Chapter-3

Acetyl Salicylic Acid induced inhibitory effect on platelet aggregation in acute myocardial infarction is nullified by dermcidin protein

Introduction

Platelet aggregation by ADP, collagen, thrombin and l-epinephrin is a normal physiologic process which is essential and helps to main blood coagulation system (Colman *et al.,* 1987). But, on the other hand the massive aggregation of platelet is occurred specifically at fissuring site or plaque rupture/erosion of the wall of the different coronary artery is reported to result thrombus formation (a micro aggregate of platelets embedded in fibrin mass) which can cause the blockade of the normal flow of blood in the heart musculature (Fuster *et al*., 1996) and led to acute coronary syndrome (ACS). When normal blood circulation is shut off, it not only sieges the availability of the oxygenated blood, but also impedes the supply of nutrients, water and metal ions which are crucial for the normal activity of the heart. Prostacyclin (Whittle *et al*., 1978), insulin (Sinha *et al*., 1999), IFN-α (Hoylaerts*et al*., 1982), and estriol (Jana *et al*., 2013) are the inhibitors of aggregation, which can inhibit excessive platelet aggregation to sustain the systemic homeostasis. Acetylsalicylic acid (aspirin) is established for its beneficial effect for the secondary prevention of cardiovascular disease as such through its ability to inhibit platelet aggregation (Pollack *et al*., 1995). The over-exceed platelet aggregation may also occlude the pericardial artery on the heart muscle resulted in cell death on the heart surface and create infarction, which appeared as a patchy black area and eventually could produce acute myocardial infarction (AMI), which when massive, could result in the death of the victims (Page *et al*., 1971).

It has been reported already by many scientists that aspirin possesses the inhibitory effect on the aggregation of platelet and doses of aspirin can improve all acute conditions associated with the disease (Page *et al*., 1971). But, however, aspirin was unable to show that kind of effect in case of AMI and unable to inhibit platelet aggregation (Poulsen *et al*., 2007), and also it was also observed that the utilization of aspirin in a proper way is very little in AMI (Borna *et al*., 2005).

We have reported from our laboratory that the appearance of a novel protein in the circulation of both ACS and AMI (Ghosh *et al*., 2011). This protein has been identified to be dermcidin, a stress induced protein of Mr 11 Kda (Ghosh *et al*., 2011).

Herein, we observed the higher concentration dermcidin in AMI (forty folds more than normal) which might be the cause of aspirin failure and we also have demonstrated here a specific way of aspirin application that a low and unique dose of aspirin might be helpful for the AMI by the removal of dermcidin from the platelets.

RESULTS

Different platelet aggregating agents (ADP, epinephrine, collagen and thrombin) instigated platelet aggregation in the presence of acetyl salicylic acid (aspirin) in normal volunteers and from AMI subjects

PRP of AMI was incubated with different concentrations of aspirin at 37°C for 30 minutes in the presence of different aggregating agents like ADP (0.2µM) or collagen (2µg/ml) or l-epinephrine (5µM) or thrombin (1 unit/ml) and failed to construct the inhibitory effect of the compound, whereas in case of normal platelet aggregation is absolutely inhibited.

