Original Article

Drug Eluting Vein Graft with Acetylsalicylic Acid–ticagrelor–unfractionated Heparin Complex Inhibits Early Graft Thrombosis

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Background: Bypass graft surgery remains an important treatment option for left main and multi-vessel coronary artery disease. Approximately 2% of saphenous vein grafts are lost immediately after the coronary artery bypass graft operations, and 12% are lost in the first month due to thrombosis.
Aims: The aim of this study is to administer one anticoagulant and two antiplatelet agents in a way that locally affects the vein graft before the bypass operation and to thereby analyse their effects on early graft thrombosis.
Study Design: Animal experimentation.
Methods: Since ticagrelor was used locally for the first time in this study, its efficacy with combinations of other drugs [only Acetylsalicylic acid (ASA), ASA and Ticagrelor, ASA-Ticagrelor-Unfractionated Heparin (UFH)] was examined on rats including control (untreated) and sham (pluronic gel) group (n=14 for each group). Before the tunica adventitia layer of the femoral veins was bypassed to the femoral artery, it was coated with the drug-eluting pluronic F-127 gel. The presence or absence of thrombus in the vein graft samples was recorded under light microscopy. In vein graft preparations where thrombus was detected, thrombus area (μm2) was calculated using Axiovision software. Immunohistochemical staining was performed with the anti-rat von Willebrand factor (VWF) polyclonal antibody kit.
Results: In terms of presence of thrombus in the preparations, the number of the preparations containing thrombus was significantly lower in the ASA+Ticagrelor+UFH group compared to the ASA, control and sham groups, according to the comparisons made on the 1st and 3rd day (p=0.001 and p=0.02). VWF staining was significantly lower in the ASA+Ticagrelor+UFH group compared with that in the other groups on the 3rd day (p=0.005).
Conclusion: Locally effective ASA-Ticagrelor-UFH complex has been shown to significantly reduce thrombus formation in vein grafts in this experimental model. Local administration of these drugs, which are routinely administered orally just before stent implantations, on the vein graft before the bypass is performed can prevent the loss of vein grafts due to thrombus, thereby reducing the mortality and morbidity of these patients.
Keywords: Acetyl salicylic acid, heparin, hydrogel, thrombosis, ticagrelor, vein bypass graft

Bypass graft surgery remains an important treatment option for left main and multi-vessel coronary artery disease (CAD) (1). Although the durability of arterial grafts obtained for a single lesion is good, vein grafts are still frequently used for multi-vessel disease, 80% of the grafts used in coronary artery bypass graft (CABG) operations are venous (2). Approximately 2% of saphenous vein grafts (SVG) are lost immediately after the CABG operation, and 12% are lost in the first month due to thrombosis (3). Although guidelines suggest using

multiple arteries for bypass vein, real-life data shows that multiple arteries could be used in only 9% of CABG operations performed in North America (4). Thus, it is emphasised that treatment modalities that will prevent clogging of the saphenous veins, which are still the cornerstones for multi-vessel bypass surgery, should be developed (5).

In percutaneous coronary interventions, it is emphasised that two antiplatelet and one anticoagulant agents are class 1 recommendations for preventing stent thrombosis, and they should be administered to the patient at the first contact with the loading dose to initiate the effect as soon as possible (1). The purpose of this study is to administer one anticoagulant and two antiplatelet agents in a way that locally affects the vein graft before the bypass and to thereby analyse their effects on early graft thrombosis.

MATERIAL AND METHODS

Experimental animals

The study was approved by the Institutional Animal Use and Care Committee of ... and was conducted in accordance with the Helsinki Declaration of World Medical Association recommendations on animal studies (Protocol no: 2018/02-01). Seventy 4–6-month-old Wistar albino male rats weighing between 300 and 350 g were used for this study. Standard rat feed and water were provided throughout the study. The rats were kept in a special steel cage at a temperature of 22 °C ± 2 °C in a special room, with a humidity of 50%–55% and a 12-h dark/light cycle.

