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Platelet Aggregation in Portal Hypertension and Its Modification by Ultra-Low Doses of Aspirin

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Key Words
Portal hypertension · Platelet aggregation · Aspirin · Ultra-low dose · Portal vein stenosis

Abstract
Aspirin (ASA) is widely accepted as antithrombotic drug, but several reports point out that its use in ultra-low doses (ULD) has prothrombotic properties. In this study, we evaluate the effect of portal hypertension in rats on platelet aggregation in an in vivo arterial thrombosis model induced by a laser beam. Portal hypertension was produced by calibrated stenosis of the portal vein. ASA in ULD was injected to both control and portal hypertensive groups. Platelet aggregation induced by ADP, prothrombin time, activated partial thromboplastin time, fibrinogen and induced hemorrhagic time test were also performed. Portal hypertensive rats showed a diminished number of emboli and duration of embolization in the laser procedure and an increase in induced hemorrhagic time. These changes were reverted by one injection of ASA at ULD. This observation could be of importance for primary prevention or the treatment of recurrence in upper digestive tract hemorrhage in portal hypertensive patients.

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Introduction

Portal hypertension is a major complication of chronic liver disease. As a consequence of portal pressure rise, collateral portosystemic circulation develops and hemorrhage becomes a frequent cause of death. Multiple factors concur to complicate this often mortal disease. In the first place, hepatic and portosystemic collateral circulation increased resistance and hyperdynamic circulation. These changes are produced by vasodilating agents, such as prostacyclin (PGI_2) and nitric oxide (NO), and a diminished response to vasoconstrictors. The alterations in vascular autonomic regulation also trigger modifications in baroreflex response and the central nervous system [1–2]. Secondly, anatomic factors in the gastroesophageal junction make the surrounding of this place the most frequent to start the hemorrhage. Thus, pharmacologic treatment is usually aimed to modify hemodynamic forces. There are also coagulation problems whose roles were never completely clarified. Traditionally, as cirrhosis is the most frequent disease causing these alterations, the hepatic synthesis of K vitamin-dependent factors was blamed. Lately, there have been some reports trying to point out the role of platelet aggregation and the importance of NO in its modifications [3]. There are few reports

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that investigate platelet aggregation in vivo in portal hypertension, and none of them utilize a prehepatic portal hypertension model. Aspirin (ASA) in ultra-low doses (ULD) was reported as a potent enhancer of platelet aggregation in several reports [4–7], in experimental and clinical research studies. The aim of this study was to evaluate in vivo the changes in platelet aggregation in a model of portal hypertension in the absence of liver damage and the modifications observed after administering ASA in ULD.

Materials and Methods

Male Wistar rats (200–250 g) were housed separately and acclimatized before use under conditions of controlled temperature (25 ± 2°C) and illumination (12-hour light/dark cycle). They were fed with standard rat chow and water ad libitum. Animals received care in compliance with the European Convention of Animal Care.

Surgical Procedures

After 1 week of acclimatization, rats were randomized and separated into two groups: (1) sham-operated rats and (2) portal hypertensive rats. Portal hypertension was induced by a calibrated portal vein stenosis, according to the procedure described in Vorobioff et al. [8].

In brief, rats were anesthetized with ketamine (90 mg/kg body weight, i.m.), and then, a midline abdominal incision was made. The portal vein was located and isolated from the surrounding tissues. A ligature of 3-0 silk was placed around the vein and snugly tied to a 20-gauge blunt-end needle placed alongside the portal vein. The needle was subsequently removed to yield a calibrated stenosis of the portal vein. Sham-operated rats underwent an identical procedure except that portal vein was isolated but not stenosed.

Animals were housed for 14 days after the operation to develop portal hypertension in the corresponding group.

Thrombus Formation Induction

After administering 200 mg/kg thiopental sodium, a median laparotomy was made. The intestinal loop was placed on the microscope table and vascular lesions were induced by Argon laser (Stabilite 2016, Spectra Physics, France). The wavelength used was 514.5 nm and the energy was adjusted to 120 mW. The laser beam is applied during 1/15 s. The dynamic course of the thrombus formation was continuously monitored and recorded by placing the laser beam coaxially into the inverted light beam path of the microscope (Axiovert, Zeiss, France). Microscopic images were recorded through a video camera (DX L107, color camera CCD) and were monitored on a television screen (Trinitron color video monitor, PVM 144 2 QM, Sony, France). A schematic view of the apparatus used has been previously described [9]. Arterioles between 15 and 25 μm in diameter were used. The parameters assessed were the number of platelet emboli removed by blood flow and the duration of embolization (time between the first and the last emboli occurring during a 10-min observation period).