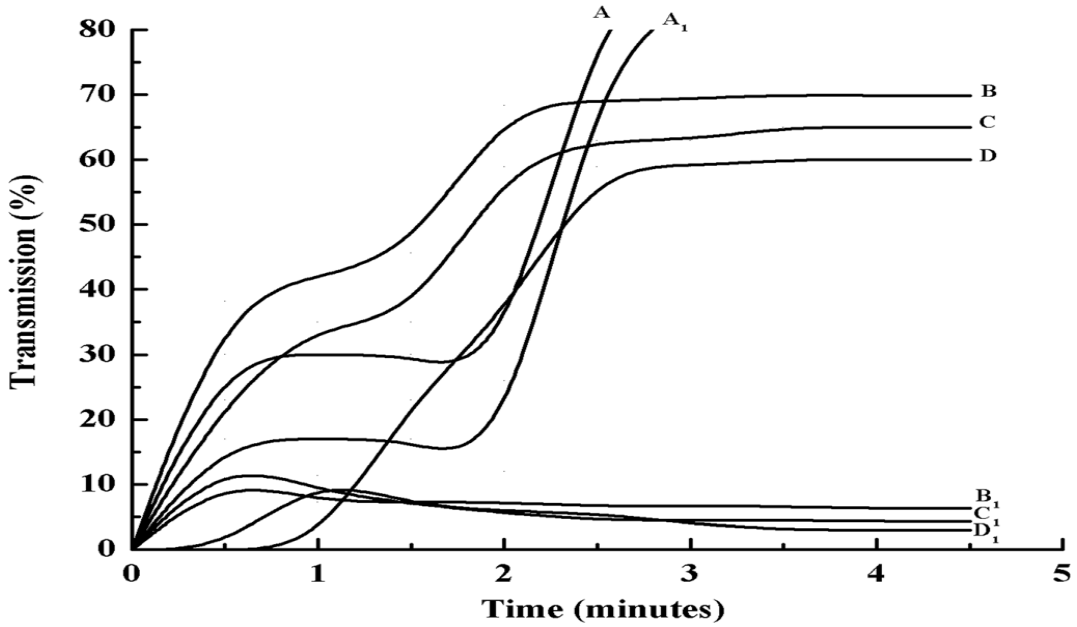

Fig-3.1: The effect of aspirin on the inhibition of platelet aggregation instigated by platelets aggregating agents in normal and AMI subjects. *The PRP from normal and AMI subjects were incubated with aspirin (80 µM) for 30 minutes at 37°C followed by platelet aggregation was initiated by adding different aggregating agents as above indicated. (A) = Aggregation induced by thrombin (1.0 Unit/ml) in aspirin treated PRP from AMI patient. (A1) = Normal PRP was treated with aspirin and aggregation induced*

by thrombin. The ascending transmission in both cases (A and A1) was due to the clotting of the PRP induced by thrombin. (B) = The platelet aggregation induced by 2.0 µM ADP in AMI PRP treated with aspirin. (B1) = The effect of treatment of normal PRP with aspirin on the aggregation of platelets induced by ADP. (C) = l-epinephrine (5.0 mM) induced platelet aggregation in PRP treated with aspirin from AMI subjects. (C1) = The effect of treatment of normal PRP with aspirin on the l-epinephrine induced platelet aggregation. (D) = Collagen induced platelet aggregation (2 mg/mL) in aspirin treated PRP from AMI subjects. (D1) = The effect of treatment of normal PRP with aspirin on the aggregation of platelets induced by collagen. AMI volunteers (n=20, M=10, F=10).

Dermcidin has a role in the resistance of aspirin effect on platelet aggregation in AMI persuaded by ADP.

To test this validation the platelets from normal volunteers was incubated with 0.4 μM dermcidin (dermcidin concentration was ≈0.4 μM in AMI plasma, as described below) for 90 minutes at 37°C, and the dermcidin treated PRP was subsequently used to determine the inhibition by aspirin of the ADP induced platelet aggregation. It was obtained that the treatment of normal PRP with dermcidin showed the platelets resistance states against the effect of aspirin (line A) when compared to control where the PRP from normal was incubated with 0.9% NaCl for 90 minutes at 37°C (line B).

