decreased significantly with gallic acid treatment ($p<0.05$; Fig 3A, 3B).

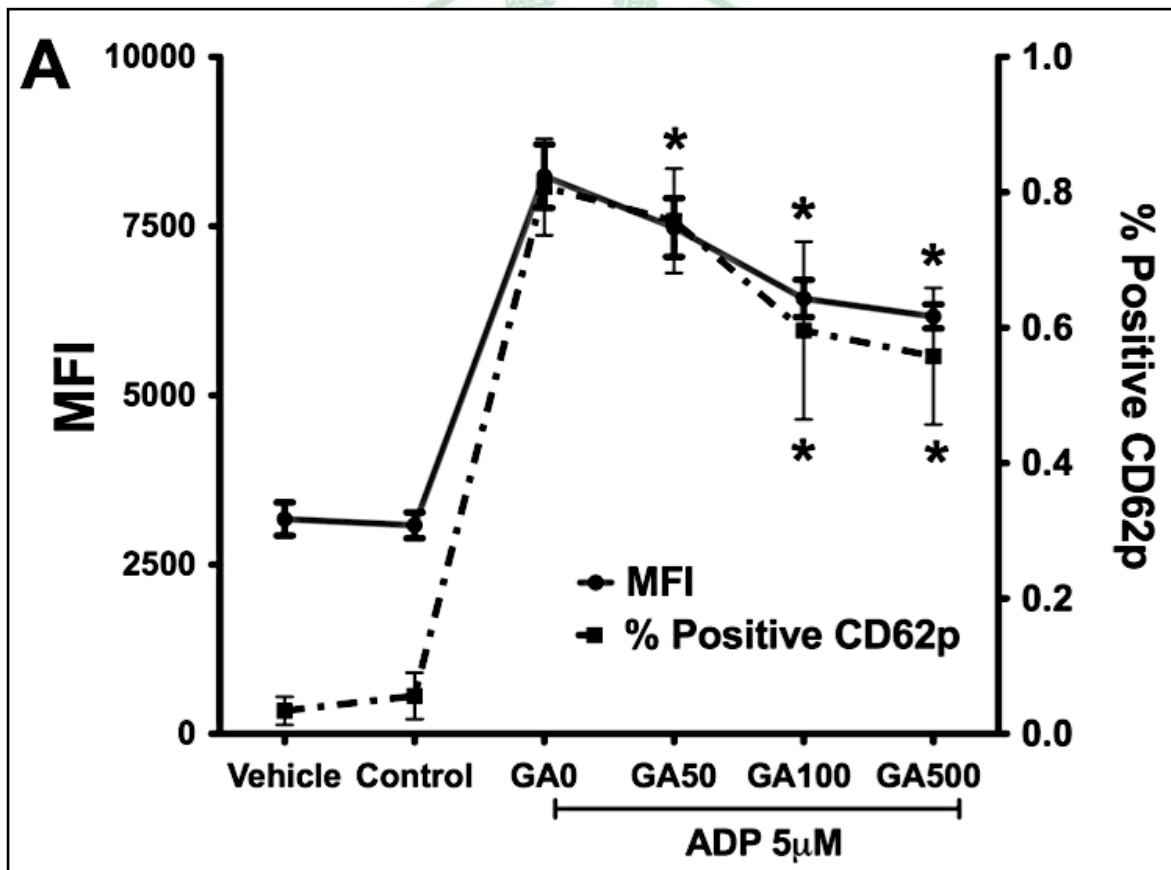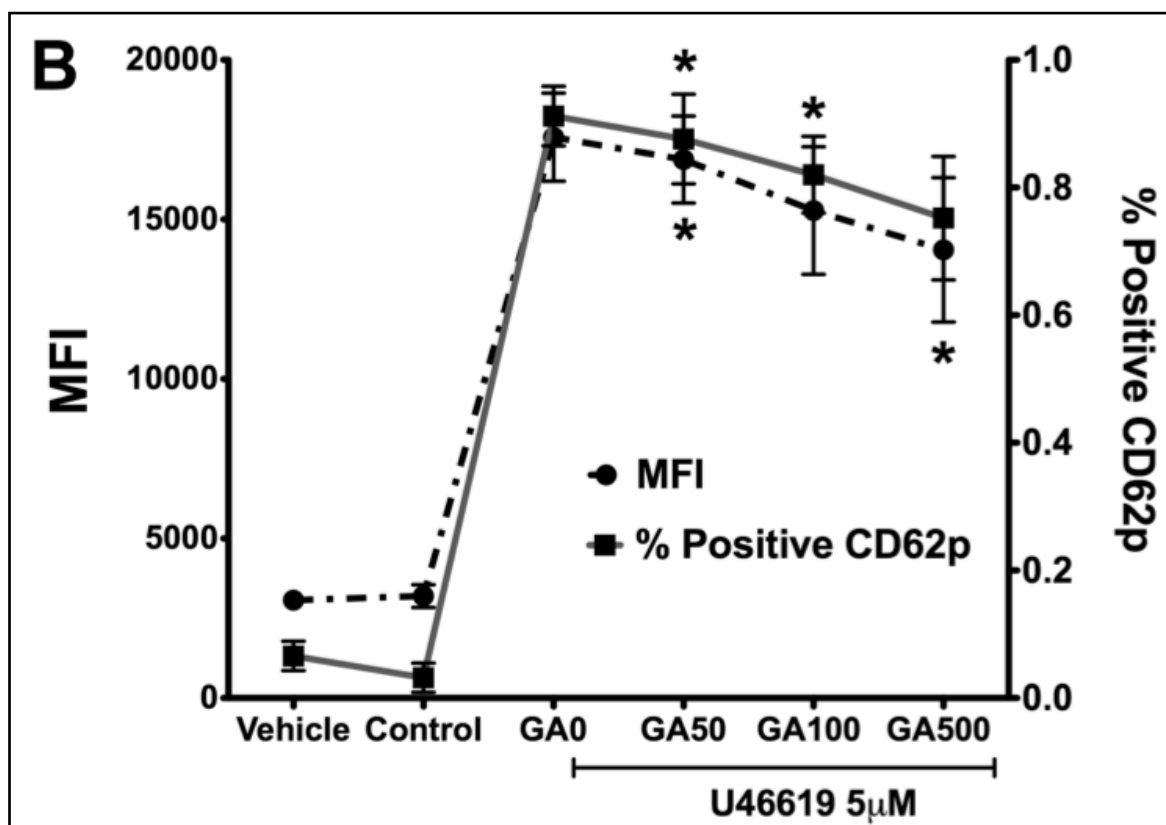

Fig. 3A, Gallic acid inhibited ADP-induced P-selectin expression.

Fig. 3B, Gallic acid inhibited U46619-induced P-selectin expression.

3.4 Effects of Gallic Acid on Intracellular Ca^{2+}, PKCα and P38MAPK of Platelets

Platelet activation and aggregation was reported to be triggered by increased intracellular Ca^{2+} levels[3,18]. We therefore examined the effects of gallic acid on intracellular Ca^{2+} level of the stimulated platelets. The representative (n=3) FACS results showed intracellular Ca^{2+} concentration after pre-incubation of gallic acid with platelets at a concentration of 100μM and 500μM then stimulated by 10μM-ADP (Fig. 4A) and 2μM-U46619 (Fig. 4B). As shown in Fig. 4, ADP and U46619 evoked a marked increase in Ca^{2+}, and this increase was markedly inhibited in the presence of gallic acid.

Hydrolysis of inositol phospholipids by phospholipase C and subsequent production of the diacylglycerol (DAG) and then activating protein kinase C (PKC) is one of the important mechanisms in platelet function[3]. P38 MAP kinase had been identified in platelets and been found to be phosphorylated by PKC and phospholipase A2, which involved platelet aggregation[19,20]. In this study, phosphorylation experiments were performed to examine the role of gallic acid in activation of PKCα and p38MAPK in