Biological Analysis

Platelet Aggregation Study

Platelet aggregation was made according to the method of Cardinal and Flower on a Chrono Log 500 VS aggregometer (Coultronics, Margency, France) on the whole blood obtained from the rat after laser experimentation. Platelet aggregation was induced by ADP final concentration of 5 μ*M* (Laboratoire Diagnostica, Stago, France). Two parameters were determined: (1) impedance, representing the maximum amplitude of aggregation expressed in ohms and (2) velocity of aggregation expressed in ohms/minute.

Coagulation Tests

At the end of each experiment, blood was collected by cardiac puncture, mixed with 3.8% sodium citrate (9 volumes blood/1 volume citrate) and centrifuged for 20 min at 4,000 rpm to obtain platelet-poor plasma. Activated partial thromboplastin time (APTT) was performed with an automated coagulation laboratory which permits the determination of APTT by the automatic addition of $CaCl_2$ to the plasma. The intrinsic factors of coagulation are activated by the ellagic acid on an extract of bovine cerebral tissue, substitute of platelet factor III. The coagulation is induced by the addition of $CaCl_2$ (Instrumentation Laboratory, Paris, France).

Prothrombin time (pTT) and fibrinogen were performed with an automated coagulation laboratory which permits the simultaneous determination of pTT and fibrinogen by using only the Ca-thromboplastin (Ca-thromboplastin high analytic sensibility: lyophilized extract of rabbit cerebral tissue from Instrumentation Laboratory).

Induced Hemorrhagic Time

An experimental model of induced hemorrhagic time (IHT) was performed 10 min before thrombosis induction by laser. The tail of the rat was immersed for 5 min at 37°C and sectioned 6 mm from the extremity. IHT measured corresponded to the time between the tail section and the end of bleeding, expressed in seconds.

Statistical Analysis

Statistical analysis was carried out with Excel (Microsoft) and expressed as mean ± standard error. Data were compared using the Student's parametric t test ($p < 0.05$ was considered significant).

Drug Tested

ASA solution was purchased from Boiron Laboratories (Sainte-Foy-les-Lyons, France). ASA in ULD was prepared as follows: 1 g of pure, finely powdered ASA was suspended in 99 ml of alcohol (70%). After being vigorously shaken, 1 ml of this dilution was mixed with 99 ml of distilled water and again vigorously shaken. The last process was repeated 13 times [4–7]. Sterilized water for injections (Aguettant, Lyon, France) was used as control.

Protocol

Rats were randomly assigned in 4 groups. Group I: sham operated + placebo (Sh); group II: sham operated + ASA (ShASA); group III: portal hypertensive + placebo (PH), and group IV: portal hypertensive + ASA (PHASA). Each group had between 5 and 9 rats in the pilot study (PS). A confirmatory study (CS) was made for laser protocol, IHT and ex vivo platelet aggregation study. In this CS, each group consisted of 25–32 rats.

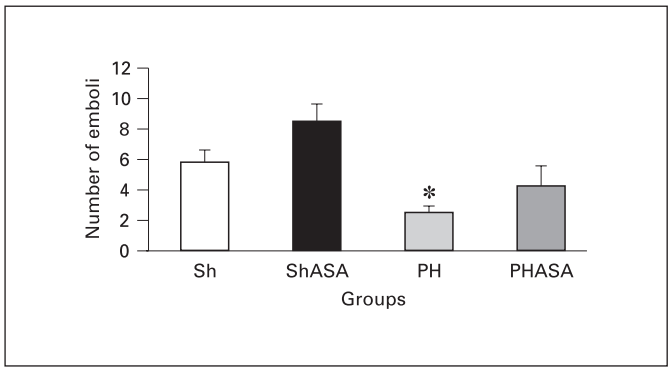

Fig. 1. PS of number of emboli. Results expressed as mean ± SEM. * $p < 0.05$ versus Sh.

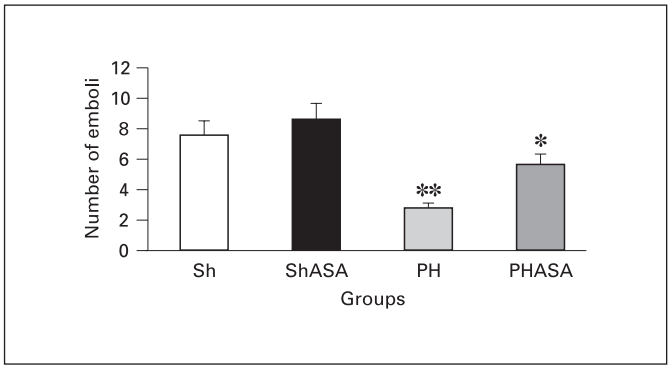

Fig. 2. CS of number of emboli. Results expressed as mean ± SEM. * $p < 0.05$ versus HP; ** $p < 0.001$ versus Sh.

