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Platelet aggregation is affected by nitrosothiols in patients with chronic hepatitis: *In vivo* and *in vitro* studies

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Abstract

AIM: To investigate the relationship among the number of platelets and plasma levels of S-nitrosothiols (S-NO), nitrite, total non-protein SH (NPSH), glutathione (GSH), cysteine (CYS), malondialdehyde (MDA), 4-hydroxininenal (4HNE), tumor necrosis factor-alpha (TNFα) and interleukin (IL)-6 in patients with chronic hepatitis C (CH).

METHODS: *In vitro* the aggregation of platelets derived from controls and CH patients was evaluated before and after the addition of adenosine diphosphate (ADP) and collagen, both in basal conditions and after incubation with nitrosoglutathione (GSNO).

RESULTS: *In vivo*, S-NO plasma levels increased significantly in CH patients and they were significantly directly correlated with platelet numbers. Patients with platelet counts < 150000/μL, had a smaller increase in S-NO, lower levels of GSH, CYS, NPSH, TNFα, and IL-6, and higher levels of nitrite, MDA, and 4-HNE relative to those of patients with platelet counts > 150000/μL. *In vitro*, the ADP and collagen aggregation time was increased in platelets from patients and not from controls; in addition, platelets from CH patients but not from controls also showed a latency time after exposure to collagen.

CONCLUSION: The incubation of platelets with GSNO improved the percentage aggregation and abolished the latency time.

Key words: Liver disease; Function of platelets; Hepatitis C; Oxidative stress; Anti-aggregant

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INTRODUCTION

Thrombocytopenia is a marker of liver cirrhosis as consequence of the portal hypertension and hypersplenism[1]. However, thrombocytopenia is also frequently observed in patients with chronic hepatitis caused by hepatitis C virus (HCV), without cirrhosis, and this phenomenon appears to be related to HCV infection rather than to other causes of chronic hepatitis (CH)[2,3]. The mechanism of this alteration is unclear. The presence of antibodies to platelets has been considered an independent ("nonpathogenic") cofactor, while plasma levels of thrombopoietin have been found to be decreased, normal, or elevated in HCV-infected patients, compared to levels found in patients having other types of liver disease[1,4,5]. More recently, Fusegawa *et al*[6] demonstrated increased platelet activation in HCV-related CH patients. This observation suggests that platelet function might be altered in these patients. In various pathological situations, platelets may be subjected to external oxidative stress by exposure to radicals released by granulocytes, endothelial cells, and/or monocytes/macrophages. These cells also release increased amounts of nitric oxide (NO) through the activation of inducible nitric oxide synthase (iNOS) by proinflammatory cytokines[7]. In these conditions, also platelets may produce NO, that, in turn, modulates itself recruitment and function. Thus, platelets and their function are likely to face physiological oxidative conditions[8]. NO and NO-donors are antithrombotic and anti-aggregant agents[9], and S-nitrosothiols (S-NO) have been synthesized and proposed as a new class of drugs with these effects[10,11]. The NO produced circulates both as nitrite/nitrate and in complexes as protein and nonprotein S-nitrosothiols, with a constant exchange occurring between these substances *in vivo*[12]. The formation of

S-NO adducts, as well as the release of NO from these compounds, is strongly influenced by the bioavailability of glutathione (GSH) and cysteine (CYS) and by the redox status of plasma. Furthermore, these pathways also affect the function of platelets, as platelets very rapidly import NO from S-NO[13-16]. Figure 1 summarizes the metabolism of NO in the circulation.

On the basis of these considerations, we performed as study's hypothesis that, in absence of hypersplenism and/or portal hypertension, the thrombocytopenia frequently observed in patients with HCV-related chronic hepatitis, could depend on other factors, as an altered metabolic pathway of NO in the circulation, secondary to a possible alteration of the redox status and/or an increase of pro-inflammatory cytokines. Therefore, we evaluated, *in vivo*, in patients with HCV-related CH and a control group, (a) the levels of S-NO and their relationships with those of nitrite, nitrate, and thiols; (b) the relationships between the foregoing and plasma levels of markers of oxidative stress and proinflammatory cytokines; and (c) the relationships among all of these factors and the number of platelets. We also evaluated the relationship between S-NO and platelet function *in vitro* using platelets derived from controls and from HCV-infected patients.

