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Original Article

Effects of rivaroxaban and apixaban on intimal hyperplasia in rabbits

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Abstract

Aim: Intimal hyperplasia causes vascular occlusion and its optimal treatment is unknown. In this study, we evaluated the effectiveness of Apixaban and Rivaroxaban treatment for preventing intimal hyperplasia in rabbits.
Material and Methods: The rabbits (n = 15) were randomly divided into three groups. Reanastomoses are applied to the carotid artery. All groups received 100 U/kg heparin sodium during the operation period. Group A (n = 5) as a control group had no medication. Group B (n = 5) was given Rivaroxaban 3 mg/kg/day. In-group C (n = 5) Apixaban was administered per orally 10 mg/kg. At the end of the treatment on the 28th day, carotid artery specimens were excised and evaluated histologically.
Results: Increased intima thickness was observed in the control group than the drug groups (P=0.019, P=0.007). It was found that there was no difference between groups in terms of lumen diameter, lumen area, tunica media area and tunica media thickness. There was difference between groups in terms of caspase 3 or TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) staining ($p<0.05$).
Conclusion: Apixaban and Rivaroxaban may have protective efficacy against intimal hyperplasia after vascular surgical intervention.

Keywords: Rivaroxaban, apixaban, intimal hyperplasia, thromboprophylaxis, factor Xa inhibitor

INTRODUCTION

Thromboembolic events such as acute coronary syndrome (ACS), stroke, deep vein thrombus, and pulmonary embolism are serious causes of death in western countries. Anticoagulant drugs are mainly used in atrial fibrillation (AF), in the presence of mechanical valves, in the case of intracardiac thrombus, deep vein thrombosis-embolism, ischemic attack, or stroke. Warfarin has been used as an anticoagulant for over 60 years. Unfortunately, narrow therapeutic range and drug interactions

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limit the use and cause dose-tracking difficulties. These reasons led to the search for new drugs instead of warfarin.

In 2010, new non-vitamin K oral anticoagulants (NOACs) drugs Rivaroxaban, Apixaban, Edoxaban have been put into clinical use. NOACs are divided into two groups. The first group is Dabigatran-direct thrombin inhibitor- and the second group is consisted of Edoxaban, Rivaroxaban and Apixaban effect directly via factor Xa (FXa). In addition, FXa triggered by tissue factors because of vascular plaque rupture, affects the conversion of prothrombin to thrombin because of catalysis [1-5]. Dabigatran inhibits direct thrombin, unrelated to vitamin K. Rivaroxaban, Apixaban and Edoxaban inhibit via FXa and provide anticoagulation. Apixaban is a direct FXa inhibitor. It shows breakthrough in the hepatic way. The half-life is about 8-15 h [3]. Rivaroxaban has a direct factor Xa inhibitor effect either. It eleminates by kidneys. Its elimination half-life is around 5-9 h [4-5]. Rivaroxaban is the only NOAC agent that studied for phase III ACS. Their advantages involve no need for close monitoring. Also drug-drug or pharmacovigilance interactions are less in reference to warfarin.

The risk of restenosis in interventional procedures varies between 30 and 50 % in the first 6 months [1-4]. In general and orthopedic surgeries, the incidence of venous thromboembolism (VTE) is reported to be 15%-60% in the absence of thromboprophylaxis [1-4]. However, this rate decrease approximately 70% with the use of thromboembolic prophylaxis [4-7]. Intimal hyperplasia and smooth muscle cell proliferation play an important role in restenosis. Apixaban and Rivaroxaban have similar affinity for human and rabbit FXa [7-12]. Various experimental studies therefore conducted in this manner. For instance, Wong et al. [12-13] compared the effects of FXa inhibitors Apixaban, Rivaroxaban and direct thrombin inhibitor Dabigatran in a rabbit model. They studied in a rabbit model because of the similar effect between rabbit and human-like factor Xa. Apixaban in rabbits showed a good correlation between concentration and inhibition of FXa activity [12-13]. Apixaban shows a similar affinity for human and rabbit FXa as rapid onset of action or pharmacodynamics response. Also, Apixaban produces rapid hepatic metabolism in rabbits [12-13].

Rabbits are widely used in the development of the thrombosis model. In this study, the protective effect of Rivaroxaban and Apixaban was evaluated.