Groups Sh and ShASA were submitted to a simulated operation. Groups PH and PHASA to portal vein ligation as described above, in surgical procedures.

One hour previous to thrombosis induction, one dose of 1 ml/kg of ASA at ULD or placebo was injected subcutaneously.

Results

In vivo Study

Number of emboli in the PS (n = 5–9/group): Sh 5.71 ± 0.83, ShASA 8.33 ± 1.20, PH 2.44 ± 0.50 and PHASA 4.17 ± 1.4. Number of emboli in the CS (n = 25–32/group): Sh 7.4 ± 1.00, ShASA 8.52 ± 5.92, PH 2.72 ± 2.14 and PHASA 5.56 ± 3.87. Portal hypertension produced a significant reduction in number of emboli in the PS ($p < 0.05$) and the CS ($p < 0.001$) when compared with control (Sh). In the CS, there was also a significant rise in number of emboli when comparing PH with PHASA.

Duration of embolization in the PS (n = 5–9/group): Sh 3.00 ± 0.43 min, ShASA 4.17 ± 0.70 min, PH 0.11 ± 0.39 min and PHASA 2.00 ± 0.81 min. Duration of embolization in the CS (n = 25–32/group): Sh 3.12 ± 0.31 min, ShASA 3.63 ± 0.51 min, PH 1.09 ± 0.19 min and PHASA 2.77 ± 0.36 min. Portal hypertension produced a significant reduction in the duration of embolization in the PS ($p < 0.05$) and in the CS ($p < 0.001$). The duration increased towards normality when comparing PHASA and PH ($p < 0.001$).

IHT in the PS (n = 5–9/group): Sh 114 ± 9.47 s, ShASA 160 ± 35.05 s, PH 289 ± 47.66 s and PHASA 156.67 ± 52.76 s. IHT in the CS (n = 25–32/group): Sh 7.4 ± 1 s, ShASA 8.52 ± 1.12 s, PH 2.72 ± 0.38 s and PHASA 5.56 ± 0.73 s. Portal hypertension produced a significant rise in IHT in both studies ($p < 0.05$). This rise was not observed in PHASA after ASA administration at ULD. It should be noted that different means of IHT were observed in the two studies; however, we found proportional differences in all the 4 groups studied in both protocols.

Ex vivo Study

The changes observed in the ex vivo coagulation study (platelet aggregation induced by ADP and expressed as amplitude and velocity, APTT, pTT and fibrinogen) were not statistically significant when comparing the different groups studied (data not shown).

Discussion

As shown in figures 1–4, induction of portal hypertension significantly reduced the number of emboli and the duration of embolization in both pilot and confirmatory series in the in vivo thrombus formation study.

The addition of ASA at ULD induced a trend to normalize both parameters in the PS. This trend turned into a significant difference in these parameters when comparing groups PH and PHASA in the CS.

In IHT, portal hypertension also showed a significant increase in tendency to bleed. This tendency was not present after ASA administration (fig. 5, 6), thus confirming its proaggregant effect.

This is the first report of changes of in vivo thrombus induction formation in a model of prehepatic portal hypertension.

Although it is generally accepted that portal hypertension induces changes in platelet function, there are not

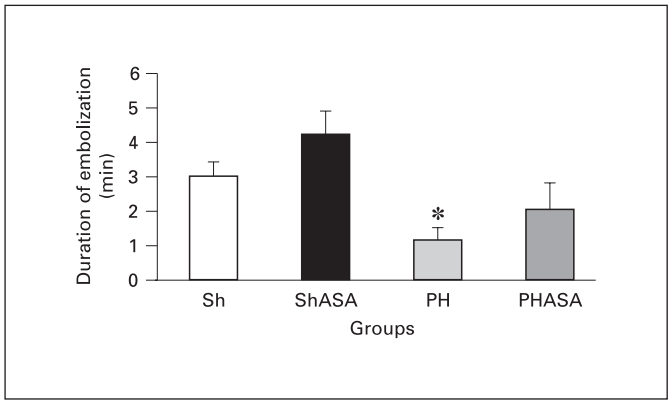

Fig. 3. PS of duration of embolization. Results expressed as mean ± SEM. * $p < 0.05$ versus Sh.