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human platelets. When ADP (2.5 μM) was added to PRP, the phosphorylated PKCα and p38MAPK increased apparently as compared with resting platelets. Gallic acid (100 μM, 500 μM) can markedly inhibited phosphorylation of PKCα and p38MAPK of platelets stimulated by ADP (Fig. 5A).
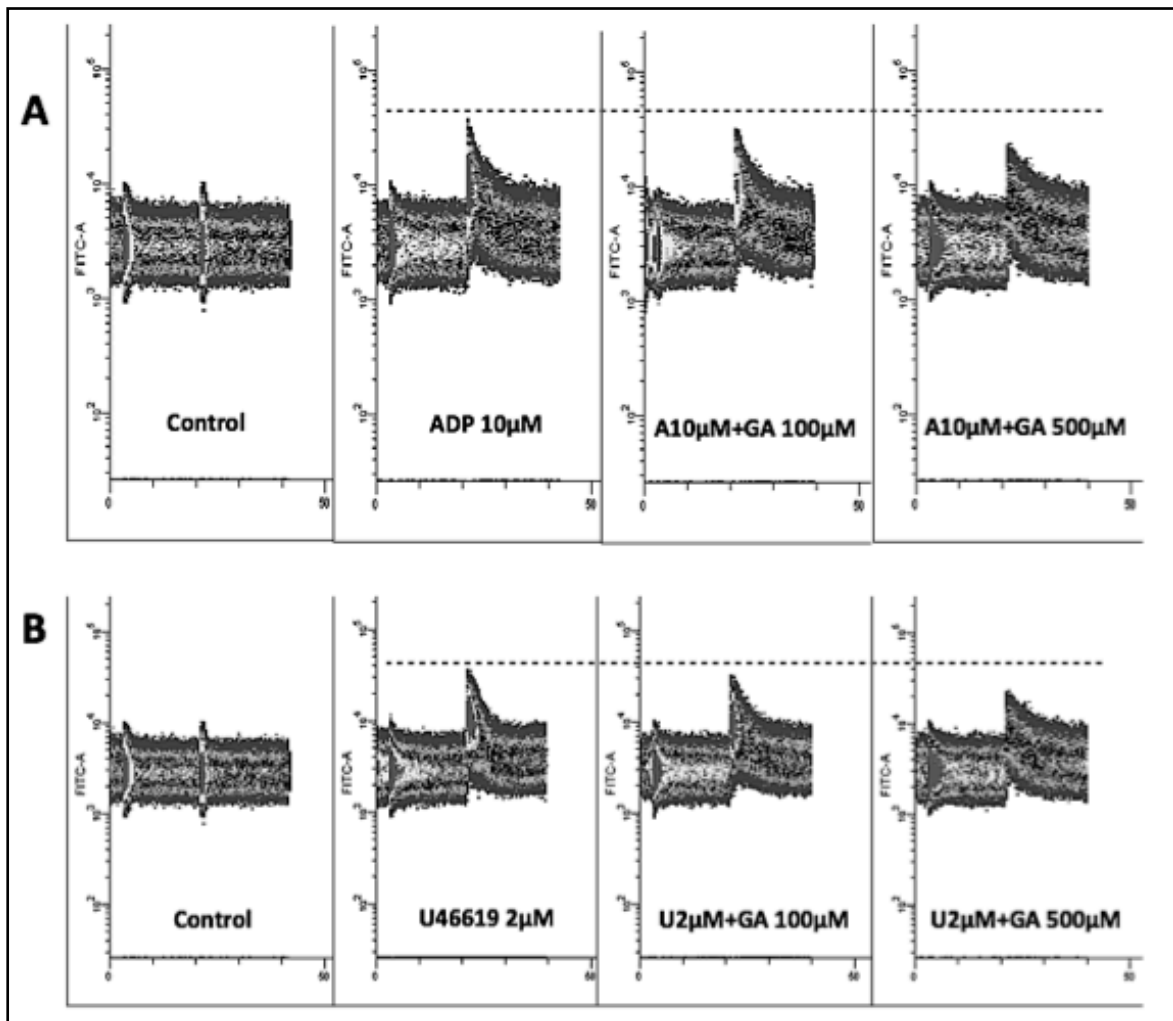
Fig. 4: Gallic acid inhibited calcium rise in platelets after stimulation with ADP (A) and U46619 (B).
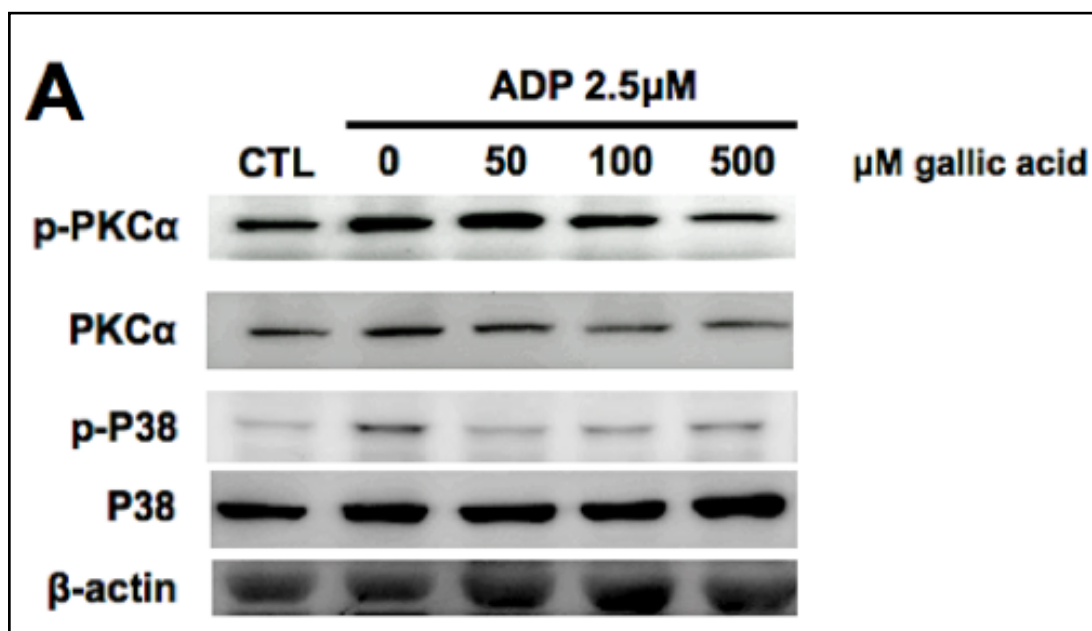
Fig. 5A, Effects of gallic acid on activation of protein kinase C alpha (PKCα), P38 mitogen-activated protein kinases (MAPK) in platelets. Platelets were pretreated with gallic acid (50 ~ 1000 μM) for 15 minutes prior to ADP 2.5 μM stimulation, and the phosphorylation of PKCα and p38 (A) was assayed by western blot (representative of 3 experiments).