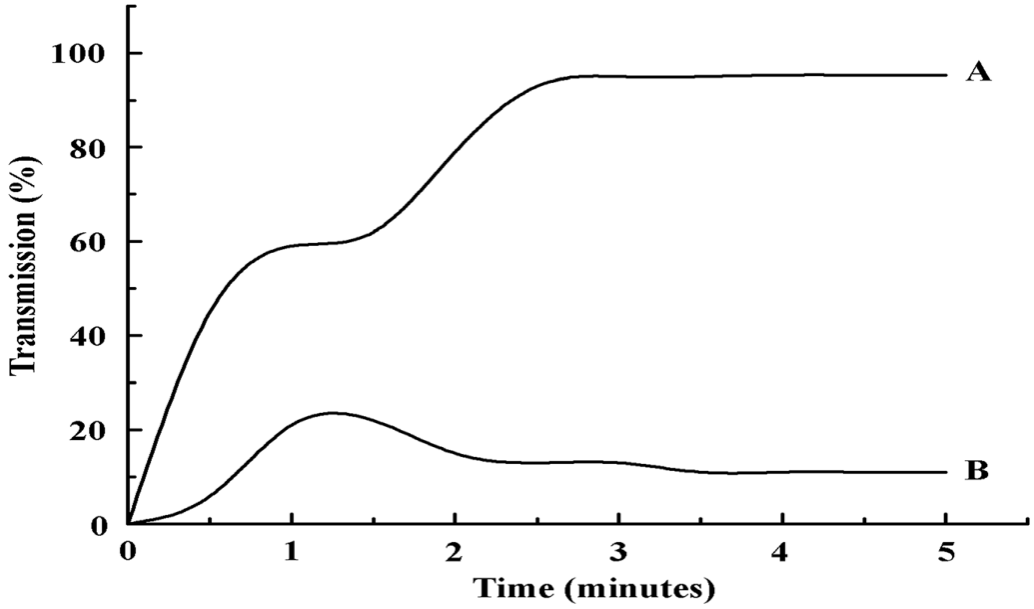

Fig-3.2: Incubation of 0.4 μM dermcidin with normal PRP and its effect on the platelets where aspirin is treated and aggregation is induced by ADP. *(A) = normal PRP where dermcidin is treated in presence of aspirin and aggregation actuated by ADP, (B) = normal PRP in the presence of aspirin and aggregation induced by ADP where dermcidin is not treated. The figure is an illustrative of at least 10 different demonstrations from 10 different volunteers by using blood samples.*

Equilibrium tie-up of dermcidin to normal platelets and it was analyzed by scatchard plot

To determine whether dermcidin has an effect on the occurrence of resistance to aspirin and the event was liaised through the binding of the stress protein to the platelets, equilibrium binding was analyzed by Scatchard plot between dermcidin and normal platelets. And a curvilinear plot was found that dictated dermcidin possessed the heterogeneous binding sites populations on the platelet surface, i.e. one is low affinity-high capacity binding sites and another is high affinity-low capacity binding sites (Kahn, N. N. & Sinha, A. K., 1990).

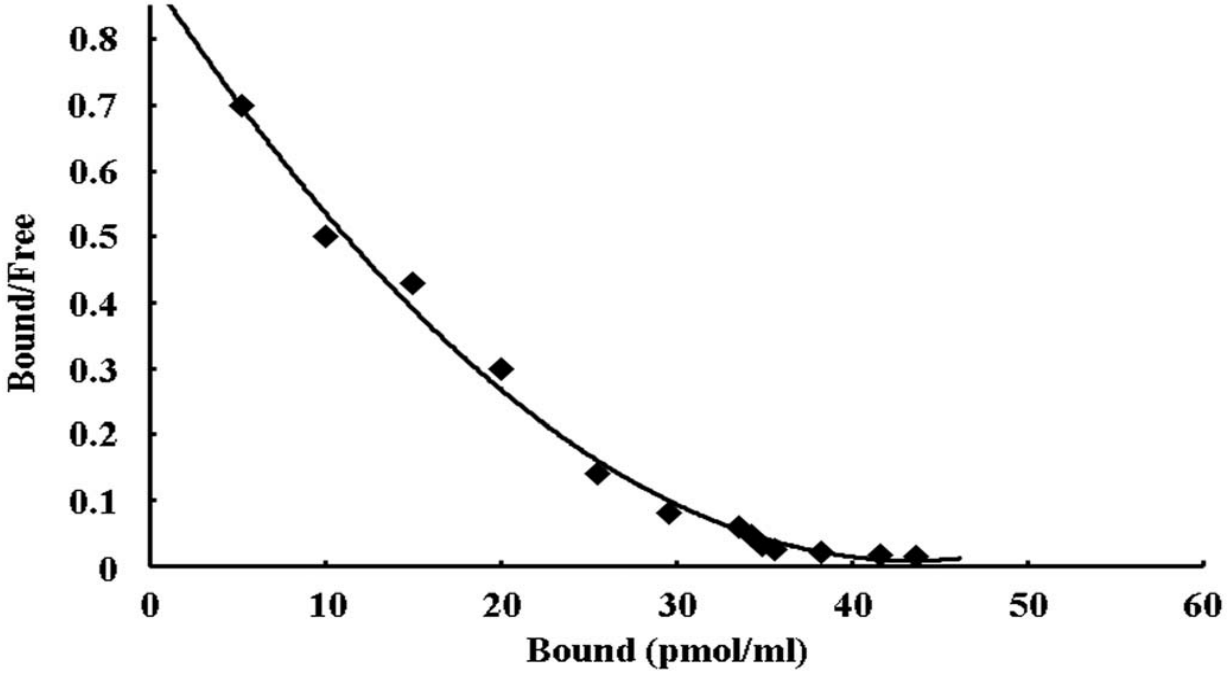

Fig-3.3: Scatchard plot analysis of dermcidin and normal platelets. *From the normal volunteers gel filtered platelets were prepared. The platelets were gel filtered by the column. The filtered platelets (3X10^8 platelets/mL) were incubated with dermcidin (0.4 µM) at 37°C for 90 minutes. The unbound dermcidin was sieved by Millipore filtration from the platelets bound dermcidin as described in the Methods section. The bound*

dermcidin was allowed to leave from the platelets by the 0.05% TritonX-100 and the binding of dermcidin was ascertained by ELISA by using dermcidin antibody.