Hydrogel and drugs

Pluronic F-127 (Sigma-Aldrich, Germany, lot no: BCBW5376), a hydrogel with controlled release for approximately 3 days, was selected as the local delivery medium. Hydrogel was prepared as described in the literature (6), and the gel preparation instructions in the product leaflet were followed. For this purpose, 2 g of pluronic F-127 was dissolved in 10 mL of dimethyl sulfoxide (DMSO) and heated at 40°C for 20 min to obtain 20% (w/v) stock solution. Acetyl salicylic acid (ASA) (Kimetsan, Turkey, KIM-ASA/01PG/180629) was chosen as the first antiplatelet agent of the study; 100 µg of ASA active agent was dissolved in 20% 1-mL pluronic-127 gel (pH 7.2) (7). Since there is no pro-drug that inhibits ADP receptors (8), ticagrelor (Sigma-Aldrich, Germany, lot no: 1390403), which can act locally, was chosen as the second antiplatelet agent; 100 µg of ticagrelor active agent was dissolved in 1 mL of 20% pluronic-127 gel (pH 7.2). Since ticagrelor was applied locally for the first time, the local dose was applied by calculating the respective amount of ticagrelor on gram tissue based on systemic ticagrelor administration performed previously in an experimental study by Preusch et al. who reported that they had used a systemic dose of 270 mg/kg (9). Our calculation of local dose was performed by considering the graft weight (0.37 mg); therefore, the local dose of ticagrelor was calculated to be 100 µg. Unfractionated heparin (UFH) (Polifarma, Turkey) was chosen as the anticoagulant. The UFH dose that was locally used in a previous study that prevented arterial thrombosis was taken as reference (10). Thus, 100 units/mL UFH was prepared in 20% pluronic-127 gel (pH 7.2).

Study groups

Since ticagrelor was used locally for the first time in this study, its efficacy with combinations of other drugs (only ASA, ASA and ticagrelor, ASA-Ticagrelor-UFH) was examined on rats including control (untreated) and sham (pluronic gel) group. Since ASA was shown to be effective locally in vein grafts (7) and we wanted to test whether drug combinations with ticagrelor have any superiority against ASA treatment alone. Wistar albino rats were randomised into five basic groups. The sample sizes of groups were determined according to the thrombus area data obtained from the study by Torsney et al. (7). The authors reported a reduction of 40% in the comparison of baseline and post-interventional results. Power analysis performed with 80% power and an alpha error of 5% according to those results revealed that groups should be comprised of at least 14 subjects.

Group I (control, n=14): Only bypass operation was performed and no local treatment was provided to this group. Tissue samples were obtained on the 1st day (n=7) and on the 3rd day (n=7).

Group II (n=14): ASA was locally applied to the vein graft on which bypass operation was performed. Tissue samples were obtained on the 1st day (n=7) and on the 3rd day (n=7).

Group III (n=14): ASA+Ticagrelor were locally applied to the vein graft on which bypass operation was performed. Tissue samples were obtained on the 1st day (n=7) and on the 3rd day (n=7).

Group IV (n=14): ASA+Ticagrelor+UFH were locally applied to the vein graft on which bypass operation was performed. Tissue samples were obtained on the 1st day (n=7) and on the 3rd day (n=7).

Group V (sham, n=14): Only pluronic gel was locally applied to the vein graft on which bypass operation was performed. Tissue samples were obtained on the 1st day (n=7) and on the 3rd day (n=7).

Anaesthesia protocol

Anaesthesia was induced by intraperitoneally administering ketamine hydrochloride (75 mg/kg) and xylazine (5–10 mg/kg) to the rats.

Bypass graft model

Bypass operation was performed by two experienced cardiovascular surgeons. Surgeons initiated the surgical procedure after establishing vision with a 3.5x magnification surgical loupe. To protect the rats from infection, preoperative cefazolin sodium (0.5g/kg) was intramuscularly administered. The modified bypass model was

performed by interpositioning the femoral vein to the femoral artery of the other leg using the end to side technique, as described previously (11). Before the tunica adventitia layer of the femoral veins was bypassed to the femoral artery, it was coated with the drug-eluting pluronic F-127 gel. This hydrogel, which is solid at room temperature due to its chemical properties, begins controlled release of the drug for three days at body temperature (6,7). Vein graft samples were obtained from the rats on the 1st and 3rd days after the procedure. Euthanasia was performed by cervical dislocation.