In this study, we aimed to investigate the effect of Factor Xa inhibitors on intimal hyperplasia and endothelial cell death, which play a role in the pathogenesis of vascular stenosis and thrombosis.

MATERIALS AND METHODS

A randomized experimental study was conducted at the animal laboratory of the University, with the approval of the ethics committee for animal research on June 10, 2020; protocol number: 20/2020. The surgeries were performed by the investigators at the institution in accordance with the "Principles of Laboratory Animal Care". The rabbit carotid artery model was chosen due to being an easily applicable and reproductive anastomosis model when compared to the rat model (3 R Principle) [2,26,29]. The rabbits that served as the control group, these animals underwent sham surgery to replicate the same handling and treatment protocols used for the rabbits that received carotid artery transection and re-anastomosis. It is important that conditions for the control animals replicate the treatment of the animals undergoing actual carotid artery surgery as much as possible to minimize variables that could impact experimental outcomes.

In addition, rabbits were selected for the experiment due to the suitability of the long artery segment and histological cross-section diameter. Rabbits were provided from the animal research center and housed in this laboratory under standard conditions. This was a 12 h dark/light cycle at the appropriate temperature (24±2°) and humidity (55-60%) was maintained. Standard nutrition was applied. These conditions were maintained throughout the experiment for 28 days. If such a protective effect of the drugs on intimal hyperplasia was expected, only a one-time point for follow-up was sufficient to detect [1-2]. Two investigators blind to rabbit's treatment performed all histological analyses described above. In addition, investigators took care of the rabbits twice a day during the experimental study.

Experimental groups

Fifteen male New Zealand rabbits (2000-3000 grams; age 10-12 weeks) were divided into 3 groups randomly. The experiment was performed on rabbits according to the guidelines provided by the experimental animal laboratory. All groups received 100U/kg heparin sodium during the operation period. Group A (n=5) set as a control group had no drug. Group B (n = 5), Rivaroxaban (Xarelto, Bayer, Germany) dispersed in 2 mL water was administered per orally with a 3 mg/kg/day gavage for 28 days. Also, 10 mg / kg (2.5 mg / ml) Apixaban (Pfizer, US) per orally in 2 ml water was administered to group C (n = 5). Drugs were given according to the dose stated in the literature during the experiment (2-3). The ear vein was used for vascular access during the experiment. Right-sided rabbit carotid arteries were transacted and re-anastomosed with 8.0 polypropylene sutures with running suture technique. At the end of the 28th day, all rabbits were sacrificed. The carotid artery segments were removed and prepared for histological and immunostaining examination.

Carotid Artery Surgery Injury Restenosis Model

Intramuscular ketamine hydrochloride 50 mg/kg (Ketalar, Pfizer) and xylazine hydrochloride 5 mg/kg (xylazine, Bayer) were used for anesthesia. Rabbits were operated under ketamine and xylazine anesthesia only, without intubating. Cefazolin sodium 50 mg/kg (Science Pharmaceutics) was used intramuscularly 1 h before the operation to prevent infections. Surgical loops were used during the interventions. The rabbits were first supinely placed during the surgical preparation process. Bleeding control was performed by using two silk 3/0 sutures at 2 cm intervals in the common carotid. The artery was surgically exposed near the trachea and approximately 1 cm excised for operation. A longitudinal incision of approximately 1 mm in length was made in the right common carotid artery using a surgical iris blade. The carotid arteries were transacted and re-anastomosed with 8.0 polypropylene in the continuous suture technique. Mean duration time of reimplantation was five minutes. At the end of the operation, sutures were released to provide blood flow again. The maintenance of arterial blood flow after the procedure was preserved with this technique. Skin closure was performed using 2.0 polypropylene. Animals were hospitalized under experimental conditions. During the experiment, the rabbits were monitored daily for the neurological signs Mancinelli described, including head tilt, circling, ataxia, paresis or paralysis, nystagmus and seizures. No neurological signs or symptoms were observed in the follow-up. Sacrifice was conducted with high doses of anesthesia. Finally, the specimens were obtained for histological examination on the 28th day.

Histological evaluation

Carotid arteries dissected from rabbits were placed in 10% buffered formaldehyde for fixation. After the tissues underwent a routine tissue follow-up procedure, embedding in paraffin blocked them. Then, 5-micrometer sections were taken from paraffin blocks with rotary microtome on a lysine slide microtome (RM 2255, Leica Instruments, Germany). The sections taken were stained with haematoxylin eosin and Masson's trichrome stain for morphometric analysis (figure 1-2-3: histological sections of group A-B-C (haematoxylin + eosin stain and Masson's trichrome stain).