3.5 Effects of Gallic Acid on Activities of Akt and GSK3β

In platelet activation, Akt/PKB activity had been noticed to related to the phosphorylation of PKC and intracellular Ca^{2+} concentration[21]. Recently, glycogen synthase kinase (GSK) 3β had been reported its significant roles as an substrate for Akt in mediating platelet activation[22]. To assess the effects of gallic acid on Akt and GSK3β, ADP (2.5 μM) was added to PRP and the level of phosphorylated Akt and GSK3β increased as compared with resting platelets. Gallic acid (1000 μM, 500 μM and 100 μM) can inhibited phosphorylation of Akt and GSK3β stimulated by ADP (Fig. 5B). From our results, gallic acid can dose-dependently decreased phosphorylation of Akt and GSK3β in human platelets.
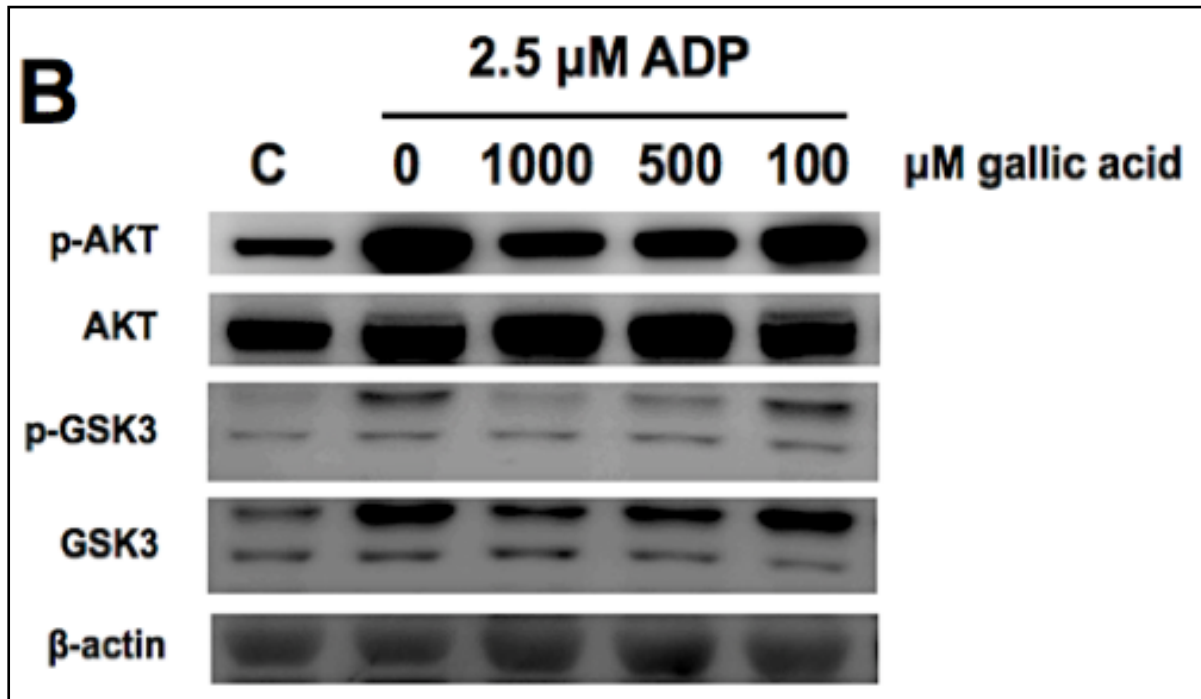
Fig. 5B, Effects of gallic acid on activation of Akt and glycogen synthase kinase 3β (GSK3β) in platelets. Platelets were pretreated with gallic acid (50 ~ 1000 μM) for 15 minutes prior to ADP 2.5 μM stimulation, and the phosphorylation of Akt and GSK3β was assessed by Western blot (representative of 3 experiments).

