Role of cytosolic calcium and actin polymerization on agonist-induced secretion by the platelets of liver cirrhosis patients

Karaciğer sirozlu hastaların trombositlerinde aktin polimerizasyonu ve sitozolik kalsiyumunun etkisi

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Abstract

Objective: Variceal bleeding in cirrhosis is mainly due to platelet activation defect and secondary to coagulation defects. Secretion is an important process which release procoagulants for hemostasis. In the present investigation we have evaluated the secretory function of platelets in liver cirrhosis and also the simultaneous changes in cytosolic calcium (Ca^{2+}) and the polymerization of actin in agonist- stimulated platelets in vitro.
Methods: Liver cirrhotic patients with (n=27) or without (n=23) bleeding complication were included in the study. Control subjects (n=50) were also utilized for the study to compare the analytical data. Platelets were activated by collagen in vitro and the secretory response was assessed by the levels of nucleotides, serotonin, pyrophosphate (PPi) and inorganic phosphate (Pi) secreted into the extracellular fluid of the platelet suspension at various time intervals. During the course of secretion the alteration in the polymerization of actin was monitored simultaneously with the changes in the cytosolic Ca^{2+} level.
Results: The secretory response of platelets to collagen was significantly low in both bleeders and non-bleeders when compared to that of normal subjects. During secretion, low level of actin polymerization and cytosolic Ca^{2+} level were observed in the platelets of bleeders than in non-bleeders and normal subjects. The low secretory capacity of cirrhosis platelets could be correlated with low levels of actin polymerization and cytosolic Ca^{2+}. The alterations were highly significant in the platelets of bleeders when compared to those of non-bleeders.
Conclusion: The defective secretory activity of platelets in cirrhosis bleeders might be partly due to low polymerization of G-actin to F-actin which is required for platelet shape change and for the release of procoagulants. Cytosolic Ca^{2+} level seems to influence actin polymerization and thereby impairs platelet secretory response to agonists in cirrhosis patients with bleeding complication. *(Turk J Hematol 2009; 26: 82-9)*
Key words: Cirrhosis, platelets, secretion, actin, polymerization, cytosolic calcium, serotonin, adenine nucleotides

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Özet

Amaç: Sirozun başlıca komplikasyonlarından biri trombosit aktivasyon bozukluğu ile ilişkili olan varis kanamasıdır. Bu araştırmada, karaciğer sirozu olan hastalardan izole edilen trombositlerin, hemostaz için prokoagülan salınımında önemli

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bir süreç olan, sekretuvar kapasitesini belirledik. Ayrıca, in vitro olarak agonistle uyarılan trombositlerdeki sitosolik kalsiyumdaki (Ca^{2+}) eş zamanlı değişiklikleri ve aktinin polimerizasyon durumunu da araştırdık. Bu çalışmanın amacı, karaciğer sirozunda trombositlerin sekretuvar fonksiyonunun aktin polimerizasyonunun seviyesinden ve sitosolik Ca^{2+} seviyesindeki değişikliklerden etkilenip etkilenmediğini değerlendirmekti.

Materyal ve Metod: Trombositler in vitro olarak kollajen ile aktive edildi ve sekretuvar cevap çeşitli zaman aralıklarında trombosit süspansiyonunun hücre dışı sıvısına salınan nükleotid, serotonin, pirofosfat (PPi) ve inorganik fosfat (Pi) seviyeleri ile değerlendirildi. Salınım boyunca, aktin polimerizasyonundaki değişimler, sitosolik Ca^{2+} seviyesindeki değişikliklerle eş zamanlı olarak izlendi.

Bulgular: İstirahat halindeki trombositlerdeki bazal serotonin seviyesi hem kanaması olan hem de kanaması olmayan bireylerde normal kişilerle karşılaştırıldığında anlamlı olarak düşüktü. Adenin nükleotidlerin seviyesi kanaması olan bireylerde anlamlı olarak düşüktü. Sirotik trombositlerin sekretuvar kapasitesi düşük olduğu bulundu ve düşük seviyelerdeki aktin polimerizasyonu ve sitosolik Ca^{2+} ile ilişkili olabilirdi. Kanaması olan bireylerin trombositlerindeki değişimler kanaması olmayan bireylerle karşılaştırıldığında anlamlı derecede yüksekti.