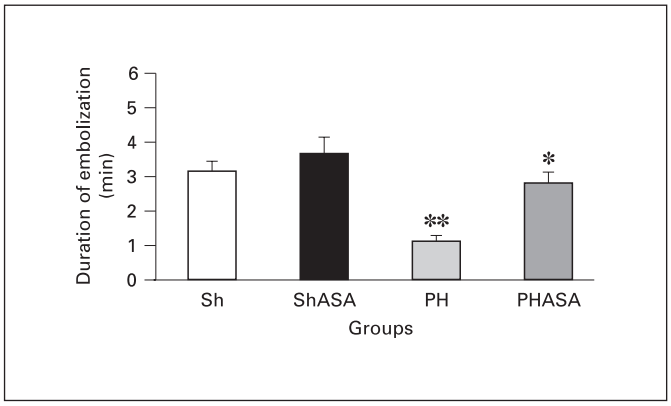

Fig. 4. CS of duration of embolization. Results expressed as mean ± SEM. * $p < 0.001$ versus PH; ** $p < 0.001$ versus Sh.

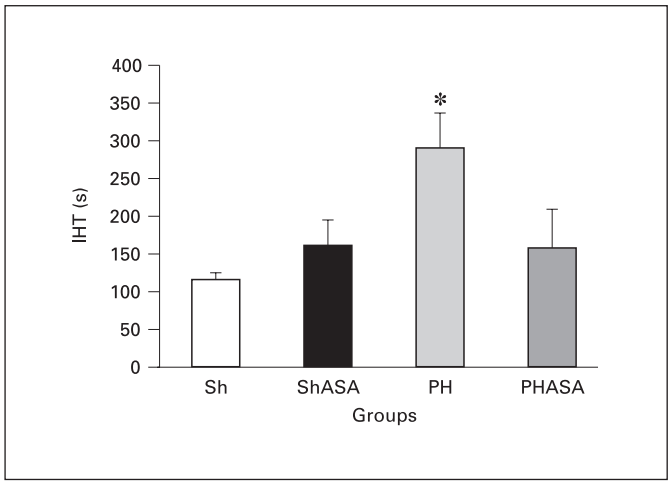

Fig. 5. PS of IHT. Results expressed as mean ± SEM. * $p < 0.05$ versus Sh.

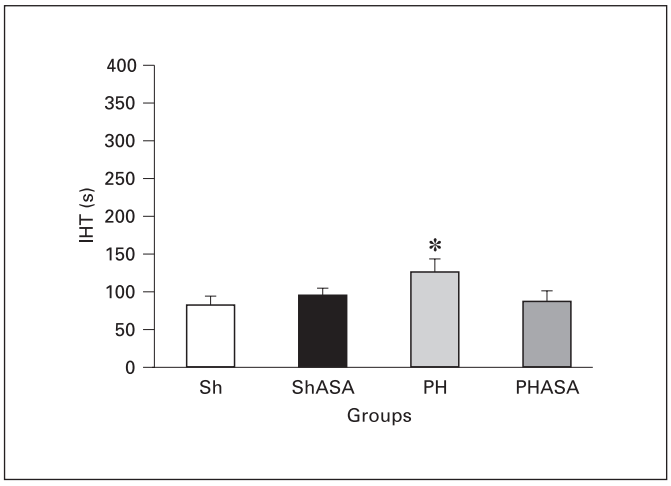

Fig. 6. CS of IHT. Results expressed as mean ± SEM. * $p < 0.05$ versus Sh.

many experimental studies directed to clarify these alterations. Albornoz et al. [3], using the Borchgrevink method for measuring platelet adhesion, documented similar changes in bile duct-ligated cirrhotic rats, observing partial reversal of these alterations after blocking NO production.

The partial portal vein ligation model of portal hypertension has been widely used to clarify portal hypertension changes, especially related to hemodynamics. Most of the changes observed in portal vein-ligated rats were also observed in cirrhotic animals. In the classical papers of Vorobioff et al. [8] and Benoit et al. [10], a relative importance of 40% for hyperdynamic circulation and 60% for increased vascular resistance of portal ligature and the portal systemic collateral vascular bed in increased portal vein pressure, as well as an approximate portal pressure rise of 50%, were established. This animal model with an almost normal liver function was chosen for the study of isolated platelet activity alterations, ruling out, where possible, changes in coagulation factors due to liver damage and because of its ability to reproduce the hyperdynamic circulatory changes first described by Kowalsky and Abelman [11, 12]. It is interesting to note that the alterations observed in platelet activity in vivo were probably initially only triggered by hemodynamic changes such as pressure alterations, increased portal inflow, endothelial stretch and shear stress.