3.6 Effects of Gallic Acid on Reactive Oxygen Species of Platelets

Gallic acid was reported to have ROS scavenging effects[23]. ROS can enhance platelet activation and thrombus formation by attenuating the function of nitric oxide[24]. We tested whether the effects of gallic acid on stimulated platelets comes from anti-ROS ability. The representative (n=3) flow histograms are presented (Fig. 6) to underscore the effect of 500 μM gallic aci
d on ROS production. The observed curve of the treatment of U46619 and gallic acid compared to U46619 treatment alone was indicative of no interference on ROS production of the platelets.

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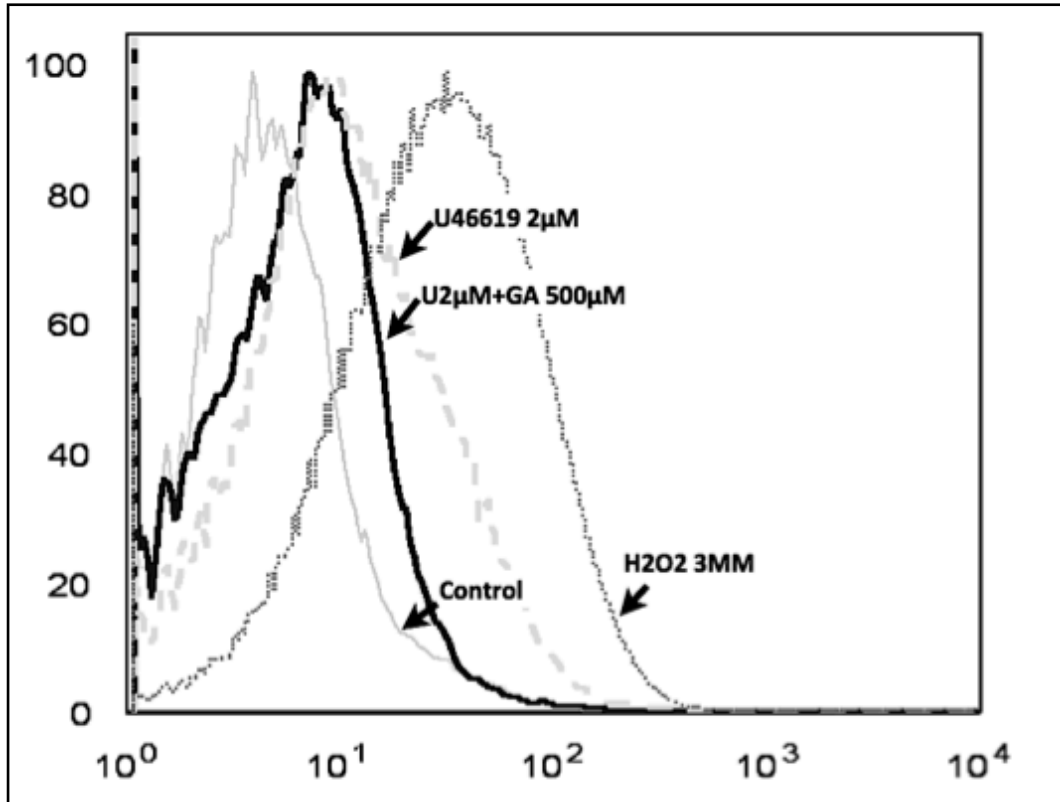
Fig. 6. The representative histograms of flow cytometry (n=3) were the effects of 500 μM gallic acid on reactive oxygen species (ROS) production. Gallic acid at a concentration of 500 μM had no inhibitory effects on platelet ROS stimulated with 2 μM U46619.