MATERIALS AND METHODS

The departmental ethics committee approved the study protocol, and all individuals gave informed consent. The study population comprised 114 patients with biopsy-proven, HCV-related chronic hepatitis without cirrhosis (CH), according to Ishak's score[17]. The absence of cirrhosis was documented, other than by liver histology, also by the absence of ultrasonographic and endoscopic findings of portal hypertension and by the normality of liver function tests (albumin, prothrombin time, total bilirubin). As controls (C), we enrolled 50 healthy volunteers (no alcohol or drug users) who were negative for markers of HIV and hepatitis virus infection (HBV, HDV, and HCV). We excluded from the study subjects with alcohol intake > 30 g/d of pure ethanol, HIV or HBV positivity, decompensated diabetes, cryoglobulinaemia, and/or other associated diseases. The exclusion of HBV-positive patients was performed because we aimed to evaluate the function of platelets in HCV-positive patients, in whom a series reports have previously focused attention on a possible relationship between HCV chronic infection and thrombocytopenia[1,4-6], and also because in our clinical practice the number of HBV-positive patients is actually low. We also excluded patients with a platelet number < 100 000/μL, because this is considered a lower limit to evaluate platelet aggregation and patients treated with steroids, beta-blockers, nitro derivatives, aspirin, antioxidants, and interferon. Other drugs, such as insulin or non-absorbable disaccharides, were allowed. Table 1 summarizes the main findings regarding the enrolled subjects.

Biochemical determinations on plasma samples

From venous samples deproteinized with 10% of sulphosalicylic acid on plasma samples collected in the morning, after overnight fasting, we determined:

Total nitrite/nitrate levels: For this purpose, as suggested by the method outlined in the commercial kit (Bioxytech nitric oxide non-enzymatic assay, OXIS International, Inc., Portland, USA), we employed granular cadmium metal for the chemical reduction of nitrate to nitrite prior to evaluating nitrites. Total nitrite concentration was evaluated by the Griess method. In an acid solution, nitrite is converted to nitrous acid (HNO_2), which diazotizes sulphanilamide. This sulphanilamide-diazonium salt is then reacted with N-(1-naphthyl)ethylenediamine (NED) to produce a chromophore, which is measured at 540 nm[18,19]. Results were expressed as μmol/L.

Total nonprotein plasma SH groups (NPSH): These were determined using Ellman's reagent; absorbance was measured at 412 nm, as we have described[20]. Results were expressed as μmol/L.

Glutathione and cysteine: These two thiols are the major constituents of NPSH. We and other groups have shown that their levels in plasma are decreased in patients with liver disease. As previously reported, we measured these two thiols by Newton's method, which makes use of the stable linkage between monobromobimane (MBBR) and SH groups, which we detected by fluorescence high pressure liquid chromatography (HPLC) after derivatization of the plasma samples in the dark for 15 min[21-23]. Results were expressed as μmol/L.

S-NO: We used a less expensive and time-consuming spectrophotometric method[24,25]. Before assaying plasma samples from our study population, we added a known amount of GSNO (6.25 μg), the major constituent of plasma nitrosothiols, to a plasma sample from each of three healthy subjects and six patients (three HCV-positive and three PBC) to evaluate our ability to recover it. Recovery of the GSNO added to the plasma samples ranged from 86% to 99%. All determinations were performed in duplicate and, in some cases, on both fresh and frozen samples. No significant differences were found in the results.

Thereafter, a plasma sample (200 μL) from each of the individuals taking part in the study was added to 40 μL of 1% ammonium sulphamate (to counteract the background nitrite concentration) and then mixed with 200 μL of 0.4 mol/L HCl containing 0.3% $HgCl_2$ and 4.6% sulphanilamide to oxidize (and render undetectable) the released NO equivalent, of which > 99% was lost. Then, 300 μL of a solution of 0.4 mol/L HCl plus 0.2% mol/L-L-naphthylenediamine dihydrochloride was added. Samples were incubated for 30 min at 25℃. The amount of S-nitrosothiols was determined by visible spectrophotometry at 550 nm using GSNO (Sigma-Aldrich, Poole, UK) as a standard, since GSNO is a stable molecule and it has been demonstrated[25] that, during the assay procedures, no appreciable decomposition of the authentic GSNO occurs. Values were expressed as μmol/L.

