Antiplatelet agents'-ticagrelol and eptifibatide-safety in experimental colitis in mice

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ABSTRACT

Background/Aims: *To evaluate the side effects of two antiplatelet agents – ticagrelor and eptifibatide – in mice with experimentally-induced inflammatory bowel disease.*

Materials and Methods: This study was designed as a controlled, animal, drug safety investigation. C57Bl/6 mice were used to establish the ulcerative colitis model by exposure to dextran sulfate sodium (DSS) and divided into three experimental groups: eptifibatide treated (150 µg/day intraperitoneally; n=10), ticagrelor treated (1 mg/day via gastric tube; n=10), and DSS control (plain drinking water; n=10). An unmodeled non-DSS group served as the experimental control. Complete blood count was taken for all mice at baseline (day 0, treatment initiation) and after four days of treatment. On day 4, all animals were sacrificed for autopsy. The primary outcome measure was bleeding, and the secondary outcomes were changes in platelet count, hemoglobin level, and hematocrit level.

Results: Neither ticagrelor nor eptifibatide treatment produced a significant effect on DSS colitis mice for the safety parameters measured. Platelet count and hemoglobin and hematocrit levels were statistically similar between the three DSS groups and the non-DSS control group (p>0.05). Autopsy found no evidence of recent bleeding in liver, spleen, central nervous system, or serous cavities.

Conclusion: The antiplatelet agents, ticagrelor and eptifibatide, were safe in DSS colitis mice, suggesting their potential in humans suffering from ulcerative colitis and supporting future safety studies.

Keywords: *Dextran sulfate, colitis, ticagrelor, eptifibatide, mice, adverse effects*

INTRODUCTION

Inflammation and coagulation are considered the most important physiological processes in the chronic pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD)—the primary types of inflammatory bowel diseases (IBD). These two processes are in constant balance, mediated by their functional interplay. Inflammation triggers coagulation and coagulation maintains and amplifies inflammation, which results in a hypercoagulable state (1).

A number of studies clearly indicated the importance of platelets (PLTs) in UC and CD exacerbation. A higher number of PLTs are encountered in patients with these diseases (2). The role of PLTs in mediating leukocyte recruitment to the inflamed colon was specifically investigated, and the results showed that platelet dysfunction could have a role in the development of lesions in these patients (3).

Platelets have been shown to synthesize large amounts of pro-inflammatory mediators, directly communicate with activated pro-inflammatory cells, and are activated in the processes of inflammation through receptors that are expressed on their surface. After activation, PLTs release histamine, prostaglandin E and D2, platelet-derived growth factor, thromboxane A2, and serotonin, which control vascular permeability and participate in vasodilatation and/or vasoconstriction regulation (4).

Randomized clinical studies have not tested the effects of drugs that affect the functioning of PLTs. There is concern surrounding the use of these medications given the common clinical manifestation of bleeding from the digestive tract, which is often the complication of the use of these drugs in other indications. However, the rationale for the use of these drugs with regard to their anti-inflammatory effect in IBD is

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that the potential for bleeding from the affected part of the digestive tract is reduced by the control of inflammation in the bowel wall.

Recent findings have shown that clopidogrel affects the inhibition of PLT activity as well as the relationship between PLTs and leukocytes and the expression of P-selectin on CD40 and lymphocytes via P2Y12 receptor inhibition, which may be a possible mechanism for controlling symptoms in patients with IBD (5, 6).

PLTs are the main source of soluble CD40L and vascular endothelial growth factor in the blood of IBD patients (7). PLTs release a multitude of molecules from their granules, including platelet factor 4, beta-thromboglobulin, interleukin-1, leukotrienes, and prostaglandins (8). PLTs show enhanced activation in IBD, even in the quiescent disease state, with the main site of activation being mesenteric blood vessels (9). T lymphocytes adhere to activated mesenteric endothelial cells expressing adhesion molecules and then bind to CD40-positive PLTs, which, in turn, activate leukocytes. The outcome of this process is rapid and remarkable amplification (10).

Improvement in our knowledge of platelet activation led to the development of several potent antiplatelet drugs with proven efficacy in the treatment and prevention of cardiovascular diseases. Glycoprotein IIb/IIIa inhibitors (GPIs) and P2Y12 inhibitors are currently used extensively in clinical practice. GPIs block glycoprotein IIb/IIIa, which mediates the final pathway of PLT aggregation. They are potent PLT inhibitors. Their most important side effect is bleeding (11). P2Y12 inhibitors block ADP-induced PLT aggregation and activation. The new drug from this class is ticagrelor—a direct acting agent that induces reversible and concentration-dependent inhibition of P2Y12 (12). P2Y12 inhibitors also increase the risk of bleeding. Anti-PLT drugs have been shown to have anti-inflammatory effects (13, 14, 15).