Changes in platelet adhesion in cirrhosis have been widely reported [13, 14]. Alterations in coagulation have

also been reported by Bajaj et al. [15] in patients with noncirrhotic portal fibrosis and extrahepatic portal venous obstruction, both conditions with portal hypertension and near-normal liver functions, in pathophysiologic states similar to the animal model used in our study. In this last paper, both groups had a decreased platelet aggregation and normal platelet malondialdehyde levels.

The model of laser thrombus induction formation has the possibility of evaluating in situ the platelet-endothelium interaction. In the past, numerous studies have shown that platelets can adhere even to an intact endothelium and substantially modulate endothelial cell function [16]. Normal endothelium is a nonadhesive nonthrombogenic surface. When it is activated, it is proadhesive and promotes the adhesion of circulating blood platelets even under conditions of high shear stress. When a lesion is provoked in endothelial surface, extracellular matrix proteins like collagen and von Willebrand factor are exposed to the blood, triggering a reaction of platelet adhesion and activation. NO is a potent vasodilating substance and inhibitor of platelet activity and is continually secreted by the endothelium in response to shear stress. Evidence suggests that elevated production of NO is essential to the development of portal hypertension [17]. PGI_2 is another important endothelium-derived vasodilating substance capable of modifying platelet function. NO and PGI_2 appear to act by separate pathways in promoting mesenteric blood flow. It is possible that the absence of the endothelium could originate a different response between platelet aggregation studies and laser thrombosis production model. Moreover, in previous studies, the presence of a vascular fragment was necessary to put in evidence the effect of ASA at ULD on platelet aggregation [5, 6]. It should be noted that Kunihiro et al. [18] found differences in platelet aggregation from blood samples obtained from systemic and portal circulation in cirrhotic patients complicated with hepatocellular carcinoma, and they attributed those differences to intraportal PGI_2. For the above cited elements, we could conclude that platelet aggregation ex vivo induced by ADP and in vivo laser induction thrombus formation may not behave in an identical way.

Although the pathophysiology of this animal model is more similar to presinusoidal cases like noncirrhotic or idiopathic portal hypertension, these observations may become useful in preventing the first episode of hemorrhage or its recurrence in patients with portal hypertension of other origins. Hemorrhage is a major cause of death in portal hypertensive patients. Drug therapy for this disease is aimed mainly at the prevention of variceal bleeding and is done by β-blockers or vasodilators, with a predominant hemodynamic action. NO blockers could modify portal pressure and increase platelet aggregation, but the inhibition of intrahepatic NO could lead to an increased hepatic resistance [17, 19]. In previous studies done in healthy volunteers with ULD of ASA, no side effects were observed [4], and the effect of ASA in ULD enhancing platelet aggregation in a laser-induced thrombosis model was documented in the normal rat [7].

In the present study, the results obtained in the PS in experimental prehepatic portal hypertension were repeated in a CS with more animals in each group. This second study did not only show the significant difference observed in laser-induced thrombus formation when comparing Sham and portal hypertensive groups, but also a significant difference between PH and ASA at ULD groups. This tendency to normal platelet aggregation in the mesenteric bed was confirmed by a trend to normalize IHT in the portal hypertensive group when treated with ASA at ULD.

The factors normally considered to enhance the hemorrhage risk in patients with portal hypertension are as different as anatomic factors [20], hyperdynamic circulation, the degree of portal hypertension [21], endotoxin-induced NO and PGI_2 [22], altered synthesis of coagulation factors induced by liver damage or by compensated mild disseminated intravascular coagulation in prehepatic and hepatic portal hypertension [23]. It is difficult to point out the relative importance of alterations of platelet aggregation in such a complex state, even though the changes observed in IHT suggest that this platelet aggregation dysfunction could be the final pathway of some of these alterations through shear stress, NO and PGI_2 production. Further studies with other portal hypertension experimental models should be performed to clarify if this effect remains when liver function is severely compromised.

Conclusion

Prehepatic portal hypertension in the rat produces an altered platelet response to endothelial damage in mesenteric circulation, as well as a prolonged IHT. ASA in ULD normalized platelet activity and IHT.

The evidence provided by this study could be of importance for the primary prevention or the treatment of recurrence of upper digestive tract hemorrhage in patients with portal hypertension.

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