Determination of nitrites and S-NO was performed on two different plasma samples, stored under identical conditions.

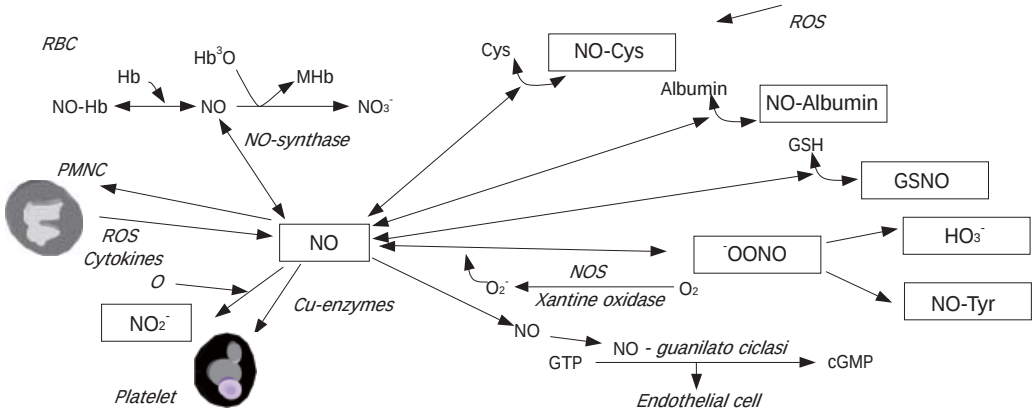

Figure 1 The metabolism of NO in the circulation: the NO produced circulates both as nitrite/nitrate and in complexes as protein and nonprotein S-nitrosothiols. The formation of S-NO adducts, as well as the release of NO from these compounds, is strongly influenced by the bioavailability of glutathione (GSH) and cysteine (CYS) and by the redox status of plasma.

Table 1 Enrolled subjects: main findings (mean ± SD)

	Controls	HCV-positive CH patients
Total number	50	114
M/F	26/24	59/55
Median age, yr (range)	53 (26-74)	54 (22-81)
ALT (IU/L, nv < 40)	12 ± 11	90.2 ± 60.4
γGT (IU/L, nv < 50)	28 ± 9	77 ± 52
Total proteins (g/dL)	6.8 ± 2.7	7.2 ± 1.2
Albumin (g/dL)	4.1 ± 1.2	3.6 ± 0.8
Total bilirubin (mg/dL)	0.8 ± 0.3	0.9 ± 0.3
Prothrombin time (s)	10.3 ± 2.1	10.5 ± 2.3
Platelets (n x 10^3/μL)	278 ± 89	159 ± 52
Ishak score for grading (median and range)	-	9 (5-11)
Ishak score for staging (median and range)	-	3 (2-3)

CH: chronic hepatitis; ALT: alanine aminotransferase; γGT: gamma-glutamyl transpeptidase.

Parameters of oxidative stress: As parameters of oxidative stress, in addition to the levels of GSH and CYS (see above), we measured those of malondialdehyde (MDA) and 4-hydroxinonenal (4-HNE), using commercial kits (Oxis International, Portland, OR, USA). Results were expressed as μmol/L.

Proinflammatory cytokines: We evaluated the plasma levels of two proinflammatory cytokines, tumor necrosis factor-alpha (TNFα) and interleukin (IL)-6, as we have previously described[26], using an immunoenzymatic reaction (ELISA test, Milenia Biotec, Germany). Values were expressed as pg/mL.