MAIN POINTS

- *The exact pathophysiology of ulcerative colitis is unknown. To improve the response rate to standard therapy in ulcerative colitis patients, it is important to take platelet function into consideration.*
- *Antiplatelet therapy is still not a part of the therapeutic armamentarium for this disease. Herein, we describe our animal study on the safety of antiplatelet therapy, by use of dextran sulfate sodium (DSS)-induced colitis mice.*
- *We found that the antiplatelet agents ticagrelor and eptifibatide produced no significant bleeding, anaemia or low platelet count in the DSS mice. Our findings raise the possibility of using antiplatelet therapy in humans with ulcerative colitis.*

In this study, we investigate the safety of ticagrelor and clopidogrel in experimental UC using a mice model.

MATERIALS AND METHODS

Animals

Forty C57BL/6 mice (inbred females, age: 2-3 months, and average body mass: 20-24 g) were obtained in-house and maintained with standard diet and water with ad libitum access, under standard environment (25±2°C). The experimental protocol was reviewed and approved by the Ministry of Agriculture, Forestry, and Water Economy of the Republic of Serbia (No. 323-07-7363/2014-05/2). Animals were treated according to the recommendations issued by Institute for Medical Research, Belgrade, Serbia.

Body Weight, Diarrhea, and Hematochezia

We examined the body weight, diarrhea, and hematochezia to assess severity of inflammation. The body weight of mice was measured on a daily basis from the beginning of the experiment. We observed mice for stool consistency (pellet, semi-formed pellet, or liquid stools) and rectal bleeding (hemoccult negative or positive, manifest bleeding) every day. Finally, we calculated disease activity index (DAI) daily as the sum of the weight loss score, the diarrheal score, and the hematochezia score based on the method used by Friedman et al. (16) as shown in Table 1. We used DAI to assess the severity of colitis.

Establishment of the Animal Model and Experimental Design

Colitis was induced in 30 mice by a five-day ad libitum exposure to drinking water spiked with 3.5% dextran sulfate sodium (DSS) (average molecular weight within the range of 35,000-55,000; TdB Consultancy AB, Uppsala, Sweden). All mice developed DSS colitis as evidenced by visible blood around the anus and present in the sawdust of the housing cage, which was used as padding.

After five days, DSS-induced mice were divided into three experimental groups (n = 10 each). The first (I) group represented the DSS control group, receiving no intervention during the subsequent four days treatment period. The second (II) group represented the ticagrelor treatment (PO) group, receiving 1 mg (in 0.5 mL) dosages per day of Brilinta® (90 mg tablet; AstraZeneca, Cambridge, United Kingdom) via gastric tube. The third (III) group represented the eptifibatide treatment (IP) group, receiving 150 µg (in 0.2 mL) dosages per day of Integrilin® via intraperitoneal injection (0.75 mg/mL; Schering Corp., Kenilworth, NJ, United States) (Figure 1).

In parallel, another group of mice (n=10), representing the experimental control (K) group, received water without DSS during the five days modeling period.

Table 1. Disease activity index (DAI).

	DAI score				
	0	1	2	3	4
Weight loss	0	1%-5%	6%-10%	11%-20%	>20%
Stoll consistency	Well-formed pellets		Pasty, semi-formed pellets		Liquid stools
Rectal bleeding	Hemoccult negative		Hemoccult positive		Gross bleeding

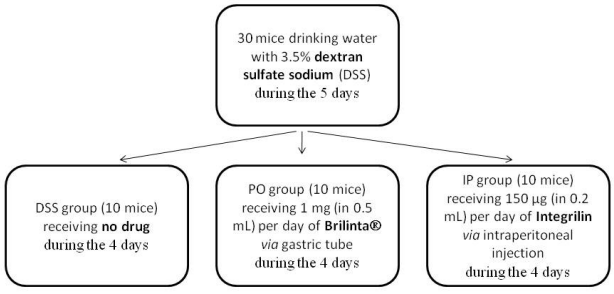

Figure 1. Experimental design.

Primary and Secondary Outcome Measures

The primary outcome measure was bleeding, and the secondary outcomes were changes in platelet count, hemoglobin (Hgb) level, and hematocrit (HCT) level. Complete blood counts were determined for each group at baseline (day 0: before treatment; DSS1, PO1 and IP1 subgroups) and at one day after the last dose (day 5; DSS2, PO2, and IP2 subgroups). Throughout the study, the mice were visited regularly by an on-site veterinarian to assess visible signs of colitis-related (rectal) bleeding.