CHAPTER 4 Discussion

Platelet aggregation and activation has been correlated to a variety of atherosclerotic diseases, including coronary artery disease, transplant vasculopathy, and carotid artery disease[25]. Anti-platelet therapies, including aspirin, cilostazol and clopidogrel had been the mainstay of treatment for the atherosclerotic diseases in nowadays. Gallic acid, a major constituent comprising red wine and tea, had been widely investigated about its cardiovascular protective properties. Our present study demonstrates for the first time that gallic acid inhibits platelet activation and platelet-leukocyte aggregation and reduces Ca^{2+} mobilization with the involving signaling pathways of decreasing phosphorylation of PKCα/p38 MAPK and Akt/GSK3β.

Platelet aggregation is the principle event in thrombus formation and plays a central role in the development of acute coronary syndrome[1,3]. Recently, a semi-synthetic antioxidant (hydroxy-tyrosyl gallate) related to gallic acid had been demonstrated to exert an inhibitory effect on platelet aggregation stimulated by thrombin[26]. Previous studies also reported the anti-aggregatory effects on platelets of red wine come from interference with the synthesis of thromboxane A2, which serves in an autocrine loop that accelerates aggregation[27-29]. Herein, we using in vitro models to show that gallic acid at concentration greater than 100 μM reduces agonist-stimulated aggregation of platelets in a dose-dependent manner.

Platelet-leukocyte aggregation was found to increase in patients with acute coronary syndrome[30]. In various inflammatory clinical entities, e.g., cardiopulmonary bypass, hemodialysis, sepsis and trauma, PLA level was higher than general population[31-33]. The role of platelet-leukocyte crosstalk in atherosclerosis and inflammation had been widely investigated. They may trigger autocrine and paracrine activation processes, leading to leukocyte recruitment into the vascular wall[34-36]. The formation of leukocyte-platelet conjugates is largely mediated by the binding of P-selectin expressed on activated platelets to PSGL-1 on leukocytes. Platelet P-selectin was also shown to have an important role in arterial thrombogenesis by forming large stable platelet-leukocyte aggregates[35]. Shedding of P-selectin into the circulation was recognized to correlate with the future risks of cardiovascular diseases[5]. Recent reports demonstrate that gallic acid, as a structure-like molecule to P-selectin, can inhibit P-selectin mediated adhesion both in vitro and in vivo[37].

Our study similarly showed that gallic acid could dose-dependently reduce ADP- or U46619- induced PLA in whole blood. In addition, gallic acid or red wine had been proved to have anti-inflammatory effects [11,15]. Therefore, the inhibitory effect of gallic acid on PLA may also be attributable to the effect on leukocytes. In our work, we further revealed that gallic acid can dose-dependently attenuates ADP- or U46619- induced P-selectin expression, which may further plays a partial role in the inhibition of PLA by gallic acid. Collectively, gallic acid in our in vitro experiments is shown to regulate platelet aggregation, PLA and platelet P-selectin expression induced by its agonists, which may partly explain the cardiovascular protective effects of gallic acid.

Intracellular free Ca^{2+} concentration controls a number of platelet functions, including aggregation and P-selectin expression, and platelet agonists increase Ca^{2+} concentration, which consists of two components: release of Ca^{2+} from intracellular stores and Ca^{2+} entry through plasma membrane channels[18,38]. Kim et al., found that pre-incubation of gallic acid with mast cells decreased the intracellular calcium level after stimulation[39]. Hence, we further explored the effect of platelet incubation with the gallic acid on Ca^{2+} concentration in these cells. Treatment of washed platelets for with gallic acid of desired concentration (100 μM and 500μM) significantly reduced ADP- or U46619-evoked Ca^{2+} release. As Ca^{2+} is a potent stimulus of platelet granule secretion, the inhibition of Ca^{2+} release may lead to inhibition of platelet granule secretion with P-selectin expression. This result did provide a mechanistic involvement by which gallic acid inhibits platelet aggregation and P-selectin expression.