In vitro evaluation of platelet aggregation function

Whole blood (16 mL) was collected by venopuncture from each subject after overnight fasting. Platelet-rich plasma (PRP) was prepared by centrifugation of blood containing 5 mmol/L EDTA, at room temperature, at 900-1000 × g for 10 min. This sample was then transferred to the counter to determine the number of platelets/mL; another aliquot of the sample was centrifuged at 5000 × g for another 10 min to obtain plasma poor in platelets (PPP).

Specimens obtained as the result of a traumatic venopuncture were not included in the study. The pH and temperature remained within the normal range for the duration of the experiments.

Platelet number among experimental groups was normalized using a correction factor (platelet number × 350 000). Platelet aggregation was assayed by using a platelet aggregometer (Aggrecorder Ⅱ PA 3220 Menarini Diagnostici, Florence, Italy). Chart recorders graphed as a function of time. The aggregation was evaluated after the addition of common aggregating agents (3 μmol/L ADP and 1 μg/mL collagen, both in basal conditions and after incubation with nitrosoglutathione (Sigma-Aldrich, Poole, UK)[27]. Collagen type Ⅱ was from Mascia Brunelli, Italy. All *in vitro* experiments were performed within 3 h of venopuncture[8,28]. Platelet aggregation induced by collagen was measured after 3 min.

Statistical analysis

SPSS 11.0 software was used for statistical analyses. Differences between data and groups were evaluated by ANOVA and Student's t-test for paired data to evaluate levels in basal conditions and after incubation of platelet aggregations with GSNO, and for unpaired data in the other experimental conditions. For plasma data, the significance of differences was also calculated with a nonparametric Wilcoxon rank test. Correlations were calculated using Pearson's bivariate correlation test. $P \leqslant 0.05$ was considered significant.

RESULTS

Plasma values

In the first part of the present study, we compared the more recent methods employed to determine plasma levels of S-NO in humans (colorimetric, HPLC with electrochemical or spectrophotometric detection)[29,30]. No significant differences were found between the methods.

In all patients we found a significant increase in S-NO plasma levels relative to the control levels ($P < 0.01$). In contrast, plasma nitrite, NPSH, GSH, CYS, MDA,

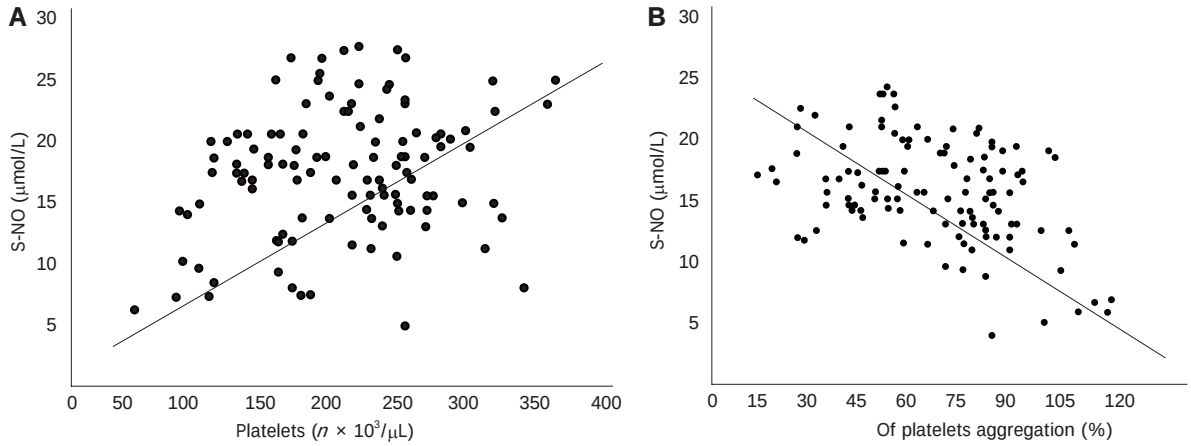

Figure 2 **A**: Correlation between S-NO and platelet number in each patient. As evident, S-NO levels were significantly directly correlated with platelet number (r = 0.58, P < 0.01). S-NO: S-nitrosothiol; **B**: Correlation between S-NO and % of platelet aggregation in each patient. As evident, S-NO levels were significantly inversely correlated with percent of platelet aggregation (r = -0.52, P < 0.05).