Histopathological evaluation

Histopathological evaluation was performed in the ... histology department by a histologist who was blinded to the experimental procedure. Vein graft tissue samples were fixed in 10% neutral buffered formalin for 48 h. After the fixation, dehydration, clearing and paraffin embedding, 5-micron-thick sections were obtained from the paraffin blocks using Rotary Microtome (Leica RM2125 RTS, Germany). Sections were placed on slides so that the endothelial surfaces of the vein grafts were facing up. The samples were evaluated under a light microscope (Zeiss, USA). Perivascular inflammation was graded by routine haematoxylin and eosin staining in tissue sections. Histopathological grading was performed by quantitative scoring with scores between 0 and 3. Scoring was performed as follows: No findings within the light microscopy field examined, 0; findings in less than 25% of the field, 1; findings in 25%–75% of the field, 2; and findings in more than 75% of the field, 3 (12). The presence or absence of thrombus in the vein graft samples was recorded under light microscopy. In vein graft preparations where thrombus was detected, thrombus area (μm^2) was calculated using Axiovision software (Zeiss, USA).

Immunohistochemical staining

Immunohistochemical staining was performed with the anti-rat von Willebrand factor (VWF) polyclonal antibody kit (Bioss, USA, lot no: AD071046) in accordance with the manufacturer's instructions. Immunohistochemical scoring of VWF was performed according to the staining intensity of the preparations (-, negative; +, positive; ++, strongly positive; +++, very strongly positive) (13).

Calculation of ADP receptor level in serum

Serum ADP receptor level was examined in the blood samples to check whether ticagrelor had entered systemic circulation in the current dose. Blood samples were obtained from the tail veins of each animal just before euthanasia. ADP receptor inhibition level was analysed using the P2YR12-ADP receptor Elisa kit (Sunredbio, China, lot no: 201904) in accordance with the manufacturer's instructions.

Statistical analysis

In summarising the data obtained from the study, descriptive statistics for continuous variables were presented as median-interquartile range according to data distribution. Categorical variables were presented as numbers and percentages. Normality of numerical variables was evaluated with One-sample Kolmogorov-Smirnov test. The Fisher–Freeman–Halton test was used for comparisons of perivascular inflammation, vWF staining and thrombus presence, while the Kruskal–Wallis test was used for comparisons of thrombus area and ADP receptor level between the groups. In the presence of significant differences with >2-group comparisons, post-hoc pairwise comparisons between groups were performed via the Bonferroni correction method. Jamovi [jamovi project (2018), Jamovi (Version 1.0.5)] and JASP Team (2018, Version 0.10.2) software were used for statistical analyses and a p-value of < 0.05 was considered statistically significant.

RESULTS

Histopathologic findings

Thrombus was observed in 4 (57%) preparations on day 1 and 4 (57%) preparations on day 3 in the ASA+T group, whereas thrombus was observed in only 2 (29%) preparations on day 1 and 4 (57%) preparations on day 3 in the ASA+T+UFH group. Moreover, all preparations in the other groups had thrombi on days 1 and 3 (p=0.001 for day 1, p=0.02 for day 3).

Considering the calculated thrombus areas for preparations that exhibited thrombus, the lowest median values were also observed in the ASA+T [day 1 (540.3 μm^2), day 3 (3522.7 μm^2)] and the ASA+T+UFH [day 1 (0 μm^2), day 3 (1092 μm^2)] groups, but there was no statistically significant difference between any of the groups (p=0.086 for day 1, p=0.174 for day 3).

In terms of perivascular inflammation, two preparations (29%) in the ASA+T+UFH group did not exhibit any inflammation on day 1, whereas all preparations had inflammation in the other groups. Moreover, none of the preparations exhibited intense inflammation (grade 3) in the ASA+T+UFH and ASA+T groups (p=0.004). One preparation (14%) did not exhibit any inflammation on day 3 in the ASA+T+UFH group. While none of the preparations exhibited intense inflammation in the ASA+T group, intense inflammation was observed in only one preparation (14%) in the ASA+T+UFH group (p=0.408) (Figure 1, table 1).

Immunohistochemical findings

No vWF staining was observed in one preparation (14%) in the ASA group, two preparations (29%) in the ASA+T group and five preparations (71%) in the ASA+T+UFH group on day 1, whereas all preparations exhibited vWF staining in the other groups. In addition, none of the preparations in the ASA, ASA+T, ASA+T+UFH groups exhibited very strong (+++) staining. None of the preparations were strongly stained (++)

in the ASA+T+UFH group, whereas all preparations exhibited strong staining in the other groups (p=0.004). There was no vWF staining in one preparation (14%) in the ASA group, three preparations (43%) in the ASA+T group and six preparations (86%) in the ASA+T+UFH group on day 3, whereas all preparations exhibited vWF staining in the other groups. None of the preparations in the ASA, ASA+T, ASA+T+UFH groups exhibited very strong (+++) staining and none of the preparations in the ASA+T and ASA+T+UFH groups exhibited strong (++) staining (p=0.005) (Table 1, figure 2).

ADP receptor levels

Considering the serum ADP receptor levels, the lowest median values were observed in the ASA+T [day 1 (88.2 pg/ml), day 3 (83.2 pg/ml)] and ASA+T+UFH [day 1 (82.7 pg/ml), day 3 (86.1 pg/ml)] groups, but there was no statistically significant difference between any of the groups (p=0.331 for day 1, p=0.793 for day 3).

DISCUSSION

The present study showed that the locally effective ASA-Ticagrelor-UFH complex reduces thrombus formation in vein grafts and results in less vWF staining compared to other treatment modalities. Studies have shown that there is intense platelet, neutrophil migration and fibrin accumulation into the vein wall in the first hours after anastomosis of the vein graft to the coronary artery, leading to intimal hyperplasia after treatment (14,15). Aiming to prevent this mechanism from the very beginning appears to slow down the subsequent processes (7). The absence of antiplatelet and anticoagulant agents during the first hours after CABG may be associated with thrombosis development (14). In a recent study, it has been shown that ASA, which is systemically administered, causes an increase in chest tube drainage and thus surgical re-exploration (16). Since systemic administration of drugs causes adverse events, researchers tried local administration of drugs to prevent SVG disease. In their study, Torsney et al. showed that local ASA application reduced thrombus formation in vein grafts. In this experimental model, they used hydrogels with up to three days of controlled release similar to the our study, and they showed that the local effect continued for approximately one month and that local ASA also reduced neointimal formation (7). In the present study, we found that the samples with the least thrombus formation were those containing the ASA-Ticagrelor-UFH complex.

Ticagrelor is not a pro-drug, it is reversibly bound to the ADP receptor and its efficacy is not affected by genetic polymorphism. Ticagrelor is the fastest agent that inhibits platelet aggregation. Its adenosine-dependent pleotropic effect is its most interesting feature (8). Grzesk et al. found that orally administered ticagrelor prevented ADP-dependent vascular smooth muscle cell contraction, unlike clopidogrel and prasugrel (17). Ticagrelor is also one drug among the ADP receptor inhibitors that reduces cardiovascular mortality without increasing bleeding complications after bypass operation (18). In the present study, no adverse effects of the drugs were detected in the subjects and no difference was noted between the groups in terms of serum ADP receptor levels at the local ticagrelor dose used in the study. Furthermore, we found that there were fewer thrombi in the ASA and ticagrelor combination treatment group. The superiority of the ASA and ticagrelor combination may be explained by pleotropic actions and the fact that ticagrelor had direct effects (because it is not a pro-drug) on tissues. Considering that the most frequently occurring pathogens in these graft infections are *S. aureus* and *S. epidermidis*, the antibacterial effect of local ticagrelor in addition to reducing thrombus formation may contribute to the reduction of graft infections as well (19,20).

Local use of UFH, the anticoagulant agent chosen for the present study, has been previously found to prevent arterial thrombosis. Andresen et al. showed that local heparin reduced arterial thrombosis in microvascular surgery more than IV heparin and that no significant difference was noted between the control group in terms of serum aPTT levels (10). vWF is an important molecule that plays a significant role in thrombus formation, is used as an endothelial activation marker and predicts mortality in patients with cardiovascular events and especially previously known CAD (21). Furthermore, some studies have found that vWF levels are a marker of damage to the vein during harvest (22). In a study by Meyer et al., strong immunohistochemical staining was determined in endothelium of the almost all SVGs for factors VIII:vWF (23). In the present study, vWF staining was at significantly lower levels in the ASA-Ticagrelor-UFH group compared to the other groups; furthermore, there was a continuous decrease in the levels of staining from the untreated group to the three-drug combination group.