Stimulation of platelets by agonists results in phospholipase C (PLC)-catalyzed hydrolysis of the plasma membrane phospholipid, phosphatidylinositol 4,5-bisphosphate, with concomitant formation of IP3 and diacylglycerol (DAG)[40,41]. DAG phosphorylates PKC, inducing protein phosphorylation, ATP release and intracellular Ca^{2+} rise, and finally activate platelets. In our work, ADP-induced PKCα phosphorylation was inhibited by gallic acid, suggesting that gallic acid-mediated antiplatelet activity involves inhibition of PKCα activation. The MAPKs consist of three major subgroups. The ERKs are involved in proliferation, adhesion and cell progression, p38 MAPK and JNKs, or stress-activated protein kinases appear to be involved in apoptosis[42]. JNK and p38 MAPK have been identified in platelets[42]. The role of p38 MAPK provides a crucial signal that is necessary

for aggregation caused by collagen or thrombin[19]. Among the numerous downstream targets of p38 MAPK, the most physiologically relevant one in platelets is cytosolic phospholipase A2 (cPLA2) which catalyzes arachidonic acid release to produce thromboxane A2[20]; thus, p38 MAPK appears to provide a TxA2-dependent platelet aggregation pathway. Pre-treatment of gallic acid with the stimulation of ADP reduced the phosphorylation of P38 MAPK in our study, which may at least partly explain the inhibitory effects of gallic acid on platelet aggregation.

Platelet stimulated by agonists, thrombin and ADP, could activate G protein-coupled receptors on the platelet surface, which have been shown to activate multiple isoforms of PI3K and Akt[43,44]. Platelets from AKT-1-deficient mice are demonstrated that failed on thrombus formation after stimulation with thrombin and collagen[45]. Glycogen synthase kinase (GSK) 3β (GSK3β) had been found in platelets and it can regulate platelet activation as an Akt effector[22,46]. GSK3β is a ser-thr kinase that is regulated by its phosphorylation on ser9[47]. Phosphorylation of this residue by the ser-thr kinase, Akt, is associated with decreased GSK3 activity, which releases a tonic inhibition of the GSK3 substrate. Therefore, the phosphorylation of GSK3β by Akt suppresses its inhibitory effect on platelet function. It was reported that decreased activity of GSK3β in haploinsufficiency mice or by treatment of platelets with the inhibitor (LiCl or SB216763) enhances agonist-induced dense granule secretion[46]. Our results disclosed that gallic acid reduced the phosphorylation of Akt and GSK3β in platelets stimulated by ADP. Taken together, from our data, gallic acid may exert its anti-platelet effects via regulating the signals of PKCα/ p38MAPK and Akt/GSK3β.

Though red wine[48] and tea[13] both had been known to have anti-oxidant properties, there were no studies about the anti-ROS effects on platelet of gallic acid. Some studies even reported that gallic acid only had a weak inhibitory effect on oxidative stress[23]. In our experiment, after pre-treatment of gallic acid, there is no influence on platelet ROS production with induction of U46619 (2 μM) or ADP (2 μM) (not shown). Earlier investigations noticed that oxidative stress could activate platelets and lead to thrombosis through consumption of nitric oxide[2]. Therefore, the inhibitory results of platelet function from gallic acid may not come from the anti-oxidative actions.

CHAPTER 5   Conclusion

Our study demonstrated that gallic acid inhibited platelet aggregation, P-selectin expression and PLA stimulated by ADP or U46619 possibly through decreasing intracellular Ca^{2+} mobilization. And the inhibition of phosphorylation of PKCα/p38 MAPK and Akt/GSK3β in stimulated platelets after gallic acid pre-treatment is the suggestive mechanisms of action. This is the first report about the properties of gallic acid on platelet inhibition and its mechanisms. These findings of gallic acid suggest a possible therapeutic application of this agent in the diseases associated with atherosclerosis.

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