Table 2 Plasma parameters (mean ± SD)

	Controls	HCV-infected patients		
		CH (all patients)	CH with platelets > 150000/μL	CH with platelets < 150000/μL
Nitrite (μmol/L)	6.51 ± 4.64	7.98 ± 3.92	5.78 ± 2.03	10.64 ± 3.51[a,b]
NPSH (μmol/L)	367 ± 24	323 ± 26	372 ± 28	254 ± 21[b,d]
GSH (μmol/L)	8.1 ± 2.4	7.3 ± 3.5	8.8 ± 2.6	2.7 ± 1.0[b,d]
CYS (μmol/L)	17 ± 4.1	14 ± 5.2	16.8 ± 5.2	10 ± 2.9[b,d]
S-NO (μmol/L)	7.4 ± 1.5	27.9 ± 8.4[b]	33.8 ± 7.5[b]	21.4 ± 6.9[a,b]
MDA (μmol/L)	0.28 ± 0.05	0.25 ± 0.90	0.21 ± 0.18	0.46 ± 0.13[a, b]
4-HNE (μmol/L)	0.20 ± 0.08	0.24 ± 0.12	0.29 ± 0.11	0.59 ± 0.31[b,d]
TNFα (pg/mL)	26.7 ± 9.1	29.9 ± 5.7	36.4 ± 3.1[b]	26.2 ± 3.9[d]
IL-6 (pg/mL)	28.9 ± 7.3	31 ± 6.1	46.4 ± 3.1[b]	26.5 ± 6.4[d]
Score of fibrosis (median and range)	0	2 (1-4)	2 (1-2)	3 (2-4)[a]
HCV-RNA (× 10^6 UI/mL)	0	6.24 ± 1.38	5.48 ± 1.32	6.18 ± 2.1

[b]P < 0.01 *vs* controls; [a]P < 0.05, [d]P < 0.01 *vs* CH with platelet counts > 150000/μL.

4-HNE, TNF-alpha, and IL6 were similar in the two groups. S-NO levels were significantly directly correlated with platelet number (r = 0.58, P < 0.01; Figure 2A) and significantly inversely correlated with percent of platelet aggregation (r = -0.52, P < 0.05; Figure 2B). In agreement with others[31], the number of platelets was significantly inversely related to the degree of histological fibrosis (r = -0.78, P < 0.01). A significant difference was observed between plasma levels of S-NO from patients with platelet counts > 150000/μL (generally considered a mean of normal values) and those with platelet counts < 50000/μL (33.8 ± 7.5 *vs* 21.4 ± 6.9 μmol/L, respectively; P < 0.01). For this purpose, we further evaluated the data by dividing HCV patients into two groups on the basis of their platelet numbers. Table 2 summarizes the results. The evaluation revealed significant differences among CH patients on the basis of their platelet numbers. In fact, in patients with a high degree of histological fibrosis, in addition to the number of platelets being significantly lower than that found in patients with a low degree of fibrosis, other parameters were significantly different. Nitrite was increased and S-NO, despite always being significantly higher than in the controls (P < 0.01), was significantly decreased relative to its level in patients with normal numbers of platelets. GSH and CYS decreased and MDA and 4-HNE increased with the progression of liver damage and reached values significantly different from those of controls (P < 0.01). In contrast, proinflammatory cytokines were higher in patients with a lower degree of fibrosis (P < 0.01 in comparison to the others). HCV RNA levels did not differ between the two CH groups. Table 2 also shows the relationship existing among thiols in the circulation and oxidative stress. In fact, with the increase of plasma markers of oxidative stress (MDA and 4-HNE), total nitrite, NPSH, GSH and CYS decrease, while S-NO increase.

Platelet aggregation

Compared with controls, maximal aggregation induced by ADP was significantly increased in CH patients (Figure 3). Moreover, the collagen addition induced a significant increase in maximal aggregation in the platelets from CH patients (Figure 3). After incubation with GSNO in agreement with others[25,32,33], maximal aggregation induced by ADP was significantly reduced in both groups, but the reduction was much more evident in the CH patients, while maximal aggregation induced by ADP was not changed by the addition of GSNO in control group and

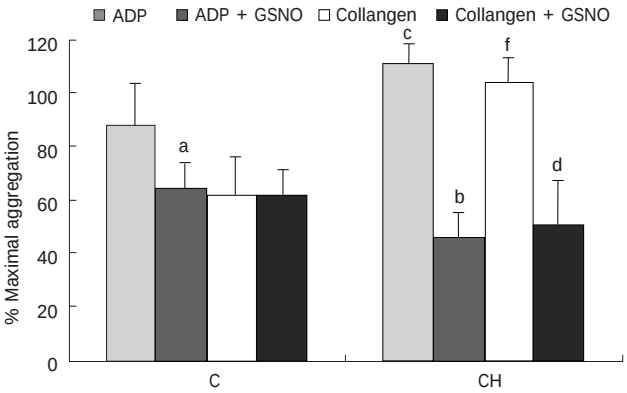

Figure 3 Percentage aggregation (mean ± SD) obtained in HCV-positive patients and controls, in basal conditions and after incubation with GSNO. Compared with controls % maximal aggregation induced by ADP was significantly increased in CH patients. Moreover, the collagen addition induced a significant increase in % maximal aggregation in the platelets from CH patients. After incubation with GSNO, % maximal aggregation induced by ADP was significantly reduced in both groups, but the reduction was much more evident in the CH patients, while % maximal aggregation induced by ADP was not changed by the addition of GSNO in control group and was significant reduced in CH patients. C: controls; CH: chronic hepatitis; GSNO: nitrosoglutathione. $^{a}P < 0.05$, $^{b}P < 0.01$ *vs* ADP; $^{d}P < 0.01$ *vs* collagen; $^{c}P < 0.05$ *vs* ADP; $^{f}P < 0.01$ *vs* collagen.

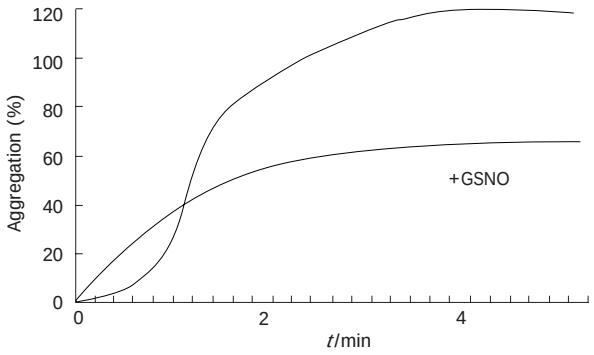

Figure 4 Aggregation curves of a single representative CH patient before and after GSNO. GSNO was able to reduce platelet % maximal aggregation and modified the curve morphology by abolishing the delay in the beginning of the aggregation. GSNO: nitrosoglutathione.

was significant reduced in CH patients (Figure 3).

Besides, the morphology of the aggregation curve induced by collagen was different in control and HCV-positive patients. In the latter a delay appeared at the beginning of the aggregation. Such a delay was completely abolished by GSNO. Figure 4 shows the aggregation curves of a single representative CH patient before and after GSNO. GSNO was able to reduce platelet maximal aggregation and modified the curve morphology by abolishing the delay in the beginning of the aggregation.

DISCUSSION

This study confirms[31] the inverse correlation between liver fibrosis and number of platelets. Thrombocytopenia in chronic hepatitis caused by HCV may be considered a consequence of portal hypertension or of myelosuppression by HCV or of reduced hepatic production of thrombopoietin[34-37]. However, our data provide additional explanations for the occurrence of thrombocytopenia in CH. In fact, we are the first to demonstrate that, as suggested by others[6], the function of platelets from patients with chronic HCV-related hepatitis may also be modified. *In vitro*, platelets from these patients display increased aggregation induced by ADP and collagen and a latency time before aggregation when compared with platelets from healthy controls. The addition of GSNO, the major constituent of circulating levels of S-NO *in vivo*, reverses these alterations, at least in part. In fact, GSNO reduces the aggregation induced by ADP in all groups, and in HCV positive patients, it not only reduces the aggregation induced by collagen but also abolishes the latency time. Our data also confirm the anti-aggregation activity of GSNO that has been documented by others *in vitro* and *in vivo*[18,25,32,33,38].