The role of polyclonal dermcidin antibody against the effect of aspirin resistance in AMI subjects due to the presence of dermcidin

To investigate the role of dermcidin further as the cause of resistance of aspirin effect in platelet aggregation in AMI, dermcidin antibody was added to the PRP of AMI and incubated for 90 minutes at 37°C. The dermcidin antibody treated PRP was subsequently challenged to the inhibitory effect of aspirin in the ADP induced platelet aggregation.

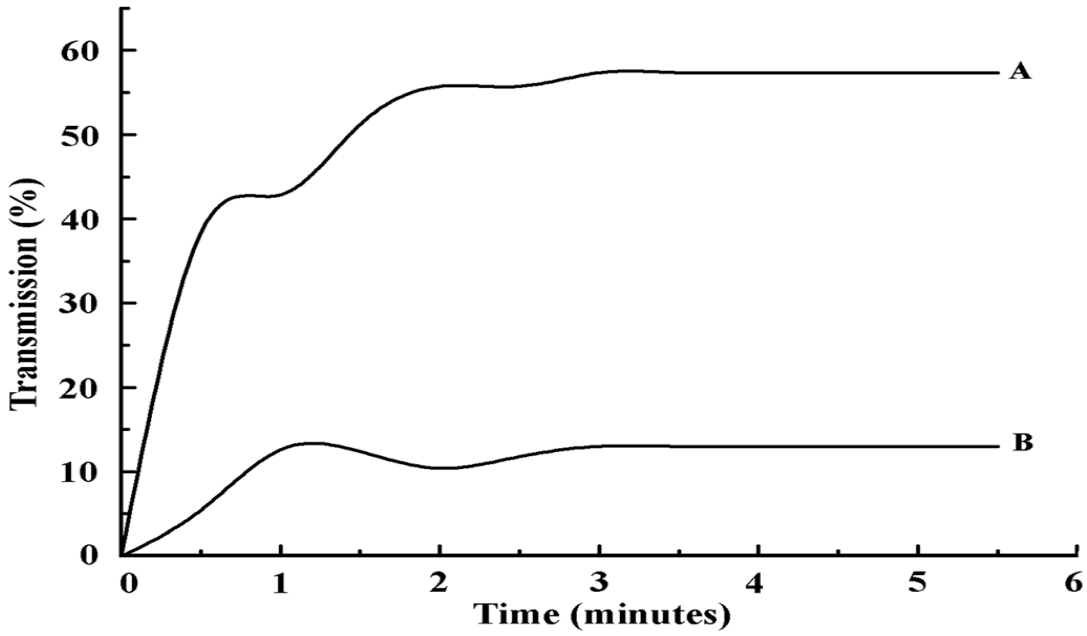

Fig-3.4: Role of dermcidin antibody *(A) represents the PRP from AMI subjects was incubated with aspirin for 30 minutes at 37°C and aggregation of platelets was induced by ADP. (B) represents the PRP from same AMI patients treated with dermcidin antibody and subsequently treated with aspirin under identical condition like A. Figures shown here are typical representative of at least 10 experiments using PRP from 10 different AMI patients (n=10, M = 5, F = 5).*

Removal of dermcidin from the surface of platelets of AMI by the stimulation of Nitric Oxide