On day five, all surviving mice were sacrificed, and autopsy was performed to establish presence of bleeding in parenchymatous organs, intracranially, and into serous cavities. PLT aggregation was measured using a multiplate PLT function analyzer (Dynabyte Medical Multiplate®, Manual version V.060403EN, Software V.2.01.b3, Munich, Germany) using adenosine diphosphate thrombin receptor-activating peptide.

Statistical Analysis

Statistical analysis of the data was completed using the PASW Statistics 18® statistical software package (SPSS (Hong Kong) Ltd., Hong Kong, China). All continuous variables were presented as means and standard deviations (SD) and compared using the Mann-Whitney U test for two independent samples without normal distribution, the Wilcoxon signed ranks test for two paired samples without normal distribution, or the Kruskal-Wallis test for more than two independent samples without normal distribution. Distribution normality was tested using the Shapiro-Wilk test, because the number of subjects was <50. All analyses were estimated at $p < 0.05$ level of statistical significance.

RESULTS

Rationale for Drug Selection

In a previous study of C57BL/6 mice, ticagrelor administration at 30-100 µg/g dose was shown to effectively and reversibly block P2Y12-dependent PLT aggregation (17). Considering the MMA's limited technical conditions for intravenous injections of drugs into mice, we opted for intraperitoneal delivery of eptifibatide. A previous study showed that intraperitoneal injection of eptifibatide into BALB/c mice, using 0.5 mg/kg/24-h dosage, slowed the progression of bone metastasis of the animals' breast cancer (18). A preliminary test was used, after which we selected the delivery method as 50 mg/g via gastric tube for ticagrelor and 150 µg via intraperitoneal injection (of 0.2 mL) for eptifibatide. As readout, we evaluated PLT aggregation at baseline, after 2 h, and 24 h of ticagrelor therapy. Similarly, we quantified PLT aggregation after 2 h, 12 h, and 24 h of eptifibatide therapy.

Pathoanatomical Effects

The veterinarian recorded less blood on the sawdust in the cage housing the mice in the ticagrelor-treated (II) group than in the cages housing either the DSS control (I) and the eptifibatide treated (III) groups. Autopsy showed congestive tissue changes in the organs of DSS mice, especially in the parenchymatous organs and particularly in the brain and liver. In general, the DSS modeled mice showed slightly dilated bowel loops with smooth and bright serosa and slightly dilated subserosal vascular beds. Some fresh blood was noted in the bowel lumen. There was no evidence of recent bleeding in liver, spleen, central nervous system, or serous cavities of any of the antiplatelet treatment groups. Histological findings of colonic mucosa in all three experimental groups after autopsy were that DSS2 (Figures 2, 3), PO2 (Figures 4, 5), and IP2 (Figures 6, 7) showed mild inflammation and ulceration.

Body Weight, Diarrhea and Hematochezia

The change in body weight is shown in Figure 8, showing a maximum weight loss below 15% in all three experimen-

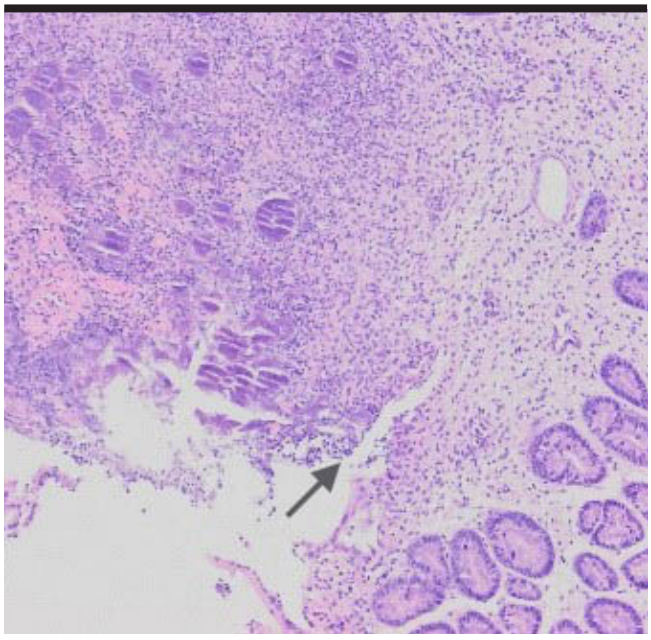

Figure 2. DSS1 group. Histological finding of colonic mucosa. Hematoxylin–eosin stain 10×. Colonic mucosa with ulceration in continuity with bottom filled by non-specific granulomatous tissue, covered with necrotic debris. Surrounding crypts are covered with epithelial cells without dysplasia (arrow).

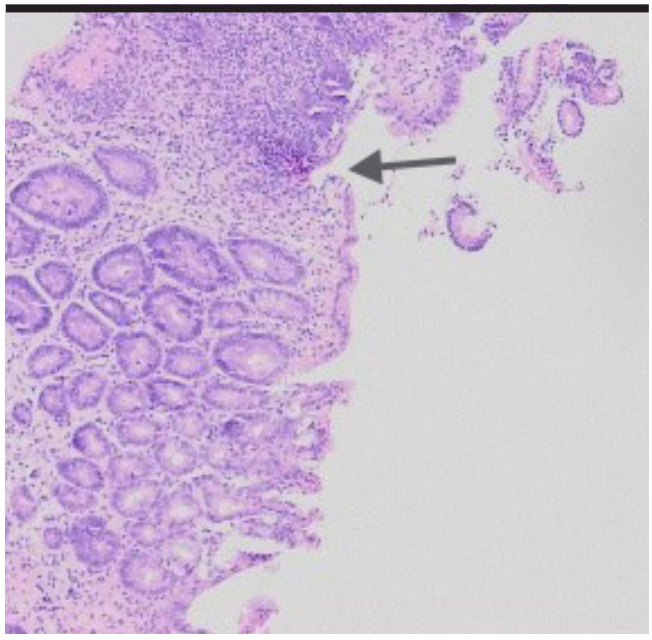

Figure 4. PO1 group. Histological finding of colonic mucosa after administering drug. Hematoxylin–eosin stain 10×. Colonic mucosa with ulceration whose bottom is filled with non-specific granulomatous tissue, covered with necrotic debris. Surrounding crypts are covered with epithelial cells without dysplasia (arrow).

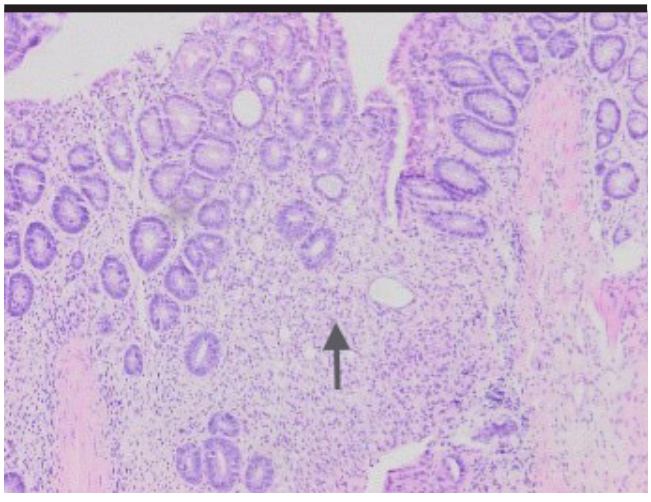

Figure 3. DSS2 group. Histological finding of colonic mucosa. Hematoxylin–eosin stain 10×. Colonic mucosa without ulceration with uniformly spaced crypts, covered by epithelial cells showing no dysplasia. There is expansion of lamina propria due to edema and mild infiltration of inflammatory cells and basal plasmacytosis (arrow).

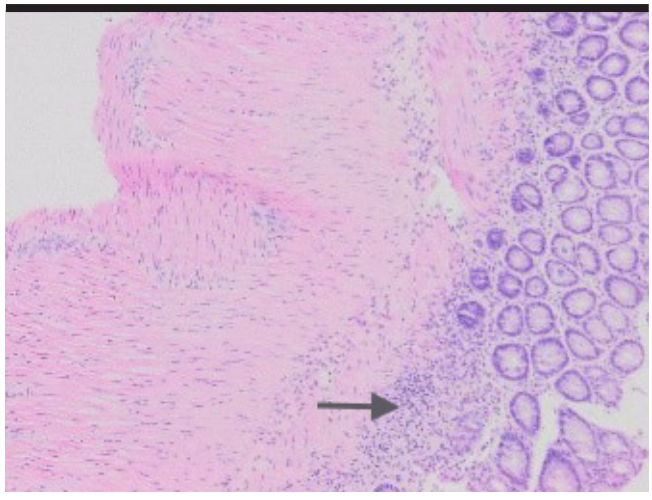

Figure 5. PO2 group. Histological finding of colonic mucosa after administering drug. Hematoxylin–eosin stain 10×. Colonic mucosa without ulceration with parallel crypts, covered with epithelial cells showing no dysplasia. There is expansion of lamina propria due to edema and lymphoplasmacytic infiltration (arrow).

tal groups. Hematochezia was observed in all three experimental groups as blood around the anus and present in the sawdust or as hemoccult positive. Blood was seen from the fourth day of experiment in all three experimental groups.

