INFLUENCE OF NITRIC OXIDE AND CGMP ON AGONIST-INDUCED PLATELET ADHESION – AN *IN VITRO* STUDY IN PLATELETS ISOLATED FROM PATIENTS WITH LIVER CIRRHOSIS

UTICAJ AZOT-OKSIDA I CGMP NA ADHEZIJU TROMBOCITA IZAZVANU AGONISTIMA – *IN VITRO* STUDIJA NA IZOLOVANIM TROMBOCITIMA PACIJENATA SA CIROZOM JETRE

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Summary

Background: Variceal bleeding, one of the major complications of liver cirrhosis, is primarily due to platelet activation defect and secondarily due to coagulation defects. Platelet adhesion is the key event in hemostasis. Since nitric oxide (NO) related stress is known to influence platelet functions in liver cirrhosis, we undertook the present study to evaluate the possible mechanism involved in the inhibition of platelet adhesion by NO.
Methods: Agonist-induced platelet adhesion *in vitro* was measured in platelets isolated from normal subjects and cirrhosis patients. The time-dependent changes in nitric oxide synthase (NOS), NO, 3',5'-cyclic guanosine monophosphate (cGMP) and cytosolic calcium (Ca^{2+}) levels were monitored during adhesion. The percentage of platelet adhesion was also monitored in the presence of an eNOS inhibitor and a cGMP inhibitor.
Results: The percentage of adhesion was significantly lower in cirrhosis platelets. Time-dependent changes in the cGMP, NO and NOS level in platelets stimulated with collagen were significantly high, with a significantly low level of elevation of cytosolic Ca^{2+} in cirrhosis as adhesion proceeded. The results showed improved platelet adhesion with inhibitors of NOS and cGMP with concomitant elevation in Ca^{2+} level.

Kratak sadržaj

Uvod: Krvarenje iz varikoziteta, kao jedna od glavnih komplikacija ciroze jetre, pre svega je posledica defekta u aktivaciji trombocita, a zatim i defekata u koagulaciji. Adhezija trombocita je ključni događaj u hemostazi. Kako je poznato da stres povezan sa azot-oksidom (NO) utiče na funkcije trombocita u cirozi jetre, sproveli smo ovu studiju sa ciljem da utvrdimo potencijalni mehanizam koji učestvuje u inhibiciji adhezije trombocita od strane NO.
Metode: Adhezija trombocita izazvana agonistima *in vitro* merena je u trombocitima izolovanim od zdravih subjekata i pacijenata sa cirozom. Tokom adhezije praćene su vremenski zavisne promene nivoa azot-oksid sintaze (NOS), NO, 3'5'-cikličnog guanozin monofosfata (cGMP) i citosolnog kalcijuma (Ca^{2+}).
Rezultati: Procenat adhezije trombocita takođe je praćen u prisustvu inhibitora NOS i inhibitora cGMP. Procenat adhezije bio je značajno niži u trombocitima sa cirozom. Vremenski zavisne promene nivoa cGMP, NO i NOS u trombocitima stimulisanim kolagenom bile su značajno velike, uz značajno nizak nivo elevacije citosolnog Ca^{2+} u cirozi tokom adhezije. Rezultati su pokazali bolju adheziju trombocita uz inhibitore NOS i cGMP praćenu povišenim nivoom kalcijuma.

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Conclusions: It is inferred that elevation in the formation of cGMP due to stimulation of NOS activity inhibits Ca^{2+} mobilization from the internal store, an essential process to trigger platelet activation. The abnormal alterations were significantly lower in cirrhosis patients without bleeding complications. So, it could be stated that the bleeding abnormality in liver cirrhosis might be due to defective platelet adhesion influenced by the NO-cGMP pathway.

Keywords: platelet adhesion, liver cirrhosis, cytosolic calcium, cGMP, nitric oxide

Zaključak: Zaključak je da pojačano formiranje cGMP usled stimulacije aktivnosti NOS inhibira mobilizaciju Ca^{2+} iz unutrašnjih zaliha, što je proces nužan za pokretanje aktivacije trombocita. Abnormalne alteracije bile su značajno manje izražene kod pacijenata sa cirozom bez komplikacija krvarenja. Dakle, može se reći da abnormalno krvarenje u cirozi jetre može biti izazvano defektnom adhezijom trombocita pod uticajem puta NO–cGMP.