Wu et al. showed that resolvin D1 loaded on a hydrogel reduced neointimal hyperplasia in vein grafts (24). Although there are many experimental studies, the only human trial has been conducted with edifoligide; however, no significant difference was noted between the two groups in terms of SVG loss (25). Drug-eluting stents are loaded with agents that reduce intimal hyperplasia (26); however, since reducing thrombus burden is also known to reduce future intimal hyperplasia (7), it appears more reasonable to first develop local treatment modalities to reduce thrombus formation to prevent SVG disease. The difference of our study from these studies is that it targets thrombus formation, the first mechanism of graft loss, and includes two antiplatelet one anticoagulant regimen, similar to the guidelines aimed at preventing stent thrombosis.

This method is cost-effective due to the very low amount of hydrogel used and very low drug doses administered. Moreover, hydrogels have been used for controlled delivery of drugs in the pharmaceutical industry for many years (6). This method is also practical, as we described in the method section. Drugs can be

prepared in hydrogel beforehand and the adventitia layer of the vein graft can be coated with hydrogel containing drugs before the bypass.

This study has some limitations. Since a hydrogel with controlled release for three days was chosen as the local distribution tool in the present study, tissue and blood samples were obtained from the subjects only on the 1st and 3rd days. Since the effect of drug combinations was investigated, subjects were randomised to too many groups. The effect of drugs on thrombus development could not be investigated in consecutive weeks for up to one month due to ethical reasons since the number of subjects would increase too much. We could not examine histopathologic markers other than vWF staining because of funding limitations.

In conclusion, locally effective ASA-Ticagrelor-UFH complex has been shown to significantly reduce thrombus formation in vein grafts in this experimental model. Local administration of these drugs, which are routinely administered orally just before stent implantations, on the vein graft before the bypass is performed can prevent the loss of vein grafts due to thrombus, thereby reducing the mortality and morbidity of these patients.

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Table 1. Comparison of perivascular inflammation, thrombus area, vWF staining, ADP receptor inhibition level and presence of thrombus between the groups.

			Group					
			Untreated (n=14)	**ASA (n=14)**	**ASA+T (n=14)**	**ASA+T+UFH (n=14)**	**Pluronic Gel (n=14)**	**p**
		Perivascular inflammation n, (%)						**0.004**
Day	**1 Day**	Grade 0	0 (0)	0 (0)	0 (0)	2 (29)	0 (0)	
		Grade 1	0 (0)	2 (29)	3 (43)	3 (43)	0 (0)	
		Grade 2	1 (14)	3 (43)	4 (57)	2 (29)	4 (57)	
		Grade 3	6 (86)	2 (29)	0 (0)	0 (0)	3 (43)	
		Thrombus area *(µm²), Median [IQR]*	12083 [764- 111806]	728.4 [63.3- 80208.6]	540.3 [0- 60012]	0 [0- 30120]	9446.2 [3525.2- 100619.4]	0.086
		vWF staining n, (%)						
		-	0 (0)	1 (14)	2 (29)	5 (71)	0 (0)	**0.004**
		+	0 (0)	2 (29)	2 (29)	2 (29)	1 (14)	
		++	3 (43)	4 (57)	3 (43)	0 (0)	4 (57)	
		++	4 (57)	0 (0)	0 (0)	0 (0)	2 (29)	
		ADP receptor levels *(pg/ml), Median [IQR]*	119.7 [63.6- 159.9]	106.5 [86.7- 113.7]	88.2 [83- 90.6]	82.7 [75.0- 97.6]	94.6 [81.1- 137.9]	0.331
		Thrombus presence n, (%)	7 (100)	7 (100)	4 (57)	2 (29)	7 (100)	**0.001**
		Perivascular inflammation n, (%)						0.408
	3 Days	Grade 0	0 (0)	0 (0)	0 (0)	1 (14)	0 (0)	
		Grade 1	1 (14)	3 (43)	4 (57)	4 (57)	1 (14)	
		Grade 2	4 (57)	2 (29)	3 (43)	1 (14)	4 (57)	
		Grade 3	2 (29)	2 (29)	0 (0)	1 (14)	2 (29)	
		Thrombus area *(µm²), Median [IQR]*	31028 [6656- 66919]	395[164.75- 60813.48]	3522.7 [0- 19559.28]	1092 [0- 20726]	28010.2 [864.43- 50278.32]	0.174
		vWF staining n, (%)						
		-	0 (0)	1 (14)	3 (43)	6 (86)	0 (0)	**0.005**
		+	3 (43)	3 (43)	4 (57)	1 (14)	4 (57)	
		++	3 (43)	3 (43)	0 (0)	0 (0)	1 (14)	
		+++	1 (14)	0 (0)	0 (0)	0 (0)	2 (29)	
		ADP receptor levels *(pg/ml), Median [IQR]*	93 [50.58- 192.88]	103.7 [76.72- 116.48]	83.2 [74.53- 91.51]	86.1 [80.62- 92.73]	76.5 [68.26- 169.63]	0.793
		Thrombus presence n, (%)	7 (100)	7 (100)	4 (57)	4 (57)	7 (100)	**0.020**

-: negative; +: positive; ++: strongly positive; +++: very strongly positive. ASA: Acetylsalicylic Acid, T: Ticagrelor, UFH: Unfractionated Heparin. Descriptive statistics were presented as median (IQR) for variables without normal distribution and Kruskal–Wallis test was used. Descriptive statistics were given as numbers (%) for categorical variables and Fisher Freeman Halton test was used. P values indicated in bold are statistically significant (P < 0.05).

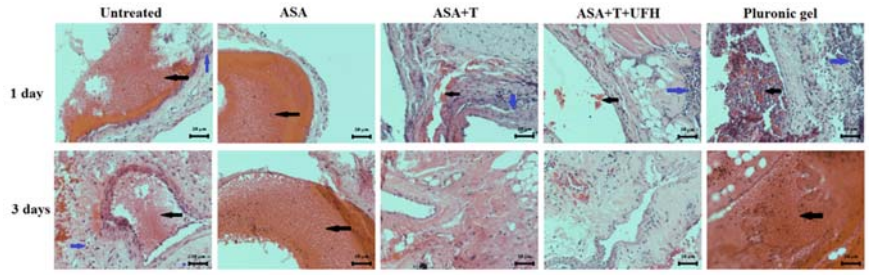

FIG. 1. Haematoxylin eosin stained microscopic images of the study groups on the 1st and 3rd day. After routine histopathological evaluation, perivascular inflammation is indicated by blue arrows and thrombus formation is indicated by black arrows. It was observed that perivascular inflammation and thrombus formation were significantly reduced with the addition of Ticagrelor (T) to Acetylsalicylic acid (ASA). From a general point of view, the lowest rate of perivascular inflammation and thrombus formation was observed in the group that contained ASA+T+Unfractionated-heparin(UFH) (Magnification x200).

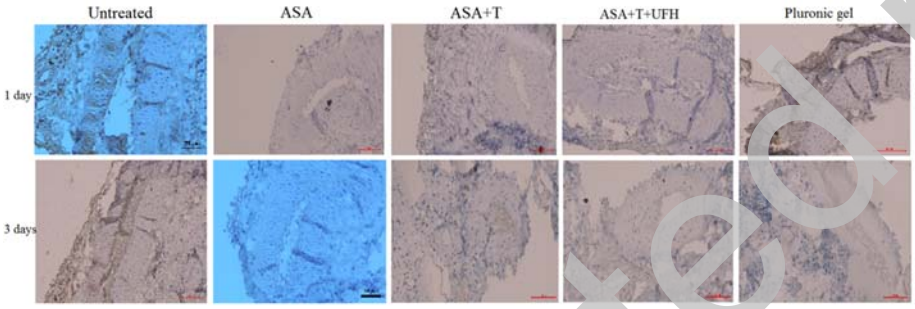

FIG. 2. Von Willebrand factor (vWF) immunohistochemical staining of the study groups on the 1st and 3rd day. It was observed that vWF staining was lower in the locally acting drug group compared to that in the untreated and pluronic gel groups. From a general point of view, the lowest staining was observed with preparations containing Acetylsalicylic-acid(ASA)+Ticagrelor(T)+Unfractionated-heparin(UFH) (Magnification x200).