Disease Activity Index Score

The DAI score was calculated daily as the sum of the weight loss score, the diarrheal score, and the hematochezia score. The results are represented in Figure 9. The DAI score began

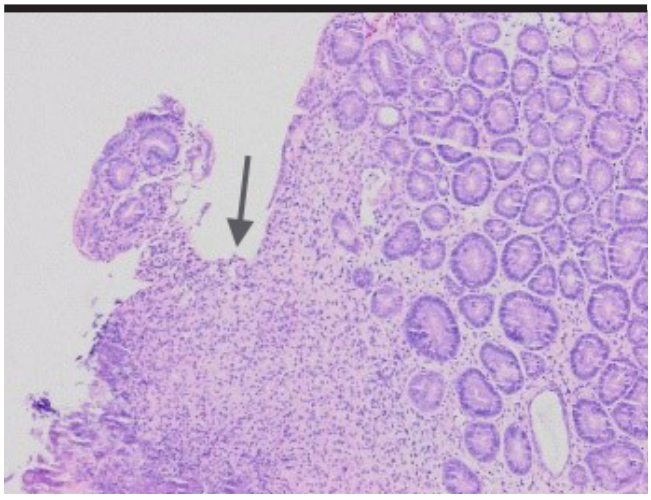

Figure 6. IP1 group. Histological finding of colonic mucosa after administering drug. Hematoxylin–eosin stain 10×. Colonic mucosa with ulceration, non-specific granulomatous tissue and necrotic debris. Crypts are covered with epithelial cells without dysplasia (arrow).

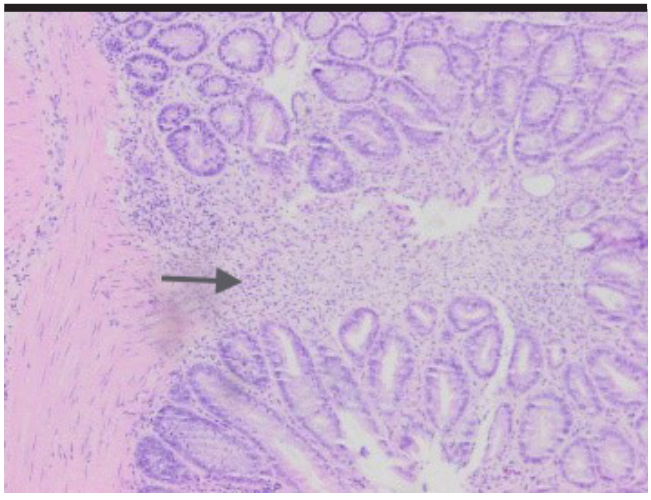

Figure 7. IP2 group. Histological finding of colonic mucosa after administering drug. Hematoxylin–eosin stain 10×. Colonic mucosa with uniformly spaced crypts and epithelial cells showing no dysplasia, without ulceration. There is lymphoplasmacytic infiltration in lamina propira (arrow).

to increase from day two of the experiment. The DAI score was not significantly different between three experimental groups (Kruskal-Wallis test; p=0.925).

Effects on Blood Parameters

The induction of DSS colitis led to significantly lower levels of Hgb and HCT (PO1, DSS1, PO1, and IP1 vs. control; Kruskal-Wallis test: p=0.007 and p=0.002, respectively) (Figures

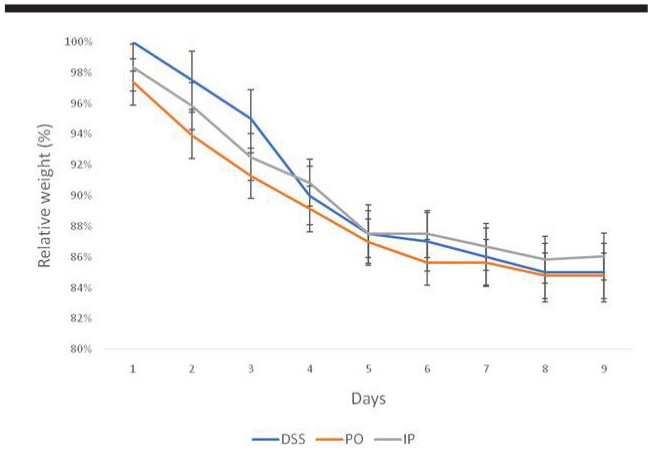

Figure 8. Change in mouse body weight. DSS, dextran sulfate sodium; PO, ticagrelor treatment; and IP, eptifibatide treatment.