It has been reported that dermcidin is a formidable inhibitor of nitric oxide syntheses (NOS) and it has also been studied that even in the absence of ADP, platelets can induce aggregation through the thromboxane A2 (Sinha A. K., 1983). So, NOS has a role in the expulsion of bound dermcidin from the platelets and the effect of aspirin on AMI platelet aggregation was studied consequently. It was found that at 15µM concentration of aspirin was able to trigger NO in platelets (Sinha *et al*., 1999; Karmohapatra *et al*., 20007) but was unable to inhibit platelet aggregation. This concentration of aspirin was able to remove platelet bound dermcidin from AMI PRP through the NO production. The 30µUnit of insulin/mL may be used instead of aspirin and was found the same dermcidin removal effect like aspirin through the stimulation of NO synthesis in (Sinha *et al*., 1999) in AMI platelets. The pre-incubation of AMI PRP with a NOS inhibitor N, ^{G}-nitro-L-arginine methyl ester (L-NAME) (0.1 mM), (Kahn *et al*., 2000) ensued in the neutralization of aspirin effect in both cases, i.e. expulsion of dermcidin from AMI platelets and in the increase of nitric oxide level. Furthermore, it was also found that the 0.8 nM NO solution in 0.9% NaCl instead of either insulin or aspirin was able to remove the dermcidin from AMI platelets. Contrarily, l-NAME (0.1 mM) had no effect on the invalidation of the removal of dermcidin from the platelets.

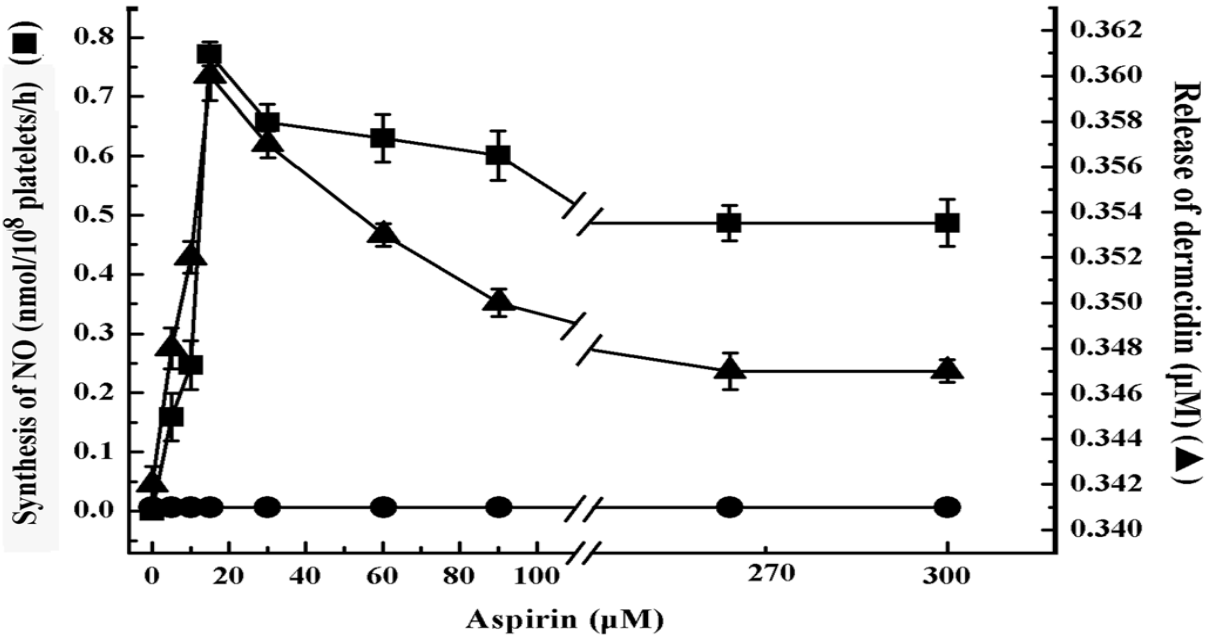

Fig-3.5: Treatment of 15μM aspirin in the PRP of AMI subjects and the expulsion of dermcidin with the increase of NO production *Solid triangles* ***(▲)*** *demonstrate here the liberation of the bound dermcidin from AMI platelets incubated with a dissimilar concentration of aspirin. NO production in the presence of different conc. of aspirin was represented by Solid squares (■). Solid circles (●) indicate PRP of AMI treated with the aspirin and 0.1 mM NAME (an inhibitor of NO synthesis) on the liberation of dermcidin from the platelets. Correlation Coefficient (''r'') represents between release of dermcidin from AMI and the increase of NO from 0 μM to 30 μM aspirin was +0.967 and ''two tailed p value'' is 0.006. The figure demonstrated is a representative of at least 10 different subjects with AMI.*

The effect of expulsion of the bound dermcidin from the platelets of AMI on the prohibitory effect of aspirin on platelet aggregation induced by ADP

For this experiment, aspirin (15μM) for 30 min or insulin (30 μUnits/mL) for 2 hours or 0.8 nM nitric oxide for 30 min were pre-incubated with PRP from the AMI patients at

37°C, and subsequently different concentration of aspirin was incubated with above stated reaction mixture and the ADP (2.0 µM) induced platelet aggregation was done. It was observed that the 10 µM aspirin was able to inhibit ADP induced aggregation in all cases. In contrary, 80µM aspirin was needed in normal to inhibit platelet aggregation. Specifically, the AMI platelets turned 8 folds more sensitive to the aspirin effect when compared to normal cases when bound dermcidin was liberated from the platelets.