Ključne reči: adhezija trombocita, ciroza jetre, citosolni kalcijum, cGMP, azot oksid

Introduction

Blood platelets play a major role in hemostasis. They respond to vascular injury by forming a hemostatic plug through a complex process involving adherence of platelets to the endothelium, activation, and recruitment of more platelets and finally release of procoagulants (1–6). A defect in any one of these processes leads to platelet dysfunction. Platelet adhesion is the initial step in the formation of a hemostatic plug. At the site of injury, platelets come into contact and adhere to the subendothelial tissues with the help of von Willebrand factor, forming bridges between the platelet surface glycoprotein Ib and the components of the subendothelium. This continues with the attachment of platelet receptors to several adhesive proteins in the matrix of connective tissue. Other platelets spread, covering the collagen surface with a single layer of platelets in a similar fashion, ultimately forming a plug to seal off the injured tissue (7).

There are various exogenous and endogenous factors that influence platelet adhesion. Evidence accumulating over the years has shown that nitric oxide (NO), a vasodilator, is involved in the inhibition of platelet functions (8, 9). NO has been implicated in the pathogenesis of various disorders, including chronic liver diseases (10). NO is synthesized from L-arginine by NO synthase (NOS). The endothelial (eNOS) and neuronal (nNOS) isoforms are known as constitutive enzymes that are calcium (Ca^{2+})/calmodulin-dependent, and inducible NOS (iNOS) is Ca^{2+}- independent (11, 12).

NO and S-nitrosothiols activate soluble guanylyl cyclase (sGC) resulting in increased 3′,5′- cyclic guanosine monophosphate (cGMP) concentrations that are associated with the relaxation of vascular tissue (13). Increase in the level of cGMP in platelets is thought to be responsible for the inhibition of platelet functions (14). The NO-mediated inhibition of platelet adhesion to the subendothelium prevents aggregation and thrombus formation (15).

Liver cirrhosis, a chronic liver disease characterized by replacement of normal liver cells by fibrous and regenerative nodules, leads to progressive loss of liver functions. One of the life-threatening complications of this irreversible disorder includes variceal bleeding due to platelet dysfunction (16). Previously, we have shown that platelet aggregation is defective in cirrhosis due to excessive generation of NO by eNOS (17).

The present study is an attempt to evaluate whether NO prevents platelet adhesion and to find out whether intracellular cGMP and Ca^{2+} levels influence platelet adhesion in liver cirrhosis patients with bleeding complications.

Patients and Methods

Subjects

Sixty liver cirrhosis patients with bleeding (n = 35, age range = 30–50 years) and without bleeding (n = 25, age range = 30–50 years) complications

Table I Clinical characteristics of cirrhosis patients and normal healthy volunteers included in this study.

Clinical findings	Cirrhosis patients	Normal subjects
Total number	60	60
Male/female ratio	38/22	35/25
Age in years (range)	30–50	35–55
Grade (according to Child – Pugh class)		
A	10	–
B	15	–
C	35	–
Platelet count		
$< 1\times10^5$ cells/μL	25	–
$> 1\times10^5$ cells/μL	20	–
$> 2.5\times10^5$ cells/μL	15	60
Prothrombin time		
< 4 s	7	60
4–6 s	15	–
> 6 s	38	–
Bleeding time		
60–90 s	4	60
91–105 s	18	–
106–125 s	38	–
% platelet aggregation		
>90%	–	60
>80%	15	–
<50%	45	–

admitted to the Department of Surgical Gastroenterology and Proctology, Stanley Medical College and Hospital, Chennai, and to the Department of Digestive Health Diseases, Government Peripheral Hospital, Chennai, were enrolled in the study. Patients were diagnosed with cirrhosis by ultrasound and Doppler ultrasound. Variceal bleeding was confirmed by endoscopy. Age- and sex-matched healthy volunteers (n = 60, age range = 35–55 years) without any liver abnormalities were treated as control subjects.

The study was ethically approved and the blood samples were collected with the consent of each patient. The details regarding the habits and symptoms including the frequency and the site of bleeding were collected. *Table I* depicts the clinical characteristics of the subjects included in the study.

Preparation and stimulation of washed platelets

Platelets were isolated from whole blood according to the method of Aster and Jandl (18). Blood obtained from the subjects was mixed with acid citrate-dextrose solution and centrifuged at $275 \times g$ for 10 min at room temperature to obtain platelet rich plasma (PRP). The PRP was again centrifuged at $400 \times g$ for 5 min to remove erythrocytes. PRP was then centrifuged at $1000 \times g$ to pellet out the platelets and washed with buffer I (0.12 mol/L NaCl, 0.0129 mol/L trisodium citrate, 0.03 mol/L glucose) followed by buffer II (0.154 mol/L NaCl, 0.01 mol/L Tris, 0.001 mol/L EDTA, pH 7.4). The platelets were finally suspended in Tyrode's buffer (137 mmol/L NaCl, 2 mmol/L CaCl$_2$, 2.7 mol/L KCl, 2.1 mmol/L MgCl$_2$, 11.9 mmol/L NaHCO$_3$, 11 mmol/L glucose, pH 7.4 containing 3.5 mg/mL of BSA). The platelet suspension was maintained at 37 °C and utilized within 1 h. The platelets isolated from the experimental subjects were adjusted to a common count for all the experiments.

Simultaneous measurement of platelet adhesion and alterations in the level of NOS, cGMP and Ca^{2+} were done in quadruplicate, i.e., suspensions of equinumber of platelets were taken in 4 sets of ELISA plates labeled as A– D and stimulated with 20 µg/mL of collagen. A–Adhesion assay, B–NOS assay, C–cGMP and D–Cytosolic Ca^{2+}. At different time intervals, the non-adhered platelets were decanted from the well and the assays were carried out in the ELISA plates. Measurement of adhesion in terms of acid phosphatase of the adhered platelets and cGMP concentration was carried out in the plate. For NOS and Ca^{2+}, initial assay procedures were carried out in the well and transferred to small tubes for further steps and finally measured in a spectrofluorimeter. Methylene blue (MB) of varying concentration was added to the resting platelets 20 min before activation. cGMP was also assayed in the presence of an eNOS inhibitor, diphenylene iodinium chloride (DIC, 0–60 µmol/L).

Measurement of cGMP

Platelets were pre-incubated at 37 °C for 5 min followed by the addition of 400 µL of ice-cold TCA, centrifugation at $2000 \times g$ for 15 min at 4 °C and the supernatant was extracted with diethyl ether. cGMP concentration in the lyophilized sample was determined using a cGMP EIA kit (Cayman Chemicals, USA).

Assay of NO and NOS activity

NO concentration in platelets was measured in terms of total nitrite using Griess reagent (19). NOS activity in platelets was assayed fluorimetrically by an NOS assay kit (FCANOS-1, Sigma Aldrich, USA) using an excitation filter of 492 nm and an emission filter of 515 nm (20).

Measurement of cytosolic Ca^{2+}

Platelet suspensions were incubated with 2 µmol/L Fura-2AM at 37 °C for 45 min, washed to remove the extracellular Fura-2AM and resuspended in buffer containing 145 mmol/L NaCl, 5 mmol/L KCl, 0.5 mmol/L MgCl$_2$, 5 mmol/L glucose and 10 mmol/L HEPES, pH 7.4. The experiments were performed in the presence of 1 mmol/L Ca^{2+}-EGTA buffer. The fluorescence signal was monitored at 510 nm with excitation wavelength of 340 nm and 380 nm in a spectrofluorimeter at 37 °C (21).

Platelet adhesion assay

Adhesion assay was carried out according to the method described by Bellavite et al. (22). The adhesive capacity of the platelets is measured in terms of acid phosphatase activity of the adhered platelets after the removal of non-adhered platelets, when they are made to adhere to artificial surfaces. Briefly, 96-well microtitre plates were coated overnight at 4 °C by adding 50 µL per well with 0.2% human serum. Before use, the wells were washed twice with PBS (0.2 mmol/L, pH 7.4). To avoid non-specific binding, 1% BSA was added to each well and incubated for 1 h at 37 °C. The plates were washed and 25 µL of agonist, collagen (20 µg/mL) was added to the wells followed by 50 µL of resting platelet suspension containing 2.5×10^9 platelets/µL. The non-adhered platelets were discarded at different time intervals (3, 6 and 9 min) by gently inverting the wells and washed twice with PBS (200 µL/well). The wells were incubated for 1 h at room temperature with 150 µL of 0.1 mol/L citrate buffer, pH 5.4 containing 5 mmol/L p-nitrophenyl phosphate and 0.1% Triton X-100. The reaction was arrested and the color was developed by the addition of 100 µL of 2 mol/L NaOH. The production of p-nitrophenol was measured with a microplate reader at 405 nm. The percentage of adherent

cells was calculated based on the standard curve obtained with a known number of platelets.

Statistical analysis

Data are presented as the mean ± SD. Paired samples were compared by Student's t- test; values of P<0.05 were considered significant. Groups of data were tested by analysis of variance and correlation analysis was carried out by Spearman's rank correlation test.

Results

Since the adhesion assay is based on the acid phosphatase activity, initially we determined the phosphatase activity of non-activated platelets. No significant alteration was seen between normal subjects and cirrhosis patients in basal acid phosphatase activity. When platelets were activated with 20 µg/mL collagen significantly lower level (p<0.001) of platelet adhesion were observed in both groups of cirrhosis patients when compared to normal healthy volunteers. A decreased level of platelet adhesion was seen in platelets from patients with liver cirrhosis correlated with higher levels of cGMP, compared to normal healthy volunteers. Platelets obtained from cirrhosis bleeders showed significant increase (p<0.001) in cGMP concentration *(Table II)* when compared to non-bleeders.

When platelets were kept on plates for 3 to 9 min with 20 µg/mL of collagen, significantly lower adhesion was observed in cirrhosis platelets. The % adhesion gradually increased from the 3rd to the 9th min in the platelets of normal healthy volunteers, but there was a delayed response in cirrhosis platelets and the maximal platelet adhesive capacity observed in the

Table II Basal levels of acid phosphatase activity and cGMP levels in the resting platelets, and adhesion capacity of platelets isolated from patients with liver cirrhosis and in normal subjects (mean ± SD).

| Parameters	Normal subjects (n = 60)	Cirrhosis patients (n = 60)	
		Non-bleeders (n = 25)	Bleeders (n = 35)
Acid phosphatase activity (µmol/10^{10})	0.30 ± 0.04	0.28 ± 0.03[NS]	0.31 ± 0.04[NS]
cGMP (pmol/ 10^9 platelets)	2.6 ± 0.31	3.1 ± 0.43[*]	3.98 ± 0.52[**#]
% platelet adhesion	97.67 ± 11.55	76.23 ± 8.0[*]	45.62 ± 5.85[**#]

**p<0.001, *p<0.05, when compared with normal subjects, #p<0.001, when compared with non-bleeders, NS – not significant
cGMP – cyclic guanosine monophosphate
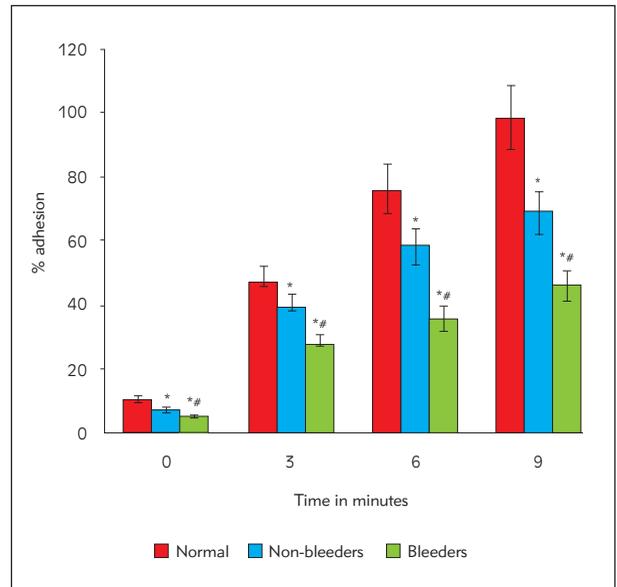
Figure 1 Time-response of collagen adhesion by platelets isolated from cirrhosis patients and normal healthy volunteers. Aliquots of platelets from normal healthy volunteers and cirrhotic patients were activated with 2 µg/mL of collagen and the adhesion capacity of the platelets was measured in terms of acid phosphatase activity of the adhered platelets. Values are mean ± SD for 60 individual experiments. Statistically significant variation is expressed as *p<0.001 when compared to normal subjects, and #p< 0.001 when compared to non-bleeders.
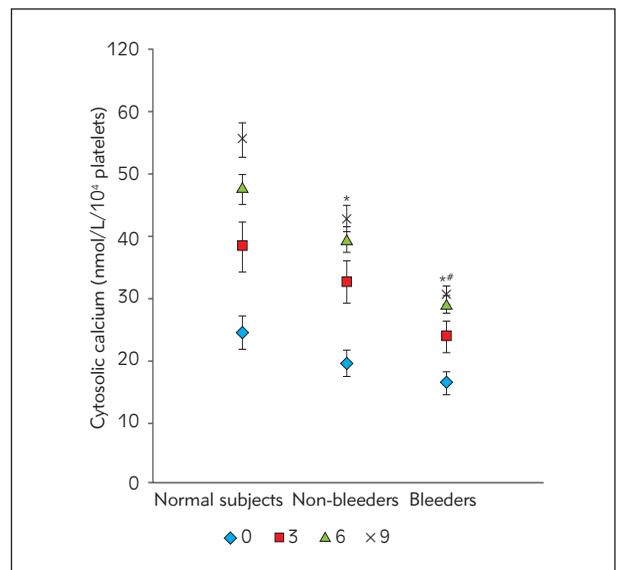
Figure 2 Time-dependent changes in the cytosolic calcium (Ca^{2+}) concentration during *in vitro* platelet adhesion in cirrhosis patients and normal healthy subjects. Platelet cytosolic Ca^{2+} in normal healthy volunteers and cirrhosis patients was measured with Fura-2AM during adhesion. Values are mean ± SD for 60 individual experiments. Statistically significant variation is expressed as *p<0.001 when compared to normal subjects, and #p<0.001 when compared to non-bleeders.
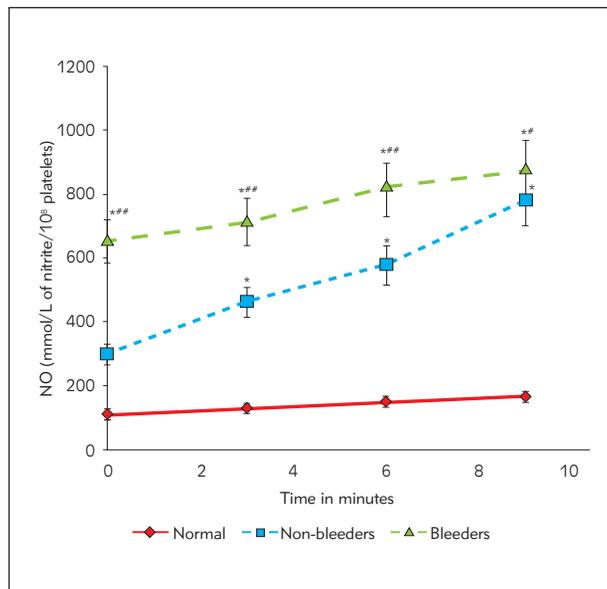
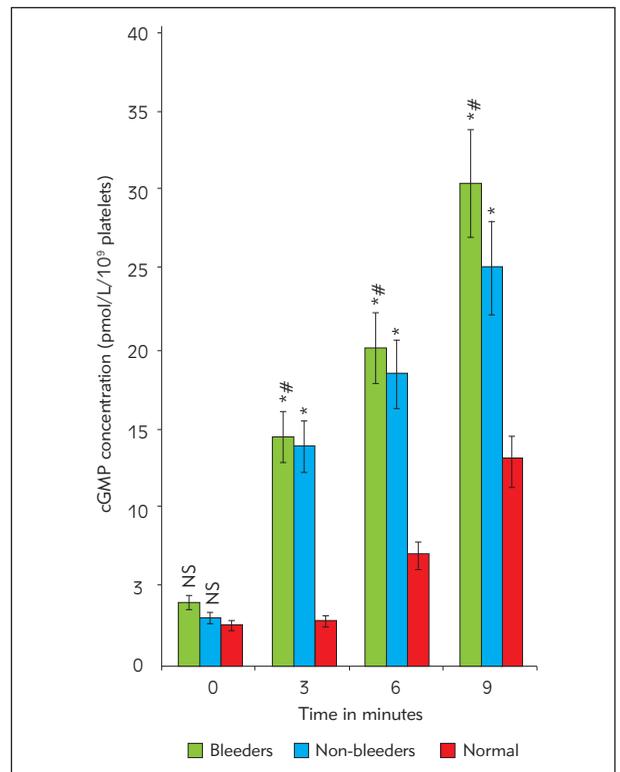**Figure 3** Time-dependent changes in nitric oxide (NO) concentration during platelet adhesion in cirrhosis patients and normal healthy subjects. NO concentration in the platelets isolated from normal healthy volunteers and cirrhosis patients was measured using Griess reagent during adhesion. Values are mean ± SD for 60 individual experiments. Statistically significant variation is expressed as *p< 0.001, when compared to normal subjects, and ##p< 0.001, #p<0.05, when compared to non-bleeders.
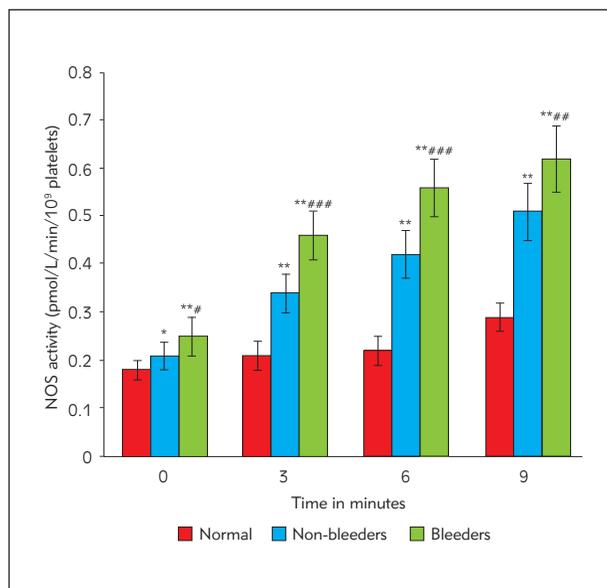
Figure 4 Time-dependent changes in the activity of nitric oxide synthase (NOS) during platelet adhesion in cirrhosis patients and normal healthy subjects. NOS activity in the platelets isolated from normal healthy volunteers and cirrhosis patients was measured spectrofluorimetrically during adhesion. Values are mean ± SD for 60 individual experiments. Statistically significant variation is expressed as **p<0.001, *p<0.05, when compared to normal subjects, and ###p<0.001, ##p<0.01, #p<0.05, when compared to non-bleeders.


Figure 5 Time-dependent elevation in cGMP concentration during platelet adhesion in cirrhosis patients and normal healthy subjects. cGMP concentration was measured using the CGMP EIA assay kit (Cayman, USA) in the platelets isolated from normal healthy volunteers and cirrhosis patients during adhesion. Values are mean ± SD for 60 individual experiments. Statistically significant variation is expressed as **p<0.001, when compared to normal subjects, and #p<0.001, when compared to non-bleeders. NS – not significant.

9th min was only half of the maximal capacity of normal platelets to adhere onto the plates *(Figure 1)*.

During the course of platelet adhesion time dependent changes in cytosolic Ca^{2+}, NO, NOS activity and cGMP concentrations were monitored. The changes in the cytosolic Ca^{2+} concentration were observed at regular time intervals using Fura-2AM during platelet adhesion *(Figure 2)*. In normal platelets, there was a proportionate increase in the level of cytosolic Ca^{2+}. There was a time-dependent, but low and delayed response in cirrhosis platelets. Platelets obtained from bleeders showed a significantly delayed elevation in Ca^{2+} concentration when compared to that in platelets from non-bleeders.

There was a significant elevation in the levels of NO *(Figure 3)*, cGMP *(Figure 5)* and NOS activity *(Figure 4)* in cirrhosis patients when compared to normal healthy volunteers. The platelets from bleeders exhibited significant elevation in NO, cGMP and NOS activity in relation to those of non-bleeders.
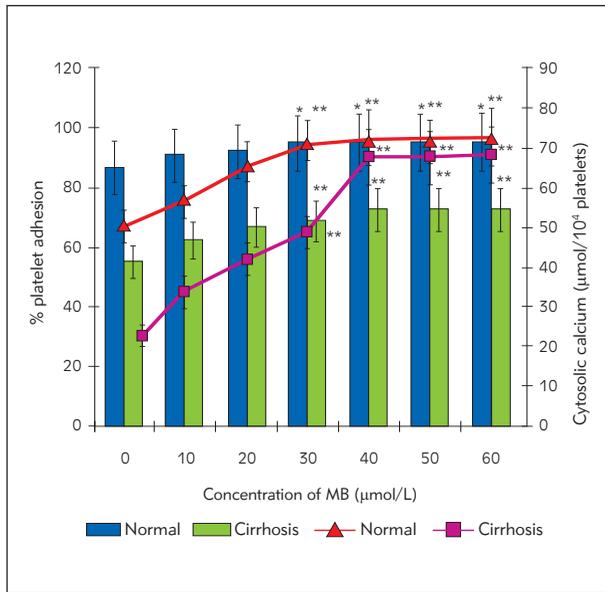
Figure 6 Dose-dependent response of methylene blue (MB) to platelet adhesion and cytosolic Ca^{2+} release in normal subjects and cirrhosis patients. Platelets were pre-incubated with MB (0–60 µmol/L) for 10 minutes and adhesion and cytosolic Ca^{2+} concentration were measured for a period of 9 min as mentioned earlier. Values are mean ± SD for 60 individual experiments. Statistically significant variation is expressed as *p<0.01, **p<0.001, when compared to the basal level.
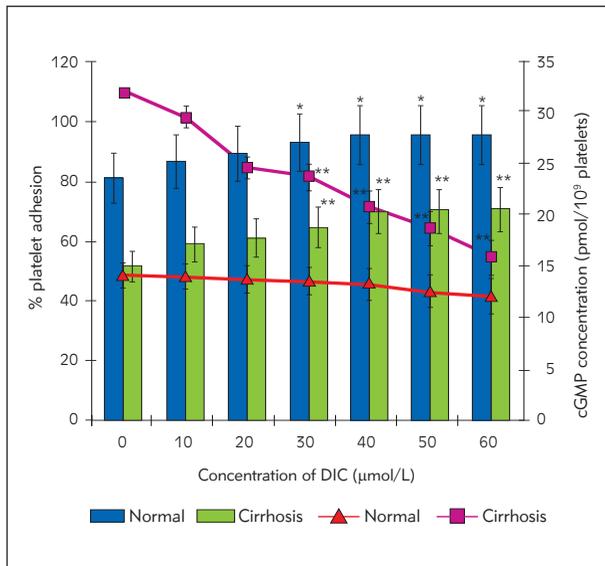
Figure 7 Dose-dependent action of diphenylene iodonium chloride (DIC) on platelet adhesion and cGMP concentration in normal subjects and cirrhosis patients. Platelets were pre-incubated with DIC (0–60 µmol/L) for 10 minutes and adhesion and cGMP concentration were measured for a period of 9 min as mentioned earlier. Values are mean ± SD for 60 individual experiments. Statistically significant variation is expressed as *p<0.01, **p<0.001, when compared to the basal level.

Table III Spearman rank correlation coefficient (r_s).

Variables	r_s		p
	Non-bleeders (n = 25)	Bleeders (n = 35)	
% adhesion Vs Ca^{2+}	+0.4021	+0.3526	< 0.05
% adhesion Vs cGMP	−0.4515	−0.3814	< 0.05
% adhesion Vs NOS	−0.3997	−0.3797	< 0.05
NO Vs cGMP	+0.4628	+0.3495	< 0.05

Platelets isolated from normal subjects and cirrhosis patients were incubated with varying concentrations (0–60 µmol/L) of MB for 20 min followed by activation with 20 µg/mL collagen. We observed a significant increase in platelet adhesion with a concomitant increase in cytosolic Ca^{2+} in cirrhosis patients. The maximum increase in Ca^{2+} release was at 50 µmol/L MB concentration (Figure 6).

In the presence of varying concentrations of DIC (0–60 µmol/L), there was a concomitant elevation in percent platelet adhesion accompanied by a decrease in cGMP concentration significant at 50 µM and no further decrease in the concentration of cGMP above 50 µmol/L (Figure 7). Normal subjects showed significant reduction in the level of cGMP in the presence of a DIC inhibitor. We found that the elevation in the level of cGMP (as shown in Figure 5) was reduced in cirrhosis bleeders.

Spearman rank correlation test for the paired values of % platelet adhesion–cGMP, and % platelet adhesion–NOS showed a significant negative correlation, and a significant positive correlation was observed for % platelet adhesion–Ca^{2+}, and NO–cGMP concentration in the cirrhosis platelets (Table III).

Discussion

Cirrhosis is one of the leading causes of death in the world and kills more than 25,000 people each year. Gastrointestinal bleeding in cirrhosis can be the result of a combination of vitamin K deficiency and thrombocytopenia, which is attributed to passive platelet sequestration in the spleen. The platelet dysfunction in cirrhosis is primarily related to the platelets and secondarily to soluble plasma factors (23–25). Patients with advanced cirrhosis show defective platelet aggregation, which is dependent, at least in part, on intrinsic platelet abnormalities. Production of endogenous NO is augmented in patients with liver cirrhosis. Increase in the level of NO corresponds to the progression of liver cirrhosis and causes defective platelet aggregation (17, 26). The influence of NO on platelet adhesion in cirrhosis platelets requires attention to reveal the mechanism involved in the pathogenesis of liver cirrhosis.

Platelet adhesion to the vascular endothelium is an essential step in the initiation of hemostasis and thrombosis. Platelet adhesion is followed by induction of signals leading to intracellular Ca^{2+} mobilization, the rearrangement of the cytoskeleton, procoagulant release, and activation of $\alpha_{IIb}\beta_3$ integrin (27–29). Therefore, we decided to evaluate the adhesive capacity of platelets in vitro, as adhesion is the key event that initiates platelet functions in cirrhosis in the patients. Platelet adhesion was significantly decreased in all the grades of cirrhosis patients. The comparison among the patients of different grades strongly suggests that the adhesive capacity was worsened along with the progress of disease in cirrhosis patients and was more prominent among the bleeders than with the non-bleeders. The time-dependent adhesive response of platelets to collagen showed a significant reduction in the percentage of platelet adhesion, which furthermore proves their defective functional ability to adhere to the endothelial collagen which gets exposed during vascular injury in vivo. In patients without bleeding complications the response to collagen over time was minimal and delayed when compared to normal subjects. In bleeding patients, the percentage of platelet adhesion was even lesser when compared with non-bleeders. In this report we have demonstrated that the number of platelets with normal adhesive capacity is significantly low in liver cirrhosis bleeders.

It is now established that NO is a key factor in the hemodynamic abnormalities associated with liver cirrhosis and chronic portal hypertension (30, 31). NO, a ubiquitous, cell permeable intercellular messenger, is synthesized by NOS in various cells and exerts its effects like vasodilatation on a diverse number of target cells such as vascular smooth muscle cells, neurons, and also in platelets (32–35). The time-dependent response to collagen revealed that as adhesion proceeded, the level of NO production was significantly higher, which demonstrates the inhibitory role of NO on platelet adhesion in cirrhosis.

Under physiological and tightly regulated conditions, nitric oxide synthases (NOS) generate NO. Our results show that the elevated NOS activity paralleled the reduced platelet adhesion in platelets isolated from cirrhosis patients. To ensure the involvement of NOS in the inhibition of platelet functions, we used varying concentrations of DIC, an eNOS inhibitor, and simultaneously studied platelet adhesion. We could find that the percentage of platelet adhesion gradually increased as the concentration of DIC increased and reached a maximum at 50 μmol/L. In the present investigation, we could observe the inhibition of platelet adhesion in cirrhosis which may probably be due to the increased activity of NOS and production of NO. Hence, we could state that the platelet functions are greatly influenced by the hyperactivity of intracellular NOS in liver cirrhosis.

In addition to platelet adhesion, NOS activity and NO generation, we measured the changes in cytosolic Ca^{2+} concentration in stimulated platelets. This was important because they provide information on the intracellular signaling pathways, and the inhibitory factor responsible for platelet adhesion. In exploring the mechanisms we observed changes in platelet cytosolic Ca^{2+} concentration concomitantly with the platelet adhesion process. During the course of adhesion we could observe a significant reduction in the mobilization of Ca^{2+} in all grades of cirrhosis when patients were compared to normal subjects, represented by low levels of cytosolic Ca^{2+}. The alterations in cytosolic Ca^{2+} level during platelet adhesion also proved that there was an oscillatory Ca^{2+} response. There was a delayed Ca^{2+} release in platelets as adhesion proceeded in cirrhosis platelets when compared to normal subjects. The variation was significant in grade B and C patients who had bleeding complications when compared to grade A patients who were non-bleeders. Defective Ca^{2+} release may be due to a high level of NO production, as NO is known to modulate Ca^{2+} mobilization (36). A low level of Ca^{2+} due to the influence of NO might be responsible for the defective platelet adhesion.

NO diffuses freely across the membrane and activates the cytosolic receptor, sGC, which causes elevation of the intracellular cGMP level (37). cGMP plays a critical role in the inhibition of platelet aggregation and adhesion of platelets (38–40). The inhibitory action of cGMP includes the inhibition of agonist-induced Ca^{2+} elevation via cGMP-dependent protein kinase (41). Although agonists like thrombin, ADP, thromboxane receptor and collagen are also known to raise platelet cGMP levels moderately and possibly to limit and regulate the extent of platelet activation (42, 43), elevation of cGMP concentrations above the normal level in platelets is reported to inhibit platelet functions (14). In the present study, there was an increase in the concentration of cGMP in the unstimulated platelets of cirrhosis bleeders when compared to non-bleeders.

The inhibitory response of platelets is mediated by the binding of NO to the heme iron of soluble guanylyl cyclase (sGC), which causes the conversion of GTP to cGMP (14, 44, 45). During the time-dependent response of platelets to collagen, the adhered platelets were simultaneously analyzed for cGMP concentration and we found that as platelet adhesion proceeded there was a steady increase in the concentration of cGMP in bleeding cirrhosis patients when compared to non-bleeders and normal subjects.

Elevation in cGMP inhibits the increase in agonist-induced cytosolic Ca^{2+} which is essential to trigger platelet activation. It has already been shown that the level of intracellular cytosolic Ca^{2+} influences actin and myosin interactions which in turn are counteracted by cGMP (46). Methylene blue, an organic spin-

trap agent, has been known to inhibit NO release and also known to act as a cGMP lowering agent (47). Therefore, we have used MB to confirm the cGMP-dependent mechanism that mediates the defective platelet adhesion in liver cirrhosis. The dose-dependent action of MB on platelet adhesion revealed cGMP-mediated alterations in the platelets of liver cirrhosis patients. At the concentration of 50 μmol/L there was a significant increase in platelet adhesion with a concomitant increase in the release of cytosolic Ca^{2+}. The elevation in cGMP concentration reduces platelet cytosolic Ca^{2+} involving the inhibition of cytoskeletal reorganization via the activation of protein tyrosine phosphatases (48), and therefore it appears likely that cGMP is a more potent inhibitor of Ca^{2+} mobilization in platelets (49, 50). Since cGMP regulates the receptor-mediated Ca^{2+} mobilization in platelets which is a crucial step that leads to platelet activation, this study proves the inhibitory action of cGMP on platelet adhesion, which might probably decrease the intracellular Ca^{2+} mobilization from the internal store.

In this study it could be speculated that the elevation of the NO level due to the upregulation of NOS is a mechanism involved in the inhibition of platelet adhesion through the cGMP-dependent pathway associated with the downregulation of Ca^{2+} release from the internal store, which results in defective platelet function. The alterations in the level of NO, cGMP, Ca^{2+} and NOS activity influence platelet adhesion and thereby contribute to platelet abnormality in liver cirrhosis.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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