Sonuç: Sirozda trombositlerin bozuk sekretuvar aktivitesi, kısmen trombosit şekil değişikliği ve prokoagülanların salınımı için gerekli olan, globüler aktinin filamentöz aktine polimerizasyonunun düşük olmasına bağlı olabilir. Sitosolik Ca^{2+} seviyesi aktin polimerizasyonunu ve böylece agonistlere karşı trombosit sekretuvar cevabını etkiliyor gibi görünmektedir.
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Anahtar kelimeler: Siroz, trombosit salınımı, aktin polimerizasyonu, sitozolik kalsiyum, serotonin, adenin nükleotidler

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Introduction

Cirrhosis of the liver is anatomically defined to be an end-stage liver disease characterized by fibrosis and structurally abnormal nodules [1]. Cirrhosis leads to multiple complications like ascites, gastrointestinal and esophageal variceal bleeding as a result of portal hypertension and platelet dysfunction [2]. Thrombocytopenia is a significant feature of chronic liver diseases including cirrhosis and attributed to passive platelet sequestration in the spleen. The platelet dysfunction in cirrhosis is probably both intrinsic to the platelets and secondary to soluble plasma factors. Witters et al have implicated the necessity of platelet research in liver diseases to provide new pathophysiological insights [3].

Blood hemostasis is affected by platelets by adhering to the sites of vascular injury, releasing compounds from their granules, aggregating together to form hemostatic platelet plug and providing procoagulant surface on their phospholipid membrane to arrest bleeding [4]. Defect in any one of the above events lead to platelet dysfunction and defective hemostasis.

Human platelets are secretory cells, and exocytosis is the mode of secretion which results in the release of α-granules, lysosomes and dense granules. The nature of α-granules and lysosomes has been reported in many (patho) physiological conditions [5,6]. Normally, the secretory organelles are randomly dispersed in the cytoplasm of resting platelets. Upon activation, platelets lose their discoid shape, develop multiple pseudopods, and concentrate the cell organelles in the centre of the cell. This alteration in the physical structure is closely associated with the polymerization of globular or monomeric actin (G-actin) to filamentous actin (F-actin) that leads to secretion of substances into the surrounding medium [7].

About 3-8 dense granules are present per platelet [8,9], which contains serotonin, adenosine tri phosphate (ATP), adenosine di phosphate (ADP), calcium (Ca^{2+}) and pyrophosphate (PPi) [10,11]. The contents, especially serotonin and ADP amplify the platelet responses induced by strong platelet agonists and stabilize platelet aggregates [12,13]. The procoagulant actions of these components are proved experimentally [14]. Many studies have shown that ATP has a complex role in the regulation of platelet aggregation [15,16]. Ca^{2+} is essential for both platelet aggregation [17] and the release reaction [18]. Addition of Ca^{2+} ionophore to platelets resulted in aggregation and release of stored contents from platelets [18].

We have previously reported that defective Ca^{2+} release from the internal stores may account for inhibition of platelet aggregation in cirrhosis [19]. Though many scientists have reported that platelet aggregation and adhesion are abnormal in liver cirrhosis of different etiology [20, 21], the study regarding the secretory functions of platelets in liver cirrhosis is limited. Hence, the present investigation was focused on evaluating the level of secretion of hemostatic elements simultaneously with changes in the level of cytosolic Ca^{2+} and actin polymerization in platelets stimulated by the agonist collagen in vitro. The study is also aimed to assess the relationship between bleeding time (BT) and the secretory capacity of platelets in liver cirrhosis.

Subjects and Methods

Patients

The study comprised of fifty patients registered in the Department of Surgical Gastroenterology and Proctology, Stanley Medical College and Hospital, and the Department of Digestive Health Diseases, Government Peripheral Hospital, Chennai. Cirrhosis was confirmed by ultrasound and Doppler use-ultrasound. Variceal bleeding was confirmed by endoscopy. Patients screened for other platelet disorders not associated with liver complications and patients under any prescribed medication were excluded from the study. Patients were grouped as bleeders (n=27) and non-bleeders (n=23) as per Child-Pugh classification. The age range of the patients was 32 to 54 years with a mean of 46±6.38 years. Age and sex-matched healthy volunteers (n=50) between 34 to 55 years

with a mean of 48±6.56 years with normal liver biochemistry were used as control subjects. The clinical characteristics of the study groups are presented in Table I.

The study protocol was ethically approved and the blood sample was collected with the consent of each patient.

Isolation of platelets

Platelets were isolated by the method of Aster and Jandl [22]. Ten millilitres of fasting blood was collected by venous arm puncture and mixed with 1.6 ml of acid-citrate-dextrose solution. The blood was centrifuged at 275g for 10 min at room temperature to obtain platelet rich plasma (PRP). The PRP was again centrifuged at 400g for 5 min to remove any contaminating red blood cell (RBC). Then the PRP was centrifuged at 1000g to pellet out the platelets and washed in platelet buffer I (0.12 M NaCl, 0.0129 M trisodium citrate and 0.03 M glucose) followed by washing in buffer II (0.154 M NaCl, 0.01 M Tris and 0.001 M EDTA, pH 7.4). The washing procedure was continued until the pellet was red cell- free. The platelet pellet was suspended in platelet storage buffer containing 0.109 M NaCl, 4.3 mM K_2HPO_4, 16 mM Na_2HPO_4, 8.3 mM NaH_2PO_4 and 5.5 mM glucose, pH 7.5 and stored at 4°C. All the estimations were performed under sterile conditions within 5 h of sample collection.

Table 1. Clinical characteristics of subjects included in this study

Clinical findings	No. of cirrhotic patients	No. of normal subjects
Total number	50	50
Male / female ratio	30/20	35/15
Age in years (range)	32-54	34-55
Grade (according to Child – Pugh class)		
A	13	-
B	12	-
C	25	-
Ascitic	35	-
Non-ascitic	15	
Hepatic encephalophathy	6	-
Platelet count		
<100x10^9 cells/L	27	-
>100x10^9 cells/L	12	-
>250x10^9 cells/L	11	50
Prothrombin time		
<4 seconds	12	50
4-6 seconds	11	-
>6 seconds	27	-
Bleeding time		
Normal (60-90 s)	11	50
Abnormal (90-125 s)	39	-

Reagents

Fura-2AM, serotonin, DNA, DNase I, guanidine hydrochloride, digitonin and EGTA were purchased from Sigma-Aldrich Chemicals, Bangalore, India. ADP, ATP, HEPES and phenyl methyl sulfonyl fluoride (PMSF) were purchased from Sisco Research Laboratories, Mumbai, India. All other chemicals and reagents used were of analytical grade.

Assay of platelet cytosolic Ca^{2+}

Platelet cytosolic Ca^{2+} was measured with the fluorescent indicator Fura-2 by the method described by Pollock et al. [23]. 2µM Fura-2 AM (Sigma-Aldrich Chemicals, Bangalore) was added to PRP and incubated for 45 min at 37°C and then centrifuged at 700 g for 20 min. The supernatant plasma was discarded and the platelet pellet was resuspended in HEPES buffer (140 mM NaCl, 5 mM KCl, 5mM KH_2PO_2,1 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES, pH 6.5) at 37°C. The platelet concentrate was repeatedly washed twice and centrifuged at 640 g for 20 min and finally the pellet was resuspended in HEPES buffer, pH 7.4 at a concentration of 1x10^4 cells/ml. Fluorescence intensities at 510nm emission were measured at 37°C using an ELICO SL174 spectrofluorometer with the excitation wavelength of 340 and 380 nm. The cells were lysed with 50µM digitonin followed by addition of 1mM $CaCl_2$ to obtain maximal fluorescence, F_{max}. Then, the lysed cells were added with 10mM EGTA in 20mM Tris base and the pH of the lysed cells was adjusted to 8.5 to yield the Ca^{2+} independent fluorescence of Fura-2, F_{min}. Ca^{2+} was then calculated according to the formula,

$$Ca^{2+} = kd \times (F - F_{min})/ (F_{max} - F)$$

Where F is the Fura-2 fluorescence of resting or stimulated platelets. k_d was taken to be 224nM [24].

Assay of serotonin

Serotonin was determined by the spectrofluorometric method [25]. Briefly, the isolated platelets were suspended in 5ml of ice-cold saline to wash out the adhering blood proteins and centrifuged at 1000g for 20 min. The washing procedure was repeated twice and the pure platelet pellet obtained was finally suspended in 3.5ml of 0.02N HCl and agitated gently to lyse the platelets. One ml of the lysate was diluted with 2ml of water and the proteins were precipitated by the addition of 0.2ml of 10% $ZnSO_4$ and 0.1ml of 1N NaOH. To the supernatant, 0.3ml of 12N HCl was added and the fluorescence was measured in an ELICO SL174 spectrofluorometer at 490 nm after excitation at 385 nm. The amount of serotonin was determined from the calibration curve obtained by using known concentrations of serotonin (Sigma - Aldrich Chemicals, Bangalore) as the standard.

Assay of adenine nucleotides

Adenine nucleotide content was measured by HPLC. The chromatographic separation was performed on a 250x4.60 mm column packed with 10-pm silica particles to which octadecyl groups has been bonded. The column was eluted with 50mM potassium phosphate buffer, pH 7.0. Samples were analyzed with isocratic elution at a flow rate of 1.2 ml/min. Twenty microliters of sample or a standard solution was

manually injected using Hamilton syringes. Chromatographic peaks in experimental samples were detected by absorbance at 254 nm and identified by comparison to known standards.

Assay of PPi and inorganic phosphate (Pi)

The enzymatic method of determination of PPi content was based on the oxidation of NADH to NAD per mole of PPi consumed which was measured spectrophotometrically at 340nm [26]. The Pi content was measured spectrophotometrically at 620nm [27].

Preparation of platelets for secretion studies

The platelet count in each group was adjusted to $2.5x10^9$ cells/µl. The platelets were activated with 2µM collagen and the reaction was terminated at different time intervals (0, 3, 6, 9 min) by mixing the sample with one third volume of ice-cold 0.633M formaldehyde in 0.05M EDTA for serotonin and one-third volume of 0.15M NaCl for adenine nucleotides and 10% TCA for PPi and Pi determinations. The contents were kept on ice for up to 60 min and then centrifuged at 12,000g for 2 min at room temperature [28]. The extracellular fluid of the platelet suspension was used for the estimation of serotonin, adenine nucleotides, PPi and Pi as described previously.

Isolation of platelet cytoskeleton

Platelet cytoskeleton was obtained from the isolated platelets [29] with slight modifications. Briefly, washed platelets (1:1 in platelet storage buffer) was added with 10 vol of Triton X-100 solubilization buffer (1% Triton X-100, 40mM KCl, 10mM imidazole-chloride, 10mM EGTA, 2mM NaN_3, pH 7.0) and mixed by inversion. The flocculent precipitate was cooled on ice for 12 min and collected by centrifugation at 3000g for 2 min. The translucent platelet membrane pellet was carefully collected from the microfuge tube and used for the estimation of G- and total actin content. The isolated platelets after activation with 2µM collagen were lysed with Triton X-100 solubilization buffer at different time intervals and processed similarly for the estimation of G-, F- and total actin content.

Assay of actin content

Measurement of unpolymerized (G- actin) and total actin was done by DNase inhibition assay [30] with slight modifications [7] to stabilize the inhibitory activity of the platelet lysate. Briefly, platelet suspension was lysed with equal volume of ice-cold Triton X-100 lysis buffer (2% Triton X-100, 10mM EGTA and 100mM Tris-HCl, pH 7.4). About 50-µl aliquot was immediately removed, mixed with equal volume of dissociating buffer (1.5 M guanidine-HCl, 20 mM Tris-HC1, 1 M sodium acetate, 6 mM $CaC1_2$, 1 mM Na-ATP, pH 8.4), and incubated on ice for 15 min. The contents were then added with 20 µl of DNase I (beef pancreas, DN 100: Sigma-Aldrich Chemicals, Bangalore) at a concentration of 0.2 mg/ml in 50 mM Tris-HC1, pH 7.5, 0.1 mM $CaCl_2$, 10 µM PMSF. Sixty µl of this mixture was removed and added to 3 ml of DNA (calf thymus, type I: Sigma-Aldrich Chemicals, Bangalore) at a concentration of 40 µg/ ml in 100 mM Tris-HC1, 4 mM $MgSO_4$, 1.8 mM $CaCl_2$, pH 7.5. Total actin concentration was determined after treating the samples at 0°C with an equal volume of 1.5 M guanidine-HCl in 1.0 M sodium acetate/l mM $CaCl_2$/1 mM ATP/20 mM Tris HCl, pH 7.5. The change in absorbance at 260 nm was recorded for 5 min.

Standard curves were generated in exactly the same way using purified rabbit skeletal muscle actin. The DNase inhibitor activity measured in the absence of guanidine- HCl represents the amount of G- actin in a sample and the activity after treating the sample with guanidine-HCl measures total actin. From the difference between the total actin and the G- actin content, F- actin content was determined. G- actin and F- actin content were expressed as % of total actin content.

Statistical analyses

The Data was analyzed by using commercially available statistic software package (SPSS for Windows V.12.0). ANOVA test was applied for statistical analysis. Spearman's rank correlation analysis was conducted for the correlation of paired values. Results were presented as mean ± SD. Statistical p-value <0.05 was used to establish significance.

Results

Concentrations of serotonin, adenine nucleotides, cytosolic Ca^{2+}, PPi, Pi and G- actin content in unstimulated platelets. The levels of serotonin, adenine nucleotides, ATP and ADP, PPi, Pi, cytosolic Ca^{2+} and cytoskeletal G-actin content of unstimulated whole platelets are presented in Table 2. There was no significant difference in the levels of adenine nucleotides, PPi and Pi between non-bleeders and normal subjects but a significant decrease in the levels of adenine nucleotides was observed in bleeders when compared with normal subjects. There was a significant decrease in the concentrations of serotonin and Ca^{2+} between patients with bleeding complication and those without bleeding complication. G- actin content determined by DNAse I inhibition assay showed no significant changes in the level of actin contents in cirrhotic patients when compared to that of normal subjects.

Secretory response of platelets to collagen

To measure the secretory response of the platelets obtained from experimental subjects, the platelets were activated with 2µM collagen and the level of secretion was measured in terms of release of serotonin, adenine nucleotides, PPi, and Pi (Figure 1). There was a significant decrease in the levels of serotonin, ADP, PPi and Pi secreted by platelets of both categories of cirrhosis patients when compared to those of normal subjects. ATP/ ADP ratio showed a significant elevation in cirrhosis patients when compared to normal subjects. The elevation was more significant in bleeders when compared to non-bleeders. The level of polymerized actin (F-actin) was found to be significantly low in cirrhotic bleeders when compared to that of normal subjects and non-bleeders (Figure 2).

Time-dependent secretory response of platelets to collagen

The response of platelets to agonist was measured in terms of serotonin and adenine nucleotides, ATP and ADP at different time intervals (Figure 3). The time-dependent secretory response of cirrhotic platelets to collagen showed that maximum secretion of serotonin and ADP was reached in normal platelets earlier than that of cirrhotic platelets. ATP secretion was found to be delayed in normal platelets whereas it showed a steady

Table 2. Adenine nucleotides, serotonin, calcium, pyrophosphate, inorganic phosphate and membrane G -actin content in the unstimulated platelets of cirrhotic patients and in normal healthy volunteers. (mean ± SD)

Parameters	Normal subjects (n=50)	Cirrhotic patients (n=50)	
		Non-bleeders (n=23)	Bleeders (n=27)
Cytosolic Ca^{2+} (nM/10^4 cells)	28±3.0	24±2.8*	17±2.0**#
Serotonin (nM/mg protein)	2.0±0.41	1.8±0.21*	1.7±0.25*#
ATP (nM/mg protein)	20.5±3.1	19.5±2.0NS	17.6±1.9*#
ADP (nM/mg protein)	12.5±1.9	11.5±1.8NS	10.0±1.6*#
ATP/ADP ratio	1.64±0.18	1.70±0.19NS	1.76±0.19NS#
PPi (nM/mg protein)	6.0±0.08	5.7±0.08NS	5.6±0.08NS
Pi (nM/mg protein)	12.6±2.1	12.0±2.0NS	11.7±2.0NS
G- actin content (% Total actin)	80±8.2	75±7.7NS	70±7.3NS

**p < 0.001, *p < 0.05, NS – non-significant, when compared with normal subjects

##p < 0.001, #p < 0.05, NS-non-significant, when compared with non-bleeders.

Ca^{2+}, calcium; ATP, adenosine triphosphate; ADP, adenosine di phosphate; PPi, pyrophosphate; Pi, inorganic phosphate; G-actin, Globular actin;

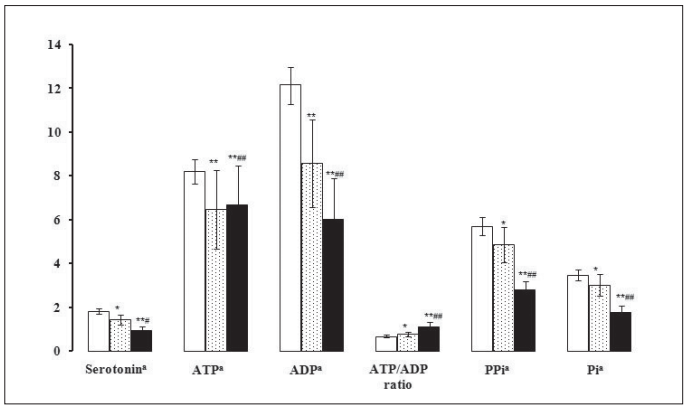

Figure 1. Levels of adenine nucleotides, serotonin, pyrophosphate and inorganic phosphate secreted by stimulated platelets. Aliquots of platelets ($2.5x10^9$ cells/µl) from normal subjects ☐ and cirrhotic non-bleeders ▨ and bleeders ■ were treated with 2µM collagen and the reaction was arrested at 9th min and the secreted adenine nucleotides, serotonin, pyrophosphate and inorganic phosphate were measured as mentioned in methods section. Values are expressed as mean ± SD for n = 20 in each group. Statistically significant variation is expressed as ** p < 0.001, *p < 0.05, NS – non-significant, when compared with normal subjects, ##p < 0.001, #p < 0.05, NS- non-significant, when compared with non-bleeders. (a - nM/mg protein)

rise in platelets isolated from cirrhosis platelets (Figure 3). In platelets of cirrhosis patients, though there was a time-dependent raise in ADP and serotonin levels, the net secretion was lesser than those of normal subjects. But the amount of ATP secretion was higher than that of normal subjects.

Time-dependent changes in the level of cytosolic Ca^{2+} and polymerization of actin during secretion

The changes in the cytosolic Ca^{2+} were monitored at regular time intervals during the course of secretion (Figure 4). In normal platelets, there was a proportionate increase in the level of cytosolic Ca^{2+} as the procoagulants release proceeded. There was a time dependent, but, low and delayed response shown by cirrhotic platelets. The level of increase was not significant in cirrhotic bleeders (Figure 4). Cirrhotic patients without bleeding complications showed elevation in the level of cytosolic Ca^{2+} during the secretion process.

Table 3. Spearman rank correlation coefficient between BT-serotonin, BT – ATP/ADP ratio, BT – Ca^{2+}, Ca^{2+} - serotonin and Ca^{2+} - F- actin

Variables	r_s
BT – serotonin	- 0.50
BT – ATP/ADP ratio	0.52
BT – Ca^{2+}	- 0.55
Ca^{2+} - serotonin	0.48
Ca^{2+} - F- actin	0.57

All correlation coefficient (Spearman rho-rs) values were significant at P <0.05

BT, bleeding time; ATP, adenosine tri phosphate; ADP, adenosine di phosphate; Ca^{2+}, calcium; F-actin, filamentous actin

During collagen-induced secretion, the time course of actin polymerization was also monitored simultaneously (Figure 5). In cirrhotic cells the level of actin polymerization was significantly lower than that of normal platelets and reflected in the level of F-actin, the polymerized form.

Spearman correlation analyses showed that there was a positive correlation between BT-ATP/ADP ratio, Ca^{2+}-serotonin and Ca^{2+}-F-actin. A significant negative correlation was observed between the paired values of BT-Ca^{2+} and BT - serotonin (Table 3).

Discussion

Platelets play pivotal roles in the hemostatic process including detection of vascular lesions, adherance at sites of injury, recruitment of additional platelets and consolidation into a hemostatic plug [31]. Most of the cirrhosis patients considered in the study had low platelet count and altered bleeding time. Thrombocytopenia is generally associated with deficient platelet function and thereby the related abnormality [3]. In our study the defective secretory response observed in all the cirrhosis

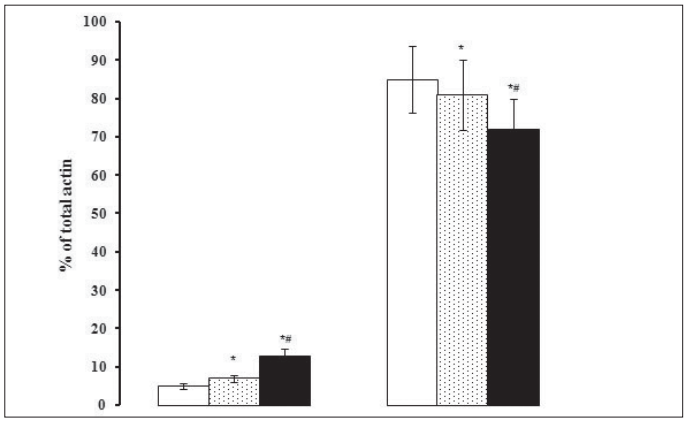

Figure 2. Levels of G- and F-actin content in stimulated platelets
Aliquots of platelets (2.5 x 10^9 cells/µl) from normal subjects ☐ and cirrhotic non-bleeders ▨ and bleeders ■ were treated with 2µM collagen and the reaction was arrested at 9^{th} min and the platelet G- and F- actin content were measured using DNase I inhibition assay. Values are expressed as mean ± SD for for n = 20 in each group. Statistically significant variation is expressed as *p<0.001 when compared with normal subjects and #p < 0.001, when compared with non-bleeders

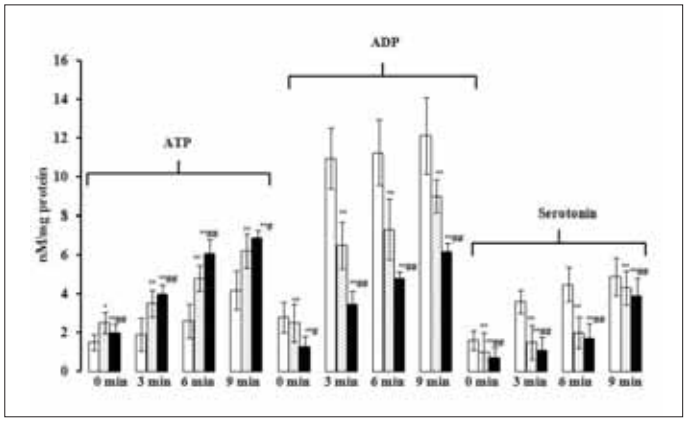

Figure 3. Time-dependent secretion of serotonin and adenine nucleotides in terms of ATP and ADP by platelets in response to collagen
Aliquots of platelets (2.5 x 10^9 cells/µl) from normal subjects ☐ and cirrhotic non-bleeders ▨ and bleeders ■ were treated with 2µM collagen and platelet secretion in terms of serotonin (nM/mg protein) and adenine nucleotides (ATP and ADP, nM/mg protein) at different time intervals was measured. Values are expressed as mean ± SD for for n = 20 in each group. ** p < 0.001, *p < 0.05, NS – non-significant, when compared with normal subjects, ##p < 0.001, #p < 0.05, when compared with non-bleeders

patients and more significantly in patients with bleeding complication could be correlated with the platelet count.

ADP, secreted from dense granules induces other platelets to degranulate and potentiate coagulation. Furthermore, ADP in conjunction with thromboxane A_2 and thrombin cause platelet contraction and the formation of a secondary hemostatic plug to bind fibrinogen which acts as a crosslink between the platelets allowing aggregation. Other molecules within platelet dense granules include ATP and ionized Ca^{2+} which are necessary for several steps of the coagulation cascade. Serotonin plays an essential role as vasoconstrictor to minimize the loss of blood. Studies in patients with dense granule abnormality indicate that deficiency in serotonin, ATP, ADP and Ca^{2+} are highly correlative and the alterations are interrelated [10].

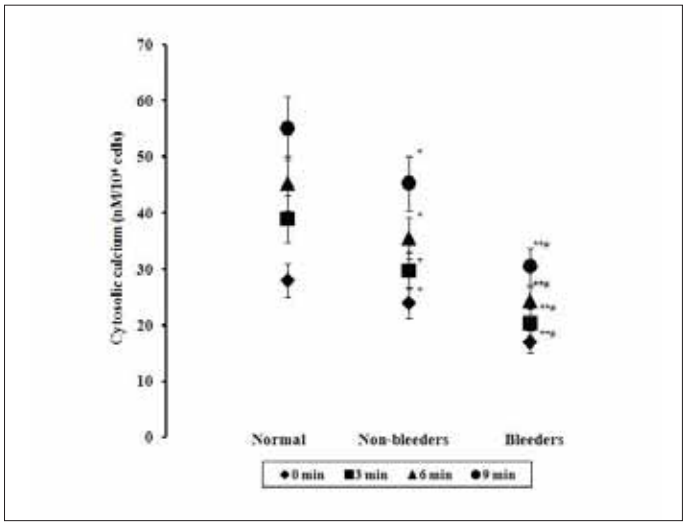

Figure 4. Time- dependent changes in the level of cytosolic Ca^{2+} during secretion by stimulated platelets
Aliquots of platelets (2.5 x 10^9 cells/µl) from normal subjects and cirrhotic non-bleeders and bleeders were treated with 2µM collagen and aliquots containing 1x10^4 platelets from normal subjects and cirrhotic non-bleeders and bleeders were withdrawn at different time intervals and calcium release using Fura-2AM was measured. Values are expressed as mean ± SD for for n=20 in each group. ** p < 0.001, *p < 0.05, when compared with normal subjects, #p < 0.001, when compared with non-bleeders

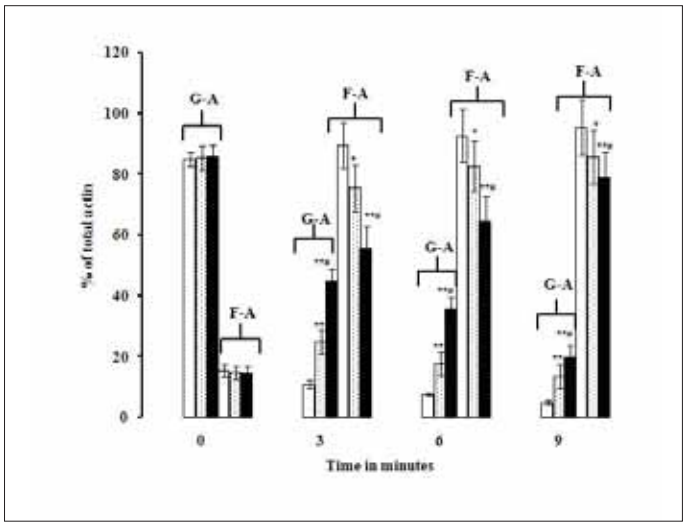

Figure 5. Time-dependent polymerization of actin by platelets in response to collagen in stimulated platelets
Aliquots of platelets (2.5 x 109 cells/µl) from normal subjects ☐ and cirrhotic non-bleeders ▨ and bleeders ■ were treated with 2µM collagen and aliquots were withdrawn at different time intervals and platelet actin polymerization was measured by DNAse I inhibition assay. (G- actin, globular actin; F- actin, filamentous actin). Values are expressed as mean ± SD for n = 20 in each group. ** p < 0.001, *p < 0.05, when compared with normal subjects, #p < 0.001, when compared with non-bleeders

We could find a significant alteration in the levels of serotonin and adenine nucleotides in resting platelets of cirrhotic bleeders. When the platelets were activated with collagen there was a significant change in the levels of secretion of serotonin, ADP and ATP in liver cirrhotic patients with bleeding complications when compared to those of non-bleeders and normal subjects. Our results show that both

serotonin and nucleotide secretion is low in cirrhotic bleeders which can be correlated with hemostatic disturbances.