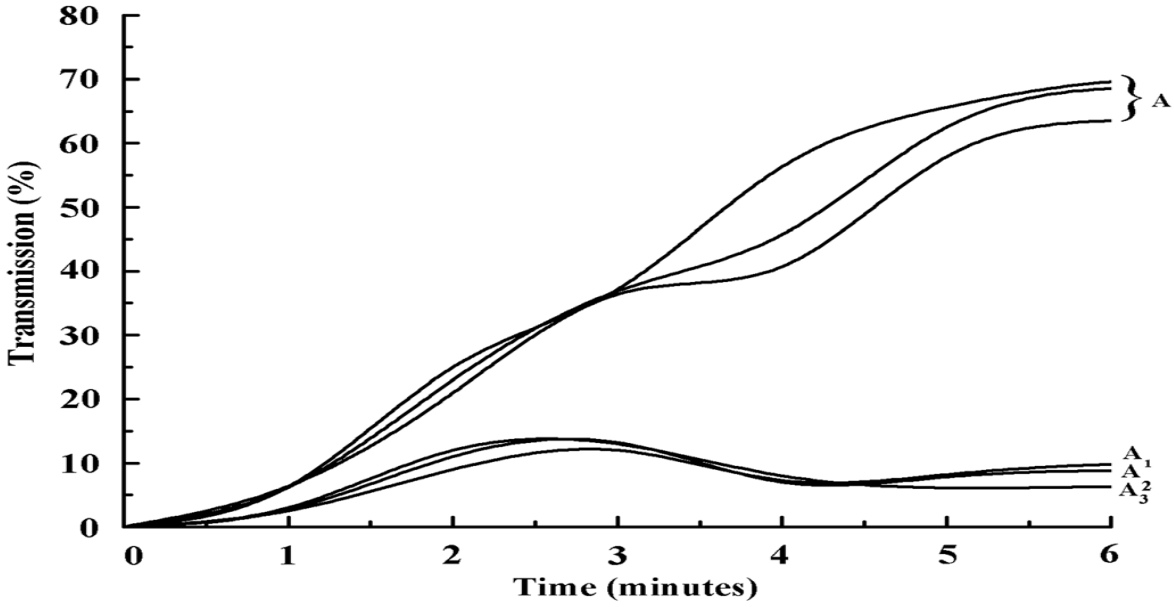

Fig-3.6: *Here, (A) indicates the AMI platelet aggregation induced by 2.0 µM ADP from 3 separate AMI subjects incubated with 80µM aspirin for 30 minutes at 37°C. A1 = The PRP from the same AMI subjects pre-incubated with 15 mM aspirin for 30 minutes at 37°C followed by treatment of the PRP with 10 mM aspirin for 30 minutes at 37°C.*

Scatchard plot analysis of the binding of dermcidin in AMI PRP pre-incubated with insulin, aspirin or with NO in NaCl solution (0.9%)

As the above experiment showed that pre-incubated AMI PRP with aspirin, insulin or with NO in 0.9% NaCl solution was found to resensitize the platelets to the aspirin effect, the binding properties of dermcidin on the AMI platelet in the presence of above stated

agents was assayed by Scatchard plot analysis. It was observed that the treatment of those agents (aspirin, insulin or NO) in platelets of AMI patients reduces the dermcidin binding sites (n) on the platelets in each case where dissociation constant (K_d = 40 nM) remained unchanged. It was found that the binding of dermcidin 128X10^3 molecules/platelet in the untreated AMI platelets decreased to 80 X10^3 molecules/platelet and to 76X10^3 molecules/platelet and to 78X10^3 molecules/platelet dermcidin binding sites when the AMI platelets were treated with aspirin, insulin and NO solution respectively.