Images were obtained from the selected areas using a computer-assisted image analysis system consisting of a microscope (Olympus BX-51, Japan) equipped with a video camera (Olympus DP70, Japan). In histomorphological measurements, lumen diameters, lumen areas, intima, media thicknesses were measured, and UTHSCA Image Tool (software version 3.0, University of Texas Health Science Center, USA) program was used to compare the groups in terms of these parameters.

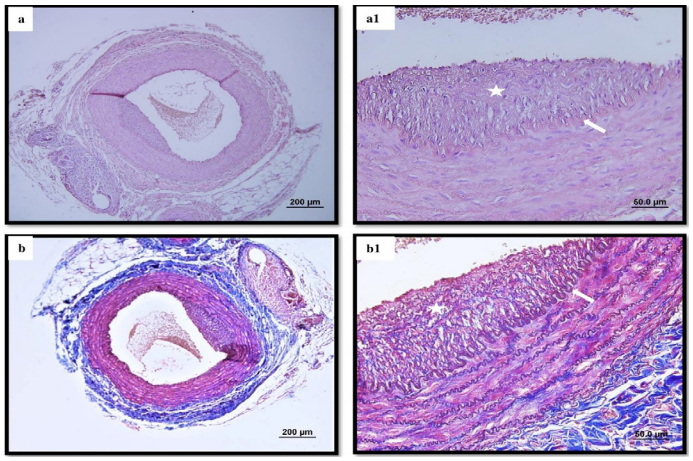

***Figure 1**. Histological sections of group A (hematoxylin + eosin stain and Masson's trichrome stain, 10X and 40X): a-10X and a1- 40X; hematoxylin + eosin stain. B-10X and b1-40X Masson's trichrome stain. Internal elastic lamina marked with white arrow and intimal hyperplasia marked with white star.*

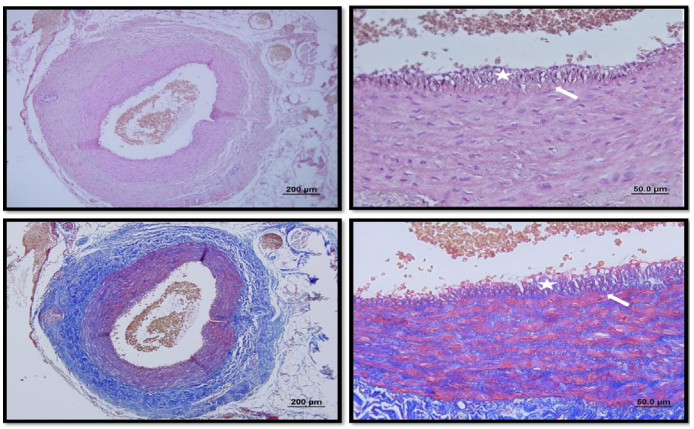

***Figure 2**. Histological sections of group B (hematoxylin + eosin stain and Masson's trichrome stain, 10X and 40X): a-10X and a1- 40X; hematoxylin + eosin stain. B-10X and b1-40X Masson's trichrome stain. Internal elastic lamina marked with white arrow and intimal hyperplasia marked with white star.*

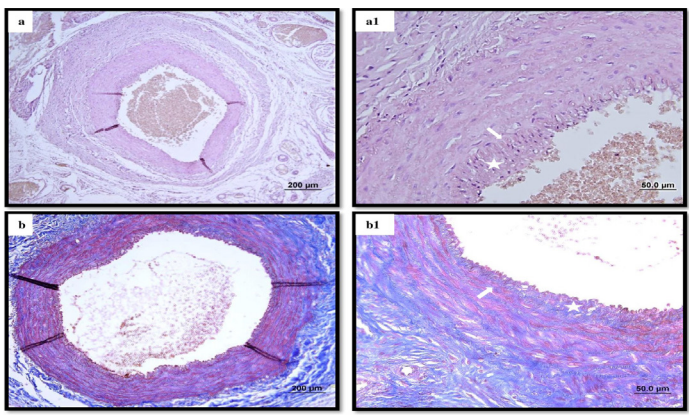

***Figure 3**. Histological sections of group C (hematoxylin + eosin stain and (Masson's trichrome stain, 10X and 40X): a-10X and a1- 40X; hematoxylin + eosin stain. B-10X and b1-40X Masson's trichrome stain. Internal elastic lamina marked with white arrow and intimal hyperplasia marked with white star.*

Immunohistochemistry

The sections taken on a lysine slide using a rotary microscope were marked using the streptavidin-avidin-biotin method for immunohistochemistry analysis. The sections taken on lysine-coated slides were kept in a 60-degree oven overnight and then passed through xylol series and deparaffinized. Then, the sections that were rehydrated by passing through alcohol series were taken into distilled water. They were then treated with 10 mM citrate buffer (Cat No.AP- 9003-125 Labvision) at 95°C for 5 min to unmask antigens by heat treatment. Then the sections were circumscribed with Dakopen (Glostrup, Denmark) and incubated in a 37-degree oven for 15 min with 3% H_2O_2 to inhibit endogenous peroxidase activity. Then, the sections were incubated with normal serum-blocking solution for 30 min and incubated with primary antibodies (Alpha-smooth muscle actin (α-SMA) (ab7817, Abcam), Caspase-3 overnight in a humid chamber. The next day the sections were washed with phosphate buffered saline (PBS) and primary antibodies removed and followed by incubation with biotinylated IgG and then with streptavidin–peroxidase conjugate (Invitrogen, Histostain-Plus Kit Broad Spectrum). After the sections were washed 3 times with PBS, the sections were incubated with 3,3'-diaminobenzidine (DAB) for 2 min (1718096, Roche, Germany) to detect immunoreactivity. Finally, after staining the sections with Mayer's haematoxylin for 10 seconds, the sections were covered with the help of entellan. All sections were digitally photographed and archived.

TUNEL

In Situ Cell Death Detection Kit Roche was used for this technique. After the sections were kept in an oven at 60 ° C for one night, they were made transparent with three changes of xylene for 30 min each. Then, it was rinsed in distilled water by providing dehydration with decreasing alcohol series. After drawing around the area to be painted with a pappen, it was kept in a sodium citrate solution in the microwave at 600 watts for 6 min It was left for 20 min to cool. It was then washed 3 times with PBS. After each wash, excess water around the tissue was removed with blotter paper. Then, the sections were kept with 3% H_2 %O_2 for 10 min to block endogenous peroxidase activity. The sections were washed again with PBS and kept for 10 min with an equilibration buffer. Then the sections were incubated with TdT enzyme solution at 37 C in the oven for 1 h. Afterward the sections were washed with PBS were kept for 10 min with working the strength stop/sash buffer. Sections washed with PBS were stained with DAB to determine the visibility of the TUNEL reaction. After washing with distilled water, the sections stained with Mayer's haematoxylin were subjected to dehydration with increasing alcohols. After the transparent process with xylene, they were closed with entellan.

Statistical Analysis

The optimal sample size for both scientific validity as well as animal welfare was concluded in accordance with the recommendations of animal research committee. We followed the principles of The ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments) during the study. We used simple randomization method; consequently, random numbers were generated using the standard (RAND) function in Microsoft Excel. Infusions of drugs, surgical process as well as data analyse were performed in a blinded fashion (surgeons, histologists and animal caretakers). Deblinding was established following data collection and analysing.

"Statistical Package for Social Sciences" (SPSS 23, Chicago, IL, USA) and Software Excel (Microsoft-USA) program were used for all statistical analyses. Whether the data were normally distributed or not was evaluated using the Shapiro Wilk test of normality. We observed that the dataset did not show normal distribution in 3 groups consisting of five rabbits, therefore, Mann-Whitney U test and correlation analysis was applied to the data set as a non-parametric test. A p value of less than 0.05 was considered significant. Research data were processed in heparin, Apixaban and Rivaroxaban groups. All values are expressed as mean ± standard deviation. A grading system was used to score the α-SMA distribution, anti-caspase-3 positive and Tunnel staining amount in the cross-section. The score was defined as follows: 1, little positive staining; 2, positive staining was moderate and ranged from grade 1 to grade 3; 3, strong positive staining evenly distributed throughout the image, and 0, no immunoreactivity. The primary endpoint of this study was defined as the thickness of intimal hyperlasia at the end of follow-up, measured by immunohