Changes in the level of cytosolic calcium, nitric oxide and nitric oxide synthase activity during platelet aggregation: an *in vitro* study in platelets from normal subjects and those with cirrhosis

SAM ANNIE-JEYACHRISTY[1], ARUMUGAM GEETHA[1,]* and RAJAGOPAL SURENDRAN[2]

[1]*Department of Biochemistry, Bharathi Women's College, Chennai 600 108, India*

[2]*Department of Surgical Gastroenterology and Proctology, Stanley Medical College and Hospital, Chennai 600 108, India*

**Corresponding author (E-mail, geethav21@yahoo.co.in*

Variceal bleeding due to abnormal platelet function is a well-known complication of cirrhosis. Nitric oxide-related stress has been implicated in the pathogenesis of liver cirrhosis. In the present investigation, we evaluated the level of platelet aggregation and concomitant changes in the level of platelet cytosolic calcium (Ca^{2+}), nitric oxide (NO) and NO synthase (NOS) activity in liver cirrhosis. The aim of the present study was to investigate whether the production of NO by NOS and level of cytosolic Ca^{2+} influence the aggregation of platelets in patients with cirrhosis of the liver. Agonist-induced aggregation and the simultaneous changes in the level of cytosolic Ca^{2+}, NO and NOS were monitored in platelets of patients with cirrhosis. Platelet aggregation was also measured in the presence of the eNOS inhibitor, diphenylene iodinium chloride (DIC). The level of agonist-induced platelet aggregation was significantly low in the platelets of patients with cirrhosis compared with that in platelets from normal subjects. During the course of platelet aggregation, concomitant elevation in the level of cytosolic Ca^{2+} was observed in normal samples, whereas the elevation was not significant in platelets of patients with cirrhosis. A parallel increase was observed in the levels of NO and NOS activity. In the presence of the eNOS inhibitor, platelet aggregation was enhanced and accompanied by an elevated calcium level. The inhibition of platelet aggregation in liver cirrhosis might be partly due to greater NO formation by eNOS. Defective Ca^{2+} release from the internal stores to the cytosol may account for inhibition of aggregation of platelets in cirrhosis. The NO-related defective aggregation of platelets in patients with cirrhosis found in our study is of clinical importance, and the underlying mechanism of such changes suggests a possible therapeutic strategy with cell-specific NO blockers.

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1. Introduction

Bleeding tendency is a well-known complication associated with cirrhosis. This disorder of haemostasis has been related to a complex coagulation defect due to impaired platelet function (Kelly and Summerfield 1987). Platelets promote haemostasis by adhering to the sites of vascular injury, releasing compounds from their granules, aggregating together to form a haemostatic platelet plug and providing a procoagulant surface on their phospholipid membrane to arrest bleeding (Bennett and Kolodziej 1992).

Nitric oxide (NO), a powerful endothelium-derived vasodilator, is a potent inhibitor of platelet function, and has a relaxant effect on vascular smooth muscle (Moncada *et al* 1991; Radomski and Moncada 1993). Endogenous NO is generated by a family of three distinct

Keywords. Cirrhosis; cytosolic calcium; nitric oxide; nitric oxide synthase; platelet aggregation

Abbreviations used: ADP, adenosine diphosphate; ATPase, adenosine triphosphatase; BT, bleeding time; cGMP, cyclic guanosine monophosphate; cNOS constitutive NOS; DIC, diphenylene iodinium chloride; eNOS, endothelial NO synthase; iNOS, inducible NOS; NO, nitric oxide; NOS, NO synthase; nNOS, neuronal NO synthase; PRP, platelet-rich plasma; RBC, red blood cell

calmodulin-dependent NO synthase (NOS) enzymes; endothelial (eNOS) and neuronal (nNOS) isoforms are constitutive (cNOSs), whereas the other one is inducible (iNOS) (Fleming and Busse 2004) and is not generally expressed in unstimulated cells. NOS catalyses the formation of NO and L-citrulline from L-arginine in the presence of oxygen and NADPH (Stuehr 1999). NO plays a biphasic role in platelet activation; a stimulatory role at low concentration and an inhibitory role at high concentration (Marjanovic *et al* 2005). NO synthesized by NOS in both platelets and vascular endothelium inhibits platelet aggregation and adhesion, exerting its action through activation of soluble guanylyl cyclase and elevation of cyclic guanosine monophosphate (cGMP) (Radomski and Moncada 1993); these, in turn, lead to decreased availability of calcium (Ca^{2+}) in the cells (Geiger *et al* 1992). Elevated cytoplasmic "free Ca^{2+}" is a major component of signal transduction following receptor stimulation in platelets (Davies *et al* 1989). Agonists activate phospholipase C, which leads to rapid depletion of inositol 1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} stores and inhibition of the cation influx (Rossier *et al* 1991). This fact has been supported by the finding that calcium adenosine triphosphatase (ATPase) inhibitors effect depletion of Ca^{2+} stores in platelets (Brune and Ullrich 1991).

Platelet-derived NO not only modulates platelet activation to strong and weak agonists but, more importantly, markedly inhibits platelet recruitment to the growing platelet thrombus (Freedman *et al* 1997). Prolonged bleeding time and platelet abnormality were reported to be associated with NO production in experimental cirrhosis (Albornoz *et al* 1999).

The present study was carried out to evaluate whether NO, NOS activity and cytosolic Ca^{2+} level play any role in the platelet activation defect in liver cirrhosis. Hence, we monitored the changes in the levels of NO, NOS and cytosolic Ca^{2+} during collagen-induced *in vitro* aggregation of platelets in patients with cirrhosis. The level of aggregation in the presence of an eNOS inhibitor was also assessed to confirm the inhibitory effect of eNOS on platelet function. The data presented here demonstrate the role of cytosolic Ca^{2+} and NO in the functional defect of platelets in liver cirrhosis.

2. Methods

Forty patients registered in the Department of Surgical Gastroenterology and Proctology, Stanley Medical College and Hospital, Chennai and Department of Digestive Health Diseases, Government Peripheral Hospital, Gandhi Nagar, Chennai were recruited in this study. The patients with cirrhosis had their diagnosis confirmed by ultrasound and Doppler use-ultrasound. Variceal bleeding was confirmed by endoscopy. Age- and sex-matched healthy volunteers (n = 40) with normal liver functions confirmed by liver biochemistry were used as control subjects. The age range of the patients was 35–50 years (mean 44.5 ± 5.26 years), and that of normal control subjects 35–55 years (mean 45.5 ± 5.64 years). Case histories collected from the patients furnished the details regarding their habits and symptoms including the status and frequency of bleeding. The clinical characteristics of patients with cirrhosis are shown in table 1. The patients were considered as grade A, grade B and grade C according to the Child–Pugh score, which is based on the levels of serum bilirubin, albumin, prothrombin time, neurological complications and the presence of ascites.

The study protocol was approved by the ethics committee and blood samples were collected with the consent of each patient.

2.1 *Determination of bleeding time*

Bleeding time, expressed in seconds, was measured from the moment of incision until the bleeding stopped (Mielke *et al* 1969).

Table 1. Clinical characteristics of patients with cirrhosis and normal subjects included in the study

Clinical findings	Patients with cirrhosis	Normal subjects
Total number	40	40
Male/female ratio	20/20	25/15
Age in years (range)	35–50	35–55
Aetiology		
Alcoholic	33	-
Metabolic	7	-
Grade (according to Child–Pugh class)		
A	2	-
B	6	-
C	32	-
Platelet count		
$<1 \times 10^5$ cells/μl	32	-
$>1 \times 10^5$ cells/μl	6	-
$>2.5 \times 10^5$ cells/μl	2	40
Prothrombin time		
<4 s	2	40
4–6 s	3	-
>6 s	35	-
Bleeding time		
60–80 s	-	40
80–90 s	2	-
100–105 s	6	-
120–125 s	32	-

2.2 *Isolation of platelets*

Platelets were isolated from the blood samples of experimental subjects. Briefly, 10 ml 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 275 *g* for 10 min at room temperature to obtain platelet-rich plasma (PRP). The PRP was once again centrifuged at 400 *g* for 5 min to remove any contaminating red blood cell (RBC). Then the PRP was centrifuged at 1000 *g* to pellet out the platelets and was subjected to washing in platelet washing buffer I (0.12 M NaCl, 0.0129 M trisodium citrate and 0.03 M glucose) followed by washing in washing 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 erythrocyte free and its purity confirmed. 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 (Aster and Jandl 1964). All the estimations were performed concurrently within 5 h of sample collection under sterile conditions.

2.3 *Estimation of nitric oxide in terms of nitrite*

NO in unstimulated and stimulated platelets was measured in terms of total nitrite (Fiddler 1977). The level of NO was expressed as nM nitrite/mg PRP protein in unstimulated platelets and nM nitrite/10^8 platelets in stimulated platelets.

2.4 *Assay of total NOS activity*

NOS activity was assayed by using an NO synthase assay kit (FCANOS-1) purchased from Sigma-Aldrich Company. The fluorimetric cell-associated NOS detection system measures the intracellular production of NO in platelets by a non-radiometric method. 4,5-diaminofluorescein diacetate penetrates the cells rapidly where it is hydrolysed by intracellular esterase to diaminofluorescein-2 which, in turn, reacts with NOS to form fluorescent triazolofluorescein. This is quantified by a spectrofluorometer using an excitation filter of 492 nm and an emission filter of 515 nm (Kojima *et al* 1998).

2.5 *Determination of platelet cytosolic Ca^{2+}*

Platelet cytosolic Ca^{2+} was measured with the fluorescent indicator Fura-2 (Astarie-Dequeker *et al* 1992). Platelets were loaded with 2 μM Fura-2 AM for 40 min at 37°C in the presence of autologous plasma, washed by centrifugation at 270 *g* for 15 min at 20°C and resuspended at a concentration of 1×10^4 cells/ml in a medium containing 145 mM NaCl, 5 mM KCl, 0.5 mM $MgCl_2$, 5 mM glucose and 10 mM HEPES, pH 7.4 at 37°C, and 30 nM free Ca^{2+} concentration adjusted with the required Ca^{2+}–EGTA buffer. Fluorescence intensities were measured at 37°C using an ELICO SL174 spectrofluorometer with the excitation wavelength alternating between 340 and 380 nm, and emission wavelength at 510 nm.

2.6 *Measurement of platelet aggregation*

Platelet aggregation was determined based on the measurement of the change in turbidity at 620 nm (Bellavite *et al* 1994). Briefly, the wells were supplemented with 100 μl of the agonist 4 μM ADP/2 μM collagen in buffer A (145 mM NaCl, 5 mM KCl, 10 mM HEPES, 0.5 mM Na_2HPO_4, 6 mM glucose, 0.2% human serum albumin in 1 litre distilled water, pH 7.4) supplemented with 3 mM $CaCl_2$ and 3 mM $MgSO_4$. The platelet count in the PRP was adjusted to 2.5×10^9 cells/μl with autologous platelet-poor plasma (PPP). After the addition of an aliquot of the platelet suspension pre-warmed at 37°C, the decrease in absorbance at 620 nm was followed over a period of 9 min in an ELISA strip reader.

Aliquots were obtained at different time intervals to monitor the changes in the levels of NO, NOS and cytosolic Ca^{2+}. Aggregation and cytosolic calcium were also measured in the presence of the eNOS inhibitor diphenylene iodinium chloride (DIC) (0–40 μM).

2.7 *Statistical analysis*

Data were analysed using a commercially available statistical software package (SPSS for Windows v. 7.5). Student *t* test was performed to find out the significance of variations between the normal subjects and patients with cirrhosis, and Spearman correlation test was conducted for correlation analyses. Results were presented as mean ± SD. A P value <0.05 was considered statistically significant.

3. Results

3.1 *Concentrations of NO, NOS and Ca^{2+}, and platelet aggregation*

The level of aggregation was significantly low in platelets of patients with cirrhosis. The percentage of collagen-induced aggregation was significantly lower than that of adenosine diphosphate (ADP) ($P<0.01$) in patients with cirrhosis. The basal PRP level of NO was significantly elevated ($P<0.01$) in platelets of patients with cirrhosis. NOS activity was also found to be higher ($P<0.01$) in unstimulated platelets. Platelet cytosolic Ca^{2+} measured by Fura-2 uptake was significantly

Table 2. Bleeding time, percentage aggregation and NO, Ca^{2+} and NOS in platelets isolated from patients with liver cirrhosis and from normal healthy volunteers
(n = 40 individual experiments, mean ± SD)

Agonists	Normal subjects	Subjects with cirrhosis	P value
ADP-induced aggregation (%)	96.43 ± 10.56	57.08 ± 6.07	0.01
Collagen-induced aggregation (%)	98.67 ± 11.42	44.57 ± 5.98	0.001
NO (nM of nitrite/mg PRP protein)	118.3 ± 21.5	686.2 ± 46.4	0.01
NOS (pM/min/10^9 platelets)	0.175 ± 0.01	0.20 ± 0.04	0.01
Ca^{2+} (nM/10^4 cells)	25 ± 4.52	15.68 ± 3.87	0.01
BT (s)	78 ± 11.4	120 ± 12.6	0.001

ADP, adenosine diphosphate; NO, nitric oxide; NOS, nitric oxide synthase; Ca^{2+}, calcium; PRP, platelet-rich plasma; BT, bleeding time.

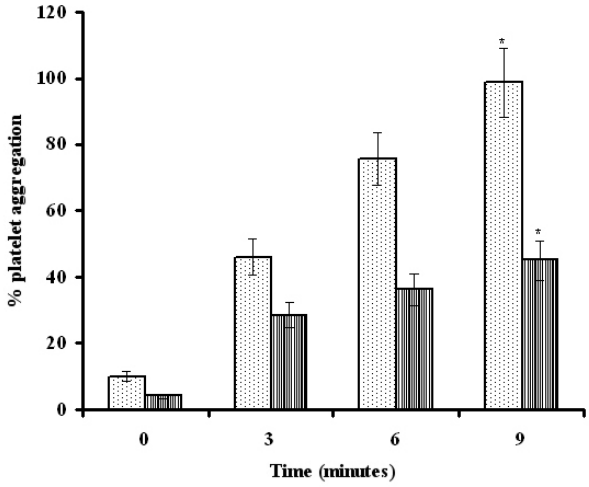

Figure 1. Percentage platelet aggregation of platelets from normal patients and those with cirrhosis.

Aliquots of platelets (2×10^9 cells) from normal subjects and patients with cirrhosis were treated with 2 μmol collagen and the aggregation capacity of the platelets was measured in terms of change in transmission at 620 nm. Values are mean ± SD for 40 individual experiments. Statistically significant variation is expressed as *P<0.001 when compared to the basal level.

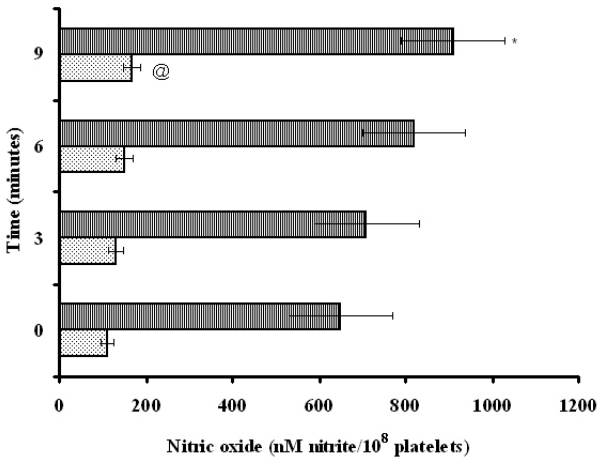

Figure 2. Time course of nitric oxide level during aggregation of platelets.

Aggregation was induced as described in figure 1. Aliquots containing 1×10^8 platelets from normal subjects and patients with cirrhosis were withdrawn at different time intervals and the NO level was measured. Values are mean ± SD for 40 individual experiments. Statistically significant variation is expressed as *P < 0.001; @, not significant when compared to the basal level.

low (P<0.01) in patients with cirrhosis compared with that in control subjects. The bleeding time was prolonged in patients with cirrhosis (P<0.001) (table 2).

3.2 *Level of platelet aggregation*

Figure 1 presents the time course of platelet aggregation in normal subjects and in patients with cirrhosis. There was a significant time-dependent increase in the percentage aggregation of normal platelets. However, no significant increase in aggregation was exhibited by platelets of patients with cirrhosis.

3.3 *Effect of NO on platelet aggregation*

Figure 2 represents the time-dependent changes in NO level during the course of platelet aggregation. Platelet

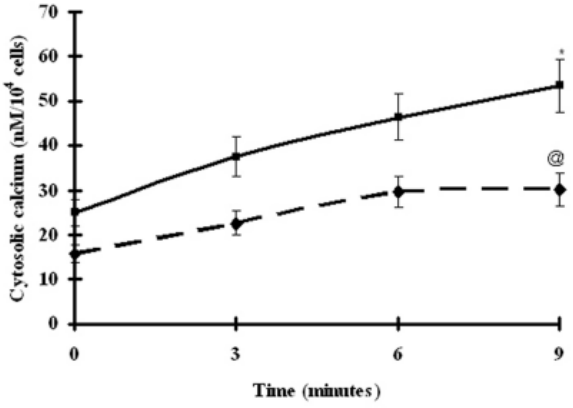

Figure 3. Time course of calcium release during aggregation of platelets.

Aggregation was induced in platelets as described in figure 1. Aliquots containing 1 × 10^4 platelets from normal subjects ——— and patients with cirrhosis — — were withdrawn at different time intervals and calcium release was measured. Values are mean ± SD for 40 individual experiments. Statistically significant variation is expressed as * $P < 0.001$; @, not significant when compared to the basal level.

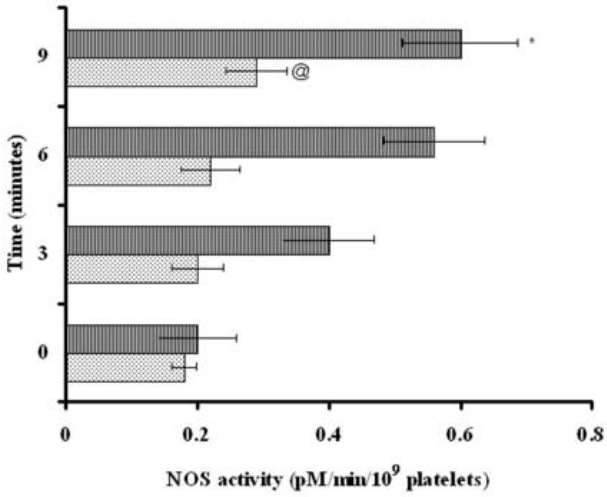

Figure 4. Time course of nitric oxide synthase activity during aggregation of platelets.

Aggregation was induced in platelets as described in figure1. Aliquots containing 1 × 10^9 platelets from normal subjects ▒ and patients with cirrhosis ▓ were withdrawn at different time intervals and NOS activity was measured. Values are mean ± SD for 40 individual experiments. Statistically significant variation is expressed as *$P < 0.001$; @, not significant when compared to the basal level.

aggregation was induced with 2 μM collagen and, at various time intervals, aliquots containing 1 x 10^8 platelets were withdrawn and the level of NO was measured. As aggregation proceeded there was a simultaneous increase in the level of NO in samples from patients with cirrhosis. The time-dependent increase was non-significant in normal platelets.

3.4 *Cytosolic Ca^{2+} level and platelet aggregation*

During collagen-induced *in vitro* aggregation, the elevation in calcium level was found to be significantly low in platelets of patients with cirrhosis (figure 3). In normal subjects, there was a proportionate increase in the level of cytosolic Ca^{2+} as aggregation proceeded.

3.5 *NOS activity on platelet aggregation*

NOS activity, which comprised the activity of both cNOS and iNOS, was found to be significantly elevated (P<0.001) in a time-dependent manner in platelets of patients with cirrhosis as aggregation proceeded (figure 4). The elevation was not significant in normal platelets.

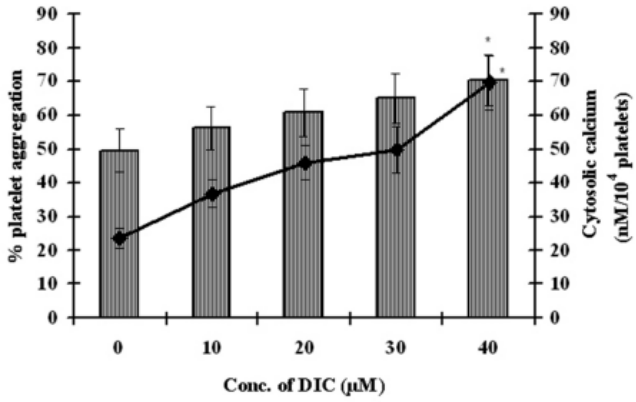

Figure 5. Dose-dependent effect of diphenylene iodonium chloride on platelet aggregation and cytosolic calcium.

Platelets from patients with cirrhosis ▓ were pre-incubated with diphenylene iodonium chloride (0–40 μM) for 10 min, aggregation and cytosolic calcium ——— were measured for a period of 9 min as mentioned in figure 1. Values are mean ± SD for 40 individual experiments. Statistically significant variation is expressed as *P<0.05 when compared to the basal level.

Table 3. Spearman rank correlation coefficient between NO–Ca^{2+}, NOS–Ca^{2+}, platelet aggregation–NOS, platelet aggregation–Ca^{2+} ($n = 40$)

Variables	r_s	*P*
BT–platelet aggregation	–0.314	<0.05
BT–NO	+0.346	<0.05
NO–Ca^{2+}	–0.4021	<0.05
NOS–Ca^{2+}	–0.4413	<0.05
Platelet aggregation–NOS	–0.4216	<0.05
% aggregation–Ca^{2+}	+0.4500	<0.05

Based on the critical values of the rank correlation coefficient (Spearman rho-r_s) null hypothesis of no correlation is rejected and it is concluded that the paired values are correlated.

BT, bleeding time; NO, nitric oxide; NOS, nitric oxide synthase; Ca^{2+}, calcium.

3.6 *Effect of eNOS inhibitor on platelet aggregation and cytosolic calcium*

In the presence of the eNOS inhibitor DIC, aggregation was found to be improved in platelets of patients with liver cirrhosis (figure 5) in a dose-dependent manner associated with elevation of cytosolic calcium level.

The Spearman rank correlation test was done for the paired values of bleeding time (BT)–platelet aggregation, BT–NO, NO–Ca^{2+}, NOS–Ca^{2+}, percentage aggregation of NOS and percentage aggregation of Ca^{2+} (table 3). A significant negative correlation in the level of NOS activity and percentage aggregation, and a significant positive correlation were observed between the level of cytosolic calcium and percentage aggregation of platelets in patients with cirrhosis.

4. Discussion

Cirrhosis is associated with marked abnormalities in the systemic circulation along with haemodynamic alterations. A direct correlation between hepatocellular failure and BT (Violi *et al* 1994) suggests that worsening of platelet function is closely related to the degree of liver failure in liver cirrhosis. In our study, a prolonged BT in patients with cirrhosis compared with that in normal subjects demonstrates that human clinical cirrhosis is associated with an aquired defect of haemostasis. This is in accordance with the results of experimental cirrhosis (Violi *et al* 1994; Blake *et al* 1990). However, many reports highlight that thrombocytopenia (Jorgensen *et al* 1984; Boks *et al* 1986) as well as impairment in the production of coagulation factors are responsible (Aoki *et al* 1993; Violi *et al* 1992) for the bleeding complications seen in patients with cirrhosis. The clinical data (table 1) show that about 90% of the patients presented with bleeding complications as well as a reduced platelet count. Hence, the abnormal bleeding time may be due to thrombocytopenia and/or platelet activation defects with abnormal aggregation and adhesion.

Platelets possess receptors for agonists including collagen and ADP, and the activation of platelets occurs through these receptors. Extracellular matrix proteins such as collagen or soluble agonists such as thrombin, ADP and epinephrine bind with the receptors and effect activation. It has been reported that platelets have a larger number of strong receptors for collagen than for ADP (Daniel *et al* 1998, Siljander and Lassila 1999). Few disorders associated with platelet abnormality have been related to defective collagen binding to receptors. Our study also showed that the percentage inhibition of platelet aggregation in the presence of collagen is significantly greater than that of ADP in patients with cirrhosis.

The role of NO in the pathogenesis of haemodynamic abnormalities has been observed in experimental cirrhosis (Pizcueta *et al* 1992a,b). The level of platelet aggregation is significantly low in cirrhosis and is negatively correlated with the degree of NO and NOS activity. The role of NOS as a modulator of platelet function is of clinical importance.

NO, a product of L-arginine by NOS and a potent anti-aggregating agent, plays an important role in the regulation of platelet functions (Radomski *et al* 1987). The influence of NO on platelet aggregation has been well demonstrated by many workers (Radomski and Moncada 1993; Brune and Ullrich 1991). The dose-dependent effect of collagen on the stimulation of NOS activity and formation of NO in the platelets has been proved (Lantoine *et al* 1995). Lantoine *et al* have also stated that cytosolic Ca^{2+} participates in the regulation of NO synthesis. The anti-aggregatory role of NO has been confirmed and its central role in the recognition of a pathological mechanism associated with platelet abnormalities has been proved (Radomski *et al* 1990).

Factors that enhance platelet-derived NO synthesis include α-tocopherol, by inhibiting protein kinase C (Freedman *et al* 1996, 2000), L-arginine by increasing NO synthesis and the intracellular thiol pool by enhancing the synthesis of S-nitrosothiol (S-NOSO), which limits the oxidative inactivation of NO (Anfossi *et al* 1999). Intracellular calcium flux and its level play an important role in the activation of platelet NOS.

The production of NO by endothelial and neuronal isoforms has been demonstrated in platelets (Burgner *et al* 1999). NO is produced by three different isoforms of NOS, which are widely expressed in all vascular cell types. NO,

mostly produced by the endothelial isoform (eNOS), plays a crucial role in vascular tone and structural regulation of the endothelial cells (Desjardins and Balligand 2006). Endothelium-derived NO has been reported to play an important role in the prevention of platelet aggregation and adhesion to the vascular wall (Rossier *et al* 1991; de Graaf *et al* 1992).

NO inhibits platelet aggregation (Stamler *et al* 1989; Cook *et al* 1990) and prevents thrombosis in a model of endotoxin-induced glomerular damage (Shultz and Raij 1992). The normal activation-dependent increase in platelet surface glycoprotein expression including P-selectin and the activation of glycoprotein IIb–IIIa complex are also inhibited by NO (Michelson *et al* 1996). The basal levels of NO and NOS in unstimulated platelets of patients with cirrhosis show a significant elevation when compared with platelets of normal subjects.

Platelet aggregation was found to be enhanced in the presence of the eNOS inhibitor DIC in a dose-dependent manner. This is an added evidence for the fact that eNOS expression inhibits platelet activation in liver cirrhosis. However, this aspect demands further study with non-specific NOS inhibitors to evaluate the fate of other isoforms in liver cirrhosis.

NO is more than a vasodilator as it exerts an antiplatelet action and plays a crucial role in the regulation of vascular haemostasis. Galley *et al* (1998) have shown that NOS activity increases with increasing Child–Pugh score and is associated with the development of systemic hypertension. In view of this role that NOS plays, the pharmacological modulation of NO synthesis is promising for the treatment of liver diseases in future, especially with the emergence of selective NO inhibitors and cell-specific NO donors.

In the present study, the basal level of cytosolic Ca^{2+} in platelets of patients with cirrhosis was significantly lower than that in normal cells. As aggregation proceeded, the level of cytosolic "free Ca^{2+}" increased in normal platelets and the increase was significantly low in platelets of patients with cirrhosis. This shows that mobilization of Ca^{2+} from the stores to the cytosol was defective. NO donors have been reported to inhibit calcium mobilization when stimulated by agonists. NO can modulate basal cytosolic Ca^{2+} in unstimulated human platelets and also decrease Ca^{2+} mobilization from NO-sensitive internal stores when stimulated. Studies with Ca^{2+} ATPase inhibitors have proved the role of NO in the modulation of Ca^{2+} handling (Le Quan Sang *et al* 1996). NO production and eNOS expression are controlled by cytosolic calcium in cultured coronary artery endothelial cells (Ni *et al* 1998). Thus, we could state that the defective aggregation and activation of platelets in patients with cirrhosis might be due to the low level of cytosolic Ca^{2+} which results in the upregulation of eNOS in platelets.

5. Conclusion

The results of this study show that the defective aggregation of platelets in liver cirrhosis might be due to elevated NO generation by eNOS. The study also demonstrates that low cytosolic Ca^{2+} generation leads to the upregulation of NOS activity in platelets, which might result in disturbed haemostasis and bleeding complications in patients with cirrhosis. It is concluded that cell-specific NO blockers can be tried to treat the platelet abnormality associated with liver cirrhosis after conducting proper preclinical trials.

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