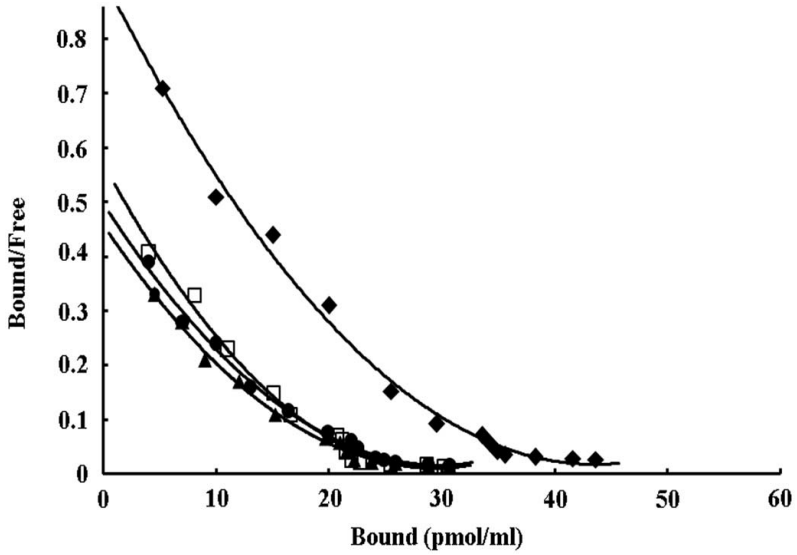

Fig-3.7: Dermcidin binding in presence of aspirin, NO or insulin was analyzed by Sctachard plot. *In control experiment, solid squares (■) indicate dermcidin binding on platelets. Hollow squares (□), solid triangles (▲) and solid circles (●) represent the binding of dermcidin in presence of 15µM aspirin, 30 µUnit of insulin/mL and 0.8 nM NO in 0.9% NaCl respectively. Details of experiment has been described in Method and Materials section; K_d and the binding site (n = dermcidin binding sites/platelet) was analyzed from scatchard plot. The Figure indicated here is an illustrative of 6 different AMI subjects which are divided in 2 different groups with 3 AMI subjects in each group.*

Discussion

Aspirin can inhibit platelet aggregation in two ways, one is through the inhibition of cyclooxygenase (Zhou *et al*., 2011) and another is by the induction of nitric oxide synthase (Ghosh *et al*., 2011), and in AMI, the use of aspirin is the corner-stone remedy. Although in AMI, >90% of manifestations have been claimed to be caused by thrombosis (Carvalho de Sousa *et al*., 1988) it was also reported that aspirin was unable to cease the platelet in AMI and the utilization of aspirin in case of AMI is very little or not known properly (Borna *et al*., 2005). And as such, it could be proposed that a third factor is involved in the resistance of aspirin in the inhibition of platelet aggregation. We have reported before that was a novel 11kDa protein dermcidin in the circulation of ACS and this protein is the inhibitor of nitric oxide synthases. It was reported that aspirin and insulin were able to inhibit the synthesis of dermcidin through the synthesis of NO (Ghosh *et al*., 2011). Dermcidin showed its inhibitory effect against nitric oxide through its binding to the "receptors" on platelet surfaces and failure of aspirin effect to neutralize the aggregation in AMI. Dermcidin, a NOS inhibitor, and also a hypertensive and diabetogenic protein, was found to be >40 folds more powerful aggregating agent than ADP induced aggregation through the impediment of synthesis of nitric oxide in platelet and as a result synthesis of thromboxane-A_2 in platelets was increased and leads to the platelet aggregation (Ghosh *et al*., 2011).

Insulin, aspirin or NO itself can induce NO which stimulated the expulsion of bound dermcidin from the high affinity binding sites of platelets, and as a result platelets of AMI became 8 folds more sensitized to the inhibitory effect of aspirin.

So, it can be inferred that aspirin itself was not the issue, but the presence of dermcidin in circulation of ACS & AMI was the cause of failure of the effect of aspirin to the inhibition of AMI platelet aggregation. So, dermcidin played the role in the

pathophysiology of deadly AMI. This protein can invalidate the aspirin effect on platelet aggregation through the mechanistic way of prostaglandin synthesis.

If these *in-vitro* study results could be extended to the AMI subjects, it might be argued that the 300 mg aspirin is not the prime dose, but the persistent use of 14 mg bolus of aspirin/70 kg body weight and after 30 min another 9 mg bolus of aspirin/70 kg weight as described might be fruitful in the expulsion of bound dermcidin from platelets to inhibit aggregation and may ameliorate the condition.