Serotonin is not synthesized in platelets but is actively taken up from the plasma and accumulates in dense granules where it is likely complexed with ATP and potentially with Ca^{2+} [32]. The serotonin released by exocytosis is relatively stable and functions as a weak agonist on serotonin receptors (-$5HT_2$-receptors) [33]. Dense granule derived serotonin, therefore acts to activate additional platelets and thus recruits them into aggregates [31]. The positive feedback effect of serotonin may be of secondary importance to its vasoconstrictive action which reduces the blood flow at the site of injury and thereby limits blood loss [32].

In normal platelets, the level of ADP secretion is significantly greater than that of ATP. In cirrhotic subjects, ATP secretion was found to be elevated when compared to that of ADP. ATP competitively inhibits ADP action [34] and thus regulates platelet activation. Elevated ATP secretion has been reported in various inflammatory and shock conditions, primarily as a consequence of nucleotide release from platelets, endothelium and blood vessel wall [35-37]. In this investigation we have shown that in cirrhotic condition, platelets secrete elevated level of ATP, which antagonize the action of ADP, and this might contribute to the platelet activation defect in liver cirrhosis. Our observation also showed that the secretory response of platelets to agonists is delayed in liver cirrhosis. It can be correlated with similar activation of platelets in vivo by the endogenous collagen exposed on the ruptured vascular walls.

Our results show that the activation of platelets by agonists was associated with parallel elevation in the level of Ca^{2+} in normal platelets. The level of elevation was not significant in the platelets of cirrhosis patients. So, the altered secretory capacity of cirrhotic platelets may be accounted by the low availability of Ca^{2+} which functions in a diverse manner to regulate the secretory process. In our previous study, we have reported that the enzyme activity is significantly low in cirrhotic platelets that cannot affect Ca^{2+} efflux required for activation [38].

The role of cytosolic Ca^{2+} in platelets has been studied in various pathophysiological conditions. It has been found that micromolar levels of Ca^{2+} ions added to the extracellular medium elicit secretion of serotonin in the presence of millimolar levels of Mg-ATP. Ca^{2+} transport inhibitors were shown to interrupt with the secretion of serotonin by platelets [39]. The state of assembly of the platelet cytoskeleton appears to be under the control of intracellular concentration of free Ca^{2+}. The ability of Ca^{2+} to control the assembly and disassembly of the platelet cytoskeleton provides a mechanism for cytoskeletal involvement in shape change and pseudopod formation during platelet activation [40]. Secretory granules bound to the end of an actin filament can move to the plasma membrane as a result of the contraction of acto-myosin that is activated by Ca^{2+} ions [41]. In this investigation we have studied the variation in the level of calcium release between bleeders and non-bleeders. When compared to non-bleeders, bleeders have shown a slow release of Ca^{2+} from the subcellular organelles to cytoplasm that might affect secretory action of platelets.

It is necessary for the platelet to possess an actin cytoskeleton that can be rapidly restructured upon activation [42]. It has also been reported that an actin-binding protein, inhibited from interacting with actin by a rise in Ca^{2+} may be a factor that permits the rapid organization of the platelet actin cytoskeleton. Few investigators have suggested that actomyosin-dependent granule centralization and membrane fusion act synergistically to facilitate granule secretion [43-45]. It has been demonstrated that agonist induced activation results in conversion of unpolymerised, monomeric, globular G- actin to filamentous F- actin [46]. The finding of the present study reveal that normal unstimulated platelets exhibit 80% of their total actin in the monomeric state but the cirrhotic platelets showed a non-significant decrease in the total and G- actin contents. When the cirrhotic platelets were stimulated, F- actin formation was significantly lower than that of normal platelets. The low level of formation of polymerized F- actin in the platelets of liver cirrhotic patients as observed in our investigation may be associated with the decreased granule secretion in cirrhotic bleeders. However, the exact role of actin polymerization in platelet granule secretion remains to be elucidated in liver cirrhosis. A study on the structural and morphological changes of cirrhotic platelets on activation has been undertaken in our laboratory to give supportive evidence for the present work.

The statistical correlation between bleeding time and the various parameters analysed reveal that bleeding time, a reliable indicator of platelet functions is negatively correlated with the secretory response evidenced by the levels of nucleotide and serotonin secretion. BT was also found to have significant positive correlation with the level of ATP, an inhibitor of platelet secretion. Cytosolic Ca^{2+} level is found to have a parallel correlation with the levels of serotonin secretion and F- actin formation. This study reveals that actin polymerization is also defective in the platelets of patients with cirrhosis and this may be responsible for the low secretory response to agonists.

Conclusion

It is concluded that the secretion of hemostatic elements by the platelets in response to the agonist collagen is defective in liver cirrhosis. Low Ca^{2+} release from the internal store and impaired actin polymerization seem to alter the normal secretory capacity of platelets in liver cirrhosis associated with bleeding complication.

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