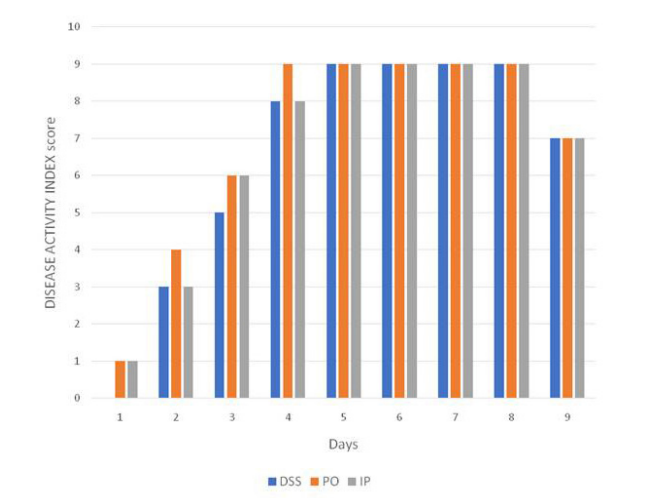

Figure 9. Disease activity index.

10, 11). However, PLT count was not significantly different between any of the DSS groups and the control group (Kruskal-Wallis test: p=0.640) (Figure 12). There were no significant differences in the drug-related changes in the Hgb, HCT, and PLT levels of the three DSS groups according to the two drugs administered (baseline vs. end of treatment; Kruskal-Wallis test: HGB, p=0.369; HCT, p=0.104; and PLT, p=0.307) (Figures 13-15).

Clinical Observations

During the first day of the experiment (day one of antiplatelet drug administration to the treatment groups), one mouse in the DSS control (I) group died. Autopsy indicated the probable cause of death to be extensive bleeding from the gut caused

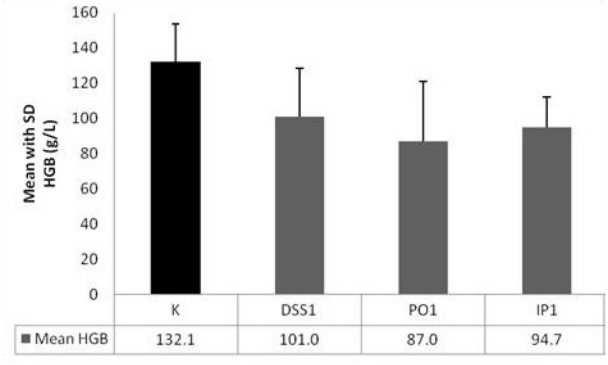

Figure 10. Hemoglobin (Hgb) values before initiation of antiplatelet drug administration. Data are presented as mean±SD. Groups DSS1, IP1, and PO1 represent DSS colitis mice before administration of drugs; K represents the experimental control group. DSS: Dextran sulfate sodium; IP: Eptifibatide treatment; PO: Ticagrelor treatment. (Kruskal-Wallis test: p=0.007)

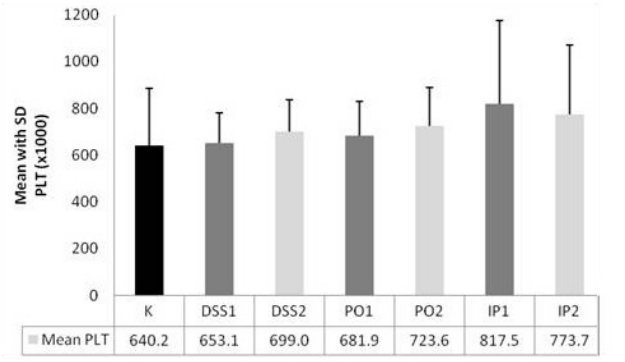

Figure 12. Platelet (PLT) count for all groups. Data are presented as mean ± SD. Groups DSS1, IP1, and PO1 represent DSS colitis mice before administration of drugs; K represents the experimental control group. Groups DSS2, IP2, and PO2 represent DSS colitis mice after administration of drugs. DSS: Dextran sulfate sodium; IP: Eptifibatide treatment; PO: Ticagrelor treatment. (Kruskal-Wallis test: p=0.640)

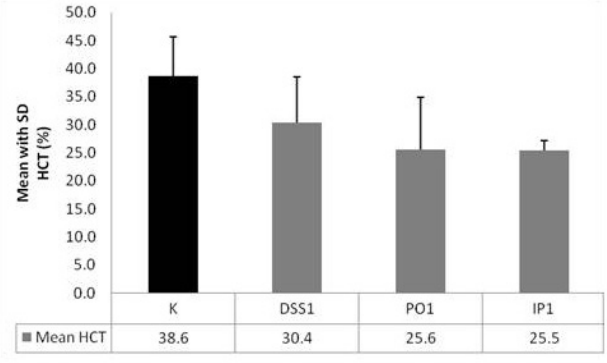

Figure 11. Hematocrit (HCT) values before initiation of antiplatelet drug administration. Data are presented as mean ± SD. Groups DSS1, IP1, and PO1 represent DSS colitis mice before administration of drugs; K represents the experimental control group. DSS: Dextran sulfate sodium; IP: Eptifibatide treatment; PO: Ticagrelor treatment. (Kruskal-Wallis test: p=0.002)

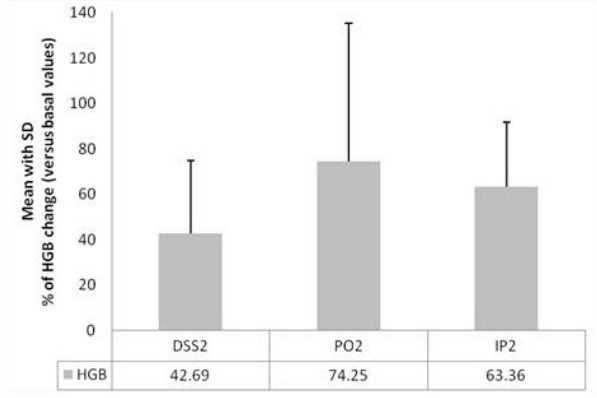

Figure 13. Percent change in values of hemoglobin (Hgb) relative to basal values. Groups DSS2, IP2, and PO2 represent DSS colitis mice after administration of drugs. DSS: Dextran sulfate sodium; IP: Eptifibatide treatment; PO: Ticagrelor treatment. (Kruskal-Wallis test: HGB, p=0.369)

by DSS colitis. On day two of treatment, one mouse in the ticagrelor-treated (II) group died. The veterinarian deemed the death to be due to manipulation of the gastric delivery tube because it occurred after tube insertion and drug administration, followed shortly by the veterinarian's attempts at reanimation. Autopsy was performed on this mouse and no bleeding was found in brain, liver, spleen, or other organs.

DISCUSSION

Several published papers on antiplatelet therapies, mostly involving animal models of sepsis, have indicated therapeutic benefit in reducing systemic inflammatory response. Among the available antiplatelet drug agents, two groups are most important: the P2Y12 inhibitors (i.e., clopidogrel and ticagrelor) and the GPIIbIIIa inhibitors. The P2Y12 inhibitors reduce PLT aggregation and PLT activation and therefore inflammation—a feature that has not yet been proven for the GPIIbIIIa inhibitors (15, 16, 17, 18, 19). P2Y12 inhibitors prevent expression of PLT-leukocyte aggregates (PLA) and P-selectin on the surface of PLTs, achieving an anti-inflammatory effect in numerous inflammatory diseases (15). Ticagrelor is known to exert a more potent anti-inflammatory

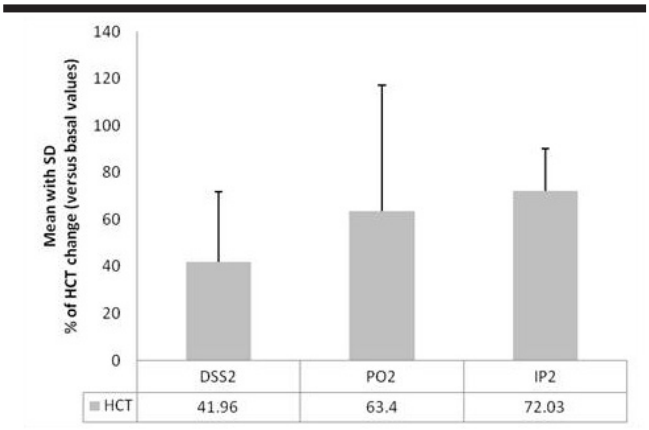

Figure 14. Percent change in values of hematocrit (HCT) relative to basal values. Groups DSS2, IP2, and PO2 represent DSS colitis mice after administration of drugs. DSS: Dextran sulfate sodium; IP: Eptifibatide treatment; PO: Ticagrelor treatment. (Kruskal-Wallis test: HCT, p=0.104)

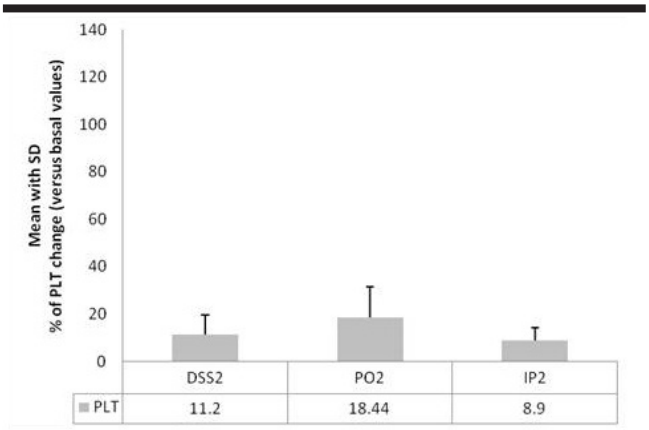

Figure 15. Percent change in values of platelets (PLT) relative to basal values. Groups DSS2, IP2, and PO2 represent DSS colitis mice after administration of drugs. DSS: Dextran sulfate sodium; IP: Eptifibatide treatment; PO: Ticagrelor treatment. (Kruskal-Wallis test: PLT, p=0.307)

effect than its counterpart, clopidogrel (20). However, Patel et al. (21) showed that clopidogrel is capable of exerting an anti-inflammatory effect in other chemically induced models of CD (2, 4, 6 - trinitrobenzene sulfonic acid) and UC (oxazolon). Their results confirmed that clopidogrel reduces activation of PLTs and their ability to interact with LCs.

In 2012, Sharron et al. (22) published a study on the use of a GPIIbIIIa inhibitor, eptifibatide, in sepsis. Their animal model based investigations implicated PLTs in the process of apoptosis induction of immune and nonimmune cells in the spleen, which they managed to inhibit by administration of eptifibatide, resulting in prolonged survival. This notion of PLT therapy was furthered by Thomas et al. (23) in a human-based study of P2Y12 inhibitors. Volunteers suffering from experimental sepsis (induced by intravenous injection of the *Escherichia coli* endotoxin) were administered ticagrelor and clopidogrel and showed a decrease in the level of pro-inflammatory cytokines. The main adverse effect of these two drugs is increased risk of bleeding. Ticagrelor also reduced production of PLA and had a more pronounced anti-inflammatory effect than clopidogrel.

In our study, the antiplatelet drug administration began immediately after the induction of DSS colitis. There were no significant changes in Hgb and HCT levels or in PLT count and no finding of recent bleeding on autopsy in the mice treated with either eptifibatide or ticagrelor. These results collectively indicate that treatment with the antiplatelet agents—eptifibatide and ticagrelor—does not increase the risk of bleeding in mice with UC.

Although our study provides insightful information on the safety of the antiplatelet agents, ticagrelor and eptifibatide, with special attention paid to bleeding events and processes, the basis was a chemically induced animal model. Certainly, more studies should be undertaken to fully elucidate the underlying mechanisms of the observed protective mechanisms before progressing to human studies. In particular, these studies should examine the various routes of administration of these drugs as our study did not use uniform delivery methods between the two drugs.

The results of previous animal studies in IBD suggest that therapy that inhibits the function of platelets, for example, via a thromboxane pathway, can be beneficial in the human population as well. In the current conventional IBD therapy, sulfasalazine has been shown to be a platelet aggregation inhibitor, which is one of the mechanisms of action of this drug. Heparin, as the primary anticoagulant that acts as a binding agent for anti-thrombin III, has a direct anti-inflammatory effect by inhibiting cathepsin G and collagen-stimulated platelet aggregation. This led to the introduction of this drug for the treatment of the most severe form of IBD in hospitalized patients as a prevention of deep venous thrombosis, which did not lead to bleeding in these patients.

It has become clear that inhibition of platelet function plays a role in preventing thrombosis, but there is increasing knowledge of the anti-inflammatory effect of this therapy, associated with the pathogenesis of IBD with intestinal microthrombosis, which plays a significant role in the development of IBD. Platelet aggregates, P-selectin expression, and release of pro-inflammatory mediators clearly indicate platelets as factors in the onset of these diseases where their inhibition can contribute to the comprehensive control of inflammation, which is the ultimate goal in treating patients with IBD.

Strengths and Limitations

This study provides information about the safety of antiplatelet agents, ticagrelor and eptifibatide, with special attention on bleeding in an animal model. Safety was accurately documented on autopsy. There was one control and three experimental groups providing reliable testing. The route of drug administration was not uniform, and the number of animals were limited.

Administering eptifibatide and ticagrelor to DSS colitis mice did not cause serious adverse events. There was no significant decrease in PLT count or Hgb and HCT levels, and no bleeding was observed into the liver, spleen, serous cavities or intracranially. These observations support the potential of antiplatelet therapy for treating UC in humans as an addition to the standard therapy. Ticagrelor could be used in the moderate form of UC and eptifibatide in the severe form, together with standard therapy as it is safe and might reduce inflammation caused by decreased platelet function.

Ethics Committee Approval: Ethics committee approval was received for this study from the Ethics Committee of Ministry of Agriculture, Forestry and Water Economy of Republic of Serbia (No. 323-07-7363/2014-05/2).

Informed Consent: N/A.

Peer-review: Externally peer-reviewed.

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