In vivo, S-NO may serve as a carrier in the mechanism of action of the endothelium-derived relaxing factor (EDRF), by stabilizing the labelled NO radical from inactivation by reactive species in the physiological milieu and by delivering NO to the heme activator site of guanyl cyclase. GSH and CYS form S-NO with anti-platelet properties associated with the stimulation of guanyl cyclase and a significant decrease in fibrinogen binding to platelets[39]. In addition, S-NO inhibits thrombin receptor-activating-peptide-induced platelet aggregation[33,40-42].

The state of NO in the circulation is dynamic, since it continuously forms S-NO adducts that are continuously exchanged with albumin or haemoglobin or released at the cellular level. The storage and biodegradability of NO in the circulation, which are strongly influenced by the redox status of plasma, significantly affect the bioavailability and effects of NO on platelets. The redox status of plasma also affects the import/export as well as the production of NO from platelets[12-14,43,44]. In fact, the dynamic process of S-thiolation in response to oxidative stress is considered an important pathway in the maintenance of the function of platelets[8,45]. S-NO may also act as antioxidant substances, as they inactivate nitrogen reactive species (nitroxyl anion, nitrosonium cation, nitrogenous oxides, peroxynitrite, *etc.*)[19]. In consideration of these complex interactions, we simultaneously evaluated plasma levels of nitrite, NPSH, GSH, CYS, and S-NO in order to have an overall picture, even if not complete, of the dynamic transport of NO in the circulation in relation to a possible alteration of the redox status.

In the present investigation, S-NO levels were increased in CH patients and directly correlated with the number of platelets. This increase is likely due to the increase in plasma levels of cytokines produced by activated neutrophils and monocytes. The activation of these circulating cells leads to an increase in the production of NO involved in exchange with NO adducts[46,47]. When we considered all patients with HCV CH together, we did not find any variation in the plasma levels of antioxidants or markers of lipid peroxidation. However, by dividing patients on the basis of their histology, we clearly documented that, in the absence of alterations in redox status, as expressed by normal plasma levels of thiols and lipid peroxidation markers, NO circulates

primarily as S-NO. In this situation, it may contribute to the maintenance of normal platelet function, probably by helping to counteract possible oxidative stress. With increased oxidative stress and/or decreased antioxidant levels, the nitrosylation pathways' activities are decreased and NO circulates as nitrite/nitrate, which are significantly increased in patients with more advanced liver fibrosis. In this condition, aggregation may be altered, resulting in the low number of platelets we recorded. In this context, the major cause of altered platelet function should be the decrease in GSH. In fact, this thiol is highly important for platelet function and when its level decreases, platelets can produce per se free radical intermediates when reacting with platelet aggregatory agents[48].

In healthy volunteers, oral administration of KNO_3 induces an increase in plasma S-NO and significantly inhibits platelet aggregation, without any effect on systemic and portal pressure[49]. Recently, S-NO have been chemically synthesized and proposed as a class of drugs that, upon decomposition, release NO and are therefore useful in patients with cardiovascular diseases[50]. They also have a detoxifying effect, as they inactivate nitrogen reactive species by continuously exchanging NO with SH groups[19].

In conclusion, our study reveals a defect in platelet aggregation in patients with HCV-related CH. This defect depends, in part, upon variations in NO-related pathways in the circulation. When the plasma redox status and bioavailability of GSH are normal, NO circulates primarily as SNO; in these conditions, the production of NO may also be increased by iNOS activated by cytokines. In this situation, S-NO could help to maintain normal platelet aggregation. Data from experiments *in vitro* confirm this hypothesis: the addition of GSNO ameliorated the aggregation defect of platelets from HCV patients. Increased oxidative stress and decreased bioavailability of GSH, as documented in patients with a high degree of fibrosis, lead to the formation of more nitrite than S-NO; all these events reduce platelet aggregation.

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