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Possible Mechanisms Of Recurrent Upper Gastrointestinal Bleeding In Patients With Peptic Ulcers.

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ABSTRACT

One of the most often causes of nonvariceal upper gastrointestinal bleeding is peptic ulcers. However, it is still unclear what mechanisms lead to un-sustained hemostasis after acute peptic ulcer bleeding. We suggested that this could be related with abnormal inflammatory reaction and aberrant platelets reactivity. To clarify this issue, we evaluated parameters of inflammation, tissues repair and platelet aggregation among patients with different outcomes of peptic ulcer bleeding. Overall, 144 patients with peptic ulcer bleeding were enrolled in this investigation. Histological evaluations of ulcer margin biopsy and platelet aggregation measurement were performed at the moment of admission. It was shown that recurrent bleeding is associated with exaggerated inflammatory reaction with severe neutrophil infiltration, tissue damage, decrease of angiogenesis and myofibroblasts reaction. The intensity of inflammation was tightly associated with increase of macrophages number that was related with higher risk of rebleeding. Platelet aggregation in bleeders was lower than in healthy volunteers. Low, reversible and prolonged platelet aggregation induced by collagen was associated with higher risk of rebleeding. This fact supports the concept on platelets contribution in ulcer's healing. Aberrant platelet aggregation could lead to lack of growth factors release and alteration of gastroduodenal mucosa healing after bleeding.

Keywords: peptic ulcers, upper gastrointestinal bleeding, platelets, hemostasis, inflammation, repair.

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INTRODUCTION

Acute, nonvariceal upper gastrointestinal bleeding(UGIB) is considered as a commonly encountered diagnosis for surgeons[1, 2]. During the last decades there has been significant improvement in UGIB management [3]. The current management of UGIB is based on assessment of clinical and laboratory signs, as well as endoscopic characteristics [4, 5].Successful initial hemostasis can be achieved in about 90% of patients, however despite advances in gastroprotection and endoscopic management there is still a high rate of rebleeding (up to 20%) [3, 6].Different clinical and endoscopic scoring systems have been developed for predicting the outcome of UGIB [4, 5, 7]. However, it is still unclear what mechanisms lead to rebleeding among patients with similar clinical and endoscopic characteristics that stimulates for identifieng causal and risk factors of primary and recurrent bleeding.One of the important causes of UGIB is peptic ulcers of stomach and duodenum[1, 8]. A wide range of risk factors of peptic ulcer bleeding development, including genetic predisposition, smoking, gender, age, socioeconomic status, alcohol consumption, gastric acid secretion, stress and body weight have been identified [9, 10, 11, 12].However, the basic mechanism of peptic ulcer bleeding development is damage of gastrointestinal tissues and vessels due to ischemia and inflammation [13, 14]. It was shown that innate and adaptive immunity reactions, induced by peptic factors and microbes, play an important role in ulcerogenesis and its complications [15].In addition to oxidative stress and damage, exaggerated inflammatory reaction can be associated with lack of repair[8, 11]. Resolution of inflammation and ulcer repair depends on such processes as cell proliferation, differentiation, migration as well as on extracellular matrix composition and remodelling [16]. The synchrony of these processes is controlled with numerous paracrine factors including gaseous mediators, prostaglandins, trefoil peptides cytokines, hormones, growth factors etc.[14, 17, 18]. The last category comprises a long list of molecules involved in healing of gastrointestinal tissues. This range of molecules includes epidermal growth factor (EGF), transforming growth factor-alpha (TGF-α), TGF-β, fibroblast growth factor (FGF), vascular growth factor (VEGF), platelet derives growth factor (PDGF) etc. [14, 16, 18]. One of the most important sources of these numerous growth factors under damage is platelets[19]. There are strong evidences that platelets play an important role in orchestrating gastrointestinal homeostasis and repair[13, 20]. Nevertheless, there is a limited number of studies assessed platelet reactivity under ulcer bleeding and their role in ulcer non-healing and un-sustained haemostasis requires further investigations.

Aim: To evaluate the interplay between inflammation, hemostasis and tissues repair among patients with different outcomes of ulcer bleeding.

PATIENTS AND METHODS

This was a single-center cohort study conducted at surgery department of M. Gorky Donetsk State Medical University. Overall,144 cases of gastroduodenal ulcer bleeding were included in this investigation. Demographic, clinical and laboratory data were collected. Patients considered eligible for enrollment had to be from 35 to 70 years of age, suffer from typical symptoms of acute bleeding from gastric and duodenal ulcers, confirmed by positive upper gastrointestinal endoscopy. Endoscopic evaluation was performed within 24 hours of admission to all patients with bleedingwith endoscopic hemostasis if indicated.Exclusion criteria were the following: age younger than 35 years or over 75 years, non-ulcer lesions, any allergy to established medications, patients having a history of alcoholism and drug abuse, coagulopathy, infarction of myocardium and ischemic stroke during the last six months, diabetes mellitus, neoplasy, renal failure, portal hypertension and cirrhosis; any medication (H2RAs, PPIs, bismuth, antibiotics, NSAIDs antiplatelet drugs/anticoagulants or corticosteroids) for 4 weeks before admission to the hospital. In addition, we excluded all the patients with severe bleeding who required blood transfusion or surgery. All enrolled patients received intravenous proton pump inhibitor (PPI) with 80 mg bolus followed by 8 mg/h continuous infusion for 48 or 72 hours [17, 12].According to the bleeding outcome all patients were subdivided into two groups: 1st group – with sustained hemostasis (n=102); group 2 included patients with in-hospital rebleeding within 72 h (n=42). Rebleeding was considered if any of the following events occurred: repeated vomiting of fresh blood, hypotensive shock, fresh melena episodes, or a decrease in the hemoglobin level. Control group included 10 healthy male volunteers who were the same age.There was no pharmaceutical industry support for this study. This study was approved by the Ethical Committee of M. Gorky Donetsk State Medical University. Written informed consent was obtained from all participants.

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Histological evaluation of ulcer margin biopsy

There were 22 cases of biopsy in 1st group and 18 cases of biopsy among patients of 2nd group. Bioptates were taken from ulcer margin area and from intact mucosa taken in stomach or duodenum at distance from ulcer in patients with Forrest class II. All biopsies were taken up to 24 hours after bleeding manifestation before treatment. After the endoscopic removal, all the specimens were immediately fixed in 10% neutral buffered formalin for ~18–24 h, processed and embedded in paraffin according to a standard protocol. Each section was cut at 5 μm and routinely stained with hematoxylin-eosin staining and immunohistochemistry. The number of polymorphonuclear leukocytes (PMNs) and macrophages (Mph, CD68 positive cells) was counted. To assess parameters of repair we evaluated number and distribution of myofibroblasts, as a key cells of granulation tissue, endothelial cells and proliferating cells in mucosa of each biopsy. For this aim the immunoreactive alpha-SMA, CD31 and Ki-67 cells were counted per field in each specimen. All histological evaluations were performed under magnification 200 in a blinded manner by two independent investigators.

Platelet aggregation assessment

To assess platelets' reactivity the whole blood was sampled at the moment of hospital admission before therapy. Blood was collected from the antecubital vein into plastic syringes containing sodium citrate at a final concentration of 0.38% with proportion 9:1 and centrifuged at 200×g for 20 minutes at 25°C to prepare platelet-rich plasma (PRP). Platelet aggregation was evaluated after induction by adenosine diphosphate (ADP; 5 μM) and collagen (1 μM) [19, 21].Measurement of platelet aggregation was carried out according to the method previously described using aggregometer Solar.

Statistical analysis:

Data were collected and analyzed using the MedCalc. Descriptive statistics were used to analyze and report the data. For the presentation of nominal data, the percentage and standard error were calculated; for the presentation of numeric data, the median and interquartile interval were calculated. Chi-square, Kruskal-Wallis, and Dunn's tests were used to determine the differences between patients of 1st and 2nd groups. Correlations were performed by the nonparametric Spearman test. P value < 0.05 was considered to indicate statistical significance[22].

RESULTS

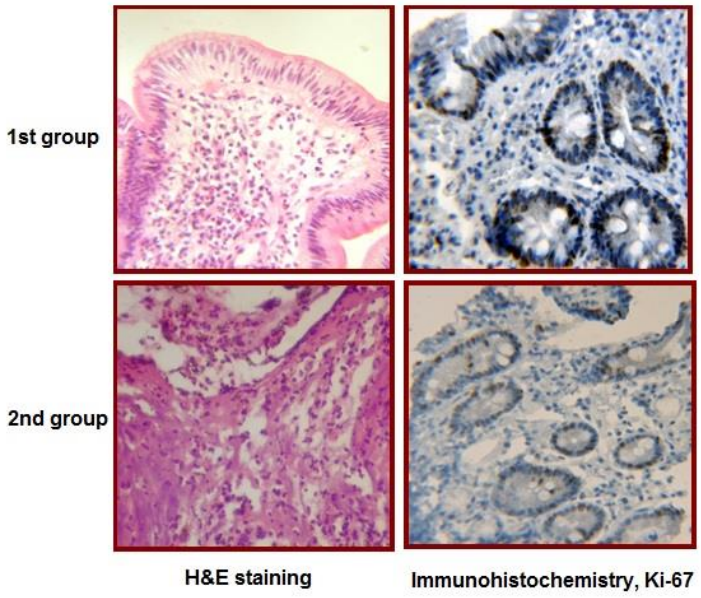

Fig 1: Damage and mucosal cells proliferation in ulcer margin after bleeding in patients with sustained hemostasis and recurrent bleeding.

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The patients of observed groups were comparable in clinical and laboratory data (table1).Morphological assessment of biopsy showed that UGIB revealed features of ischemia and inflammation at the margin of gastroduodenal ulcers. Comparative assessment of histology in biopsy of 1st and 2nd groups patients showed that rebleeders exhibited more severe PMNs infiltration (P = 0.017), that was accompanied with edema and necrosis of muscularis mucosae (Fig. 1). Interestingly that the local PMNs infiltration was not related with the count of peripheral blood neutrophils and leucocytes in general.

Seeking for the causes of intensive inflammatory reaction with secondary tissue damage we assessed the number and distribution of macrophages. It was found that the number of CD68 positive cells was much higher in ulcer margin comparing with unaffected gastroduodenal mucosa (P = 0.008).In addition, spatial pattern of Mph distribution was also different in patients of the1st and 2nd groups. In patients of the 1st we found that Mph were arranged mostly under altered epithelium and around dilated vessels. Numerous CD68 positive cells were found in areas of PMNs recruitment, as well as around lymphoid infiltration that was most often at gastric mucosa. Naturally that number of CD68+ cells in ulcer edge of 2nd group patients was significantly higher than in 1st group(*P* = 0.011). Moreover, an increased number of Mph determined the higher risk of recurrent ulcer bleeding (OR 3.5 (95% CI 1.9–4.87), P=0.001).Numerous Mph were found in depth of mucosa – next to muscularis mucosae and along inflammatory infiltrates. The strong correlation was found between the count of macrophages and neutrophils (P = 0.0013). Therefore, these findings allowed us to suggest that one of the possible causes of rebleedingcould be aberrant reaction of macrophages leading to increased PMNs recruitment and damage of gastroduodenal tissues and vessels.

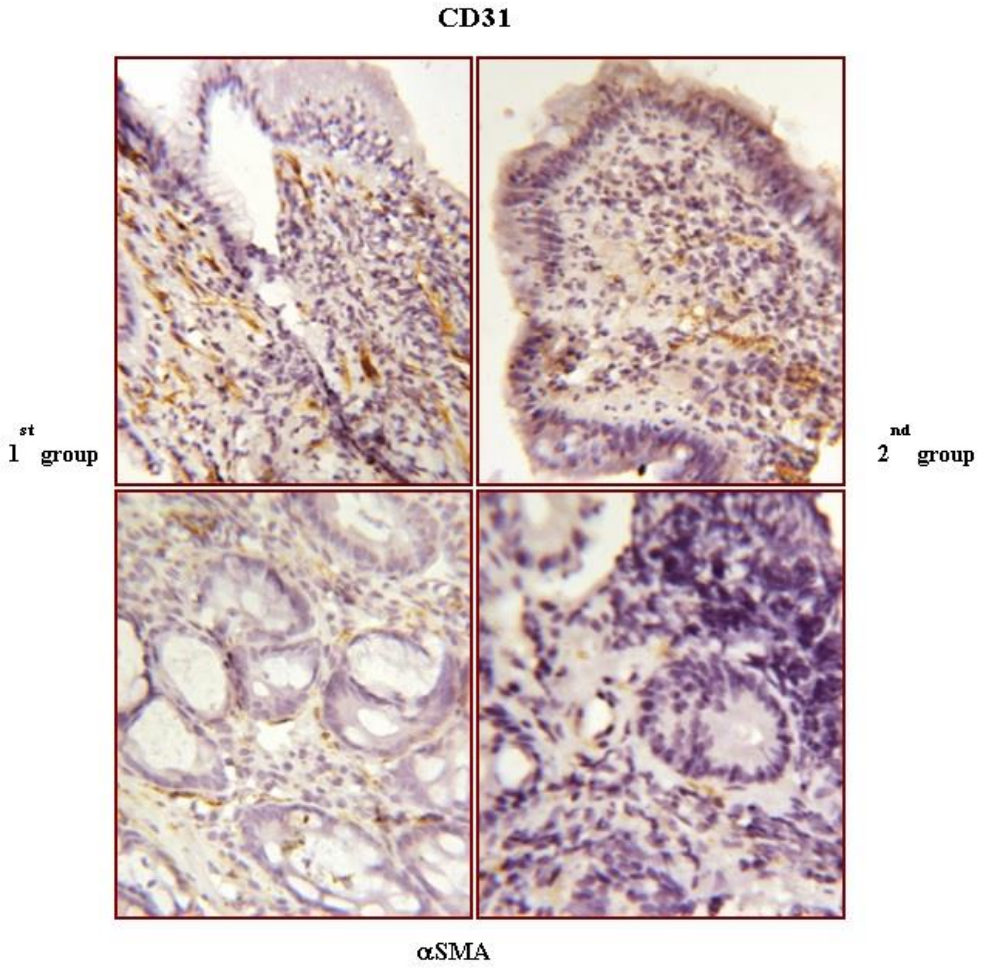

Fig 2: Angiogenesis and myofibroblasts at the ulcer margin among patients of the 1st and 2nd groups
The number of CD31 and SMA positive cells is high in ulcer margin of 1st group patients, but much lower among patients with rebleeding (2nd group)

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To assess the relationship between inflammation and ulcer healing and its association with UGIB outcome we compared the signs of repair in ulcer margin of patients with healing and recurrent bleeding. It was found that even during acute inflammatory reaction there were some features of repair including angiogenesis and granulation tissue formation at the ulcer margin in biopsy of patients of the1st group patients. Numerous α-SMA positive cell we found in biopsies of ulcer margin in the 1st group. These cells were detected predominantly superficially – in areas of epithelial desquamation and under covering epithelium in gastric and duodenal mucosa that was associated with features of active epithelization. Evaluation of CD31 positive cells in 1st group patients showed the signs of angiogenesis along myofibroblasts traffic and around areas of inflammatory infiltration in ulcer margins. In contrast, the count of CD31+ (P = 0.04) and α-SMA+ (P = 0.031) cells in biopsies of rebleeders was significantly lower comparing with the 1st group, reflecting inhibition of angiogenesis and granulation tissue formation (Fig. 2). Nevertheless,there were no significant differences in Ki-67 positive cells count regarding outcome. The most of Ki-67 positive cells were in covering epithelium. We found negative correlation between CD68 and α-SMA positive cells count in ulcer margins among rebleeders (r=-0.623; p = 0.016) with no significant relationship between CD68+ and CD31+ cells. These facts could demonstrate predominance of M1 type macrophages in ulcer margins of rebleeders that could provoke exaggerated inflammation rather than resolution of inflammatory reaction and activation of repair.

It was found that median of collagen and ADP induced platelets aggregation was lower in patients with UGIB comparing with control (table 2).Such factors as gender, age, comorbidity and severity of hemorrhage did not affect the aggregation of platelets in patients with bleeding. In addition, we did not reveal differences in platelet functioning between patients with sustained hemostasis and recurrent bleeding in first 3 days after hospital admission. However, it should be noticed that aggregation of platelets in patients with UGIB varied from 0 to 100%. That is why we compared frequency of bleeding outcome in patients with low (0-29%), middle (30-69%) and high (70-100%) aggregation of platelets (Fig. 3). Interestingly that the highest rate of rebleeding was among patients with low collagen-induced aggregation of platelets (χ^2 = 7.482; P = 0.0237). However, there were no the same pattern for ADP-induced aggregation (P = 0.275). One more interesting fact – we found a different character of aggregation curve in bleeders. Most of rebleeders demonstrated aberrant type of aggregation curve (Fig. 4). In addition to low value, platelets' response to collagen and ADP among rebleeders was either prolonged (with long latent period) or reversed. Such changes of aggregation curve were related with the clinical outcome - high frequency of bleeding recurrence was observed among patients with long latent period of aggregation induces by collagen (P = 0.02).Interestingly that platelets aggregation, induced by collagen, but not by ADP, correlated with CD31 positive cells (r=0.682; p = 0.012) and α-SMA+ cells count (r=0.794; p = 0.0027).

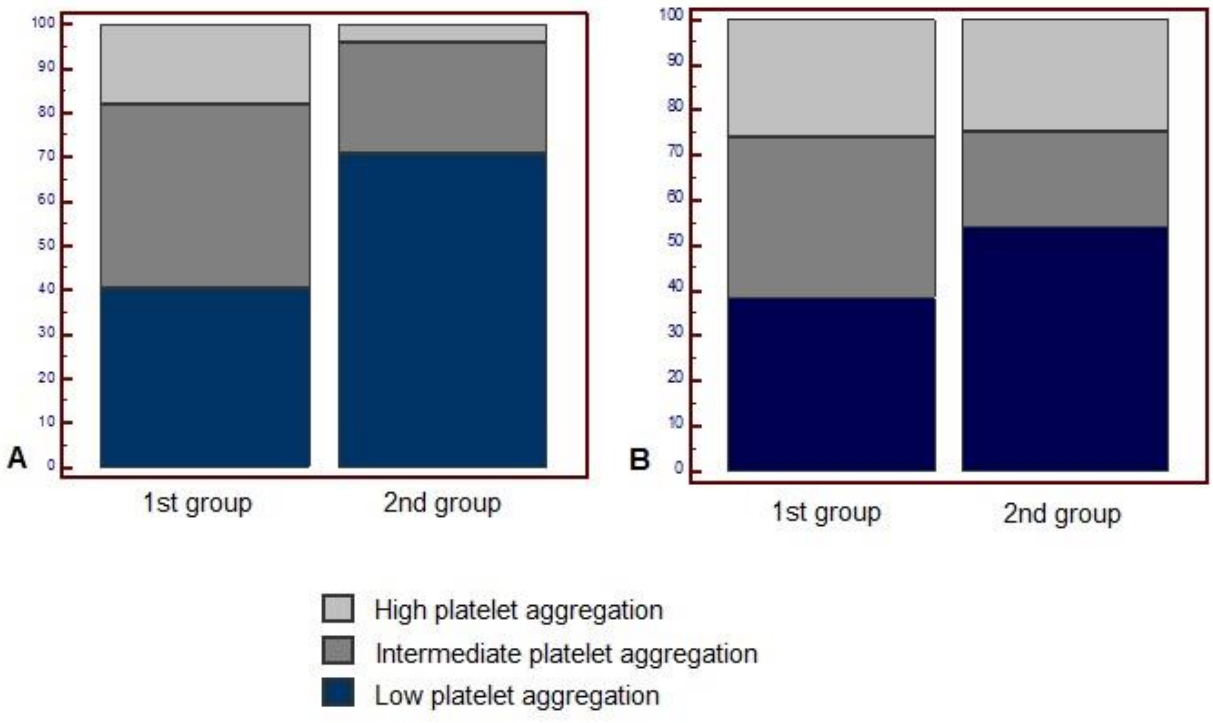

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Fig 3: Proportional distribution of platelet aggregation among patients with sustained hemostasis (1st group) and recurrent bleeding (2nd group)

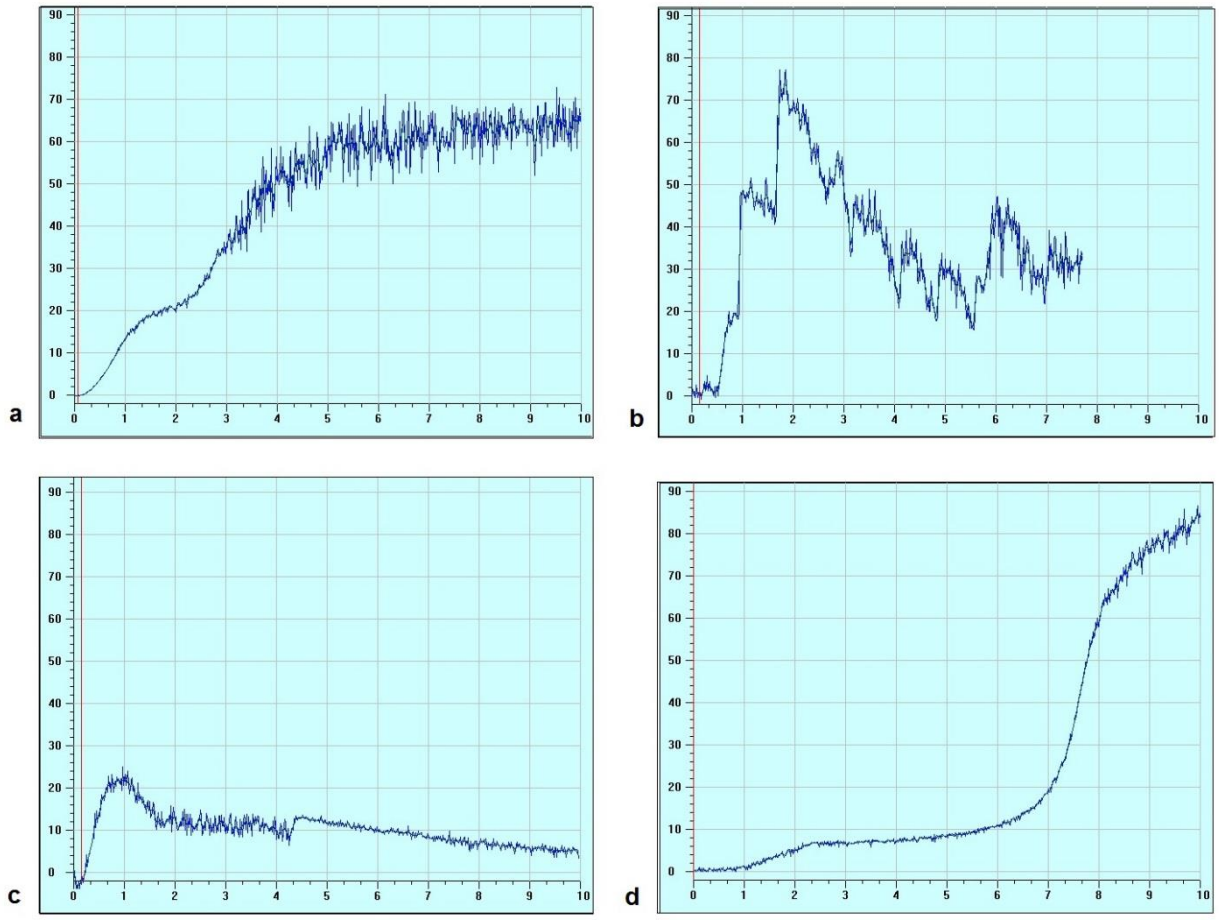

Fig 4: Types of aggregation curve in control and patients with peptic ulcer bleeding. Platelet aggregation was induced by collagen. Axis x shows time after induction (minutes), axis y corresponds to platelet aggregation (%).

a – platelet aggregation in control group, b-d – low, reversible and prolonged platelets' aggregation in patients with ulcer bleeding. Reversible and prolonged platelet activation was associated with higher frequency of rebleeding.

DISCUSSION

As itis known, bleeding is a result of injury in GD-wall as well as large vessels damage caused by different aggressive factors, including *H.pylori*, reactive oxygen radicals, H^+, enzymes, bile acids etc.[2, 11, 15].Regardless the nature of causal and risk factors of ulcerogenesis, the key mechanisms of tissue injury are such stereotypical processes as ischemia and inflammation. Histopathological evaluation of ulcer margin in this study demonstrated features of both ischemia and inflammation in patients with acute bleeding. Tissue damage with oedema and local inflammatory reaction with PMNs infiltration were identified in ulcer margin. Actually ulcer margin is considered as a main source of granulation tissue formation and epithelisation of ulcer. Not surprisingly that severity of PMNs infiltration was related with the risk of rebleeding among patients with UGIB, as neutrophils recruitment is associated with activation of respiratory burst, releasing of lipid mediators of inflammation as well as proteolytic enzymes secretion and secondary tissues damage [18, 23, 24]. The question is why patients with the same clinical and endoscopic data demonstrated different intensity of PMNs infiltration? In fact, the diversity of neutrophilic infiltration among bleeders could be related with severity of primary tissue damage in gastroduodenal area, different vascular reaction and ischemia in the ulcer margin [25]. However, there are some intrinsic factors determining the intensity of inflammatory reaction [9]. One of

them is macrophages reaction. It is widely accepted, that Mph play an important role in both up- and down-regulation of inflammation releasing self-limitation of inflammatory reaction in space and time [26, 27]. Such a dual role of macrophages in ulcer healing is related to the conception about M1–M2 dichotomy that explains an intrinsic property of macrophages to control the transitions from inflammation to healing[27].

The results of our study showed that intensity of inflammatory reaction was tightly associated with increase of Mph number in ulcer margin. In addition, Mphs count was much higher in 2nd group patients, predicting the risk of rebleeding. Activation of macrophages is considered to be a key factor for orchestrating inflammation and repair of gastroduodenal mucosa after damage [26, 28].Revealed association between PMNs infiltration and CD68+ positive cells number allows us to suppose that rebleeding development was due to exaggerated pro-inflammatory M1macrophage phenotype activation and disability to switch M1 type macrophages to M2 phenotype. What factors induce up-regulation of macrophages and their pro-inflammatory type? In addition to classical inductor of Mph activation – bacterial LPS, ischemia up-regulates macrophages through induction of hypoxia-inducible transcription factors (HIFs) 1 and 2 expression[26, 29]. Previously it was shown that exposition of human Mph to hypoxia led to up-regulation of nuclear factor-κB (NF-κB) and its signaling pathways[30]. That was associated with increased production of interleukins (IL)-1β and IL-8, chemokines and their receptors that could explain increased PMNs recruitment into ulcer area under macrophages hyperactivation [30]. Concerning this concept, patients with repeated UGIB had a failure to down-regulate the initial response that results in chronic pathogenesis and tissue damage in patients with peptic ulcer bleeding. This could demonstrate disability of resident and newly recruited Mph to switch inflammatory phase to healing, resulting in progressive secondary alteration and rebleeding development.

On another side, it is widely accepted that inflammation is associated with acceleration of haemostasis and platelets aggregation. Their activation normally realises two main tasks – clot formation preventing bleeding and secretion of numerous growth factors promoting tissues repair and angiogenesis [8, 19, 21]. The co-migration of platelets and PMNs has been observed in different pathological states including coronary artery diseases, inflammatory bowel disease etc. [31, 32]. In contrast, patients with UGIB demonstrated absolutely different character of relation between platelets and PMNs that stimulates for discovering mechanisms of platelet-leukocytes interplay in UGIB [13]. Herein we established the relations between platelets aggregation and ulcer healing features. The results of our study demonstrated that platelets aggregation in bleeders was lower than in healthy volunteers. In addition, low, reversible and prolonged platelet aggregation induced by collagen (but not ADP) was associated with higher risk of rebleeding. Indeed, platelets can stimulate angiogenesis and promote gastric ulcer healing through releasing of numerous growth factors [20]. In addition, there are clinical and experimental evidences that antiplatelet therapy attenuates gastric healing that is related to block of platelet aggregation [33]. As far as platelets aggregation is tightly associated with granules secretion, it is natural to suggest abnormalities in collagen-induced signaling and degranulation mechanisms in platelets of 2nd group patients [21, 31]. This fact reflects the different role of platelet receptors and their signalling pathways in determination of bleeding outcome. As it is well known, platelets express several collagen receptors. In addition to glycoprotein Ib and integrin alphaIIbbeta3, interacting with collagen indirectly via von Willebrand factor, platelets express several collagen receptors [33]. They are integrin alpha2beta1, playing an important role in platelet adhesion, and GPVI that is required for collagen-induced platelet activation. Signal transduction induced by binding with ligand, is realised via several pathways including Src kinases, phosphoinositide 3-kinases, and ITAM signalling cascade [21, 33]. All these signalling pathways are important for secretion of platelet granules that is the key event in release of growth factors. This could explain the established relation between abnormal collagen induced platelet aggregation and un-sustained haemostasis in bleeders. Further studies are warranted to discover mechanisms of macrophages and platelets dysfunction in patients with peptic ulcer complicated with acute bleeding.

CONCLUSION

Thus, UGIB was associated with altered platelets aggregation, inflammation and repair. Exaggerated inflammation in ulcer margin due to hyper reaction of macrophages, dip in platelets aggregation and reversible type of aggregation were associated with high risk of ulcer rebleeding.

LIMITATIONS

The limitation of this study is the variability of the subject's characteristic, which includes patients' gender, location and size of ulcers. It was uncertain whether patients were infected with *H. pylori*. In addition, limited number of histological examinations could also affect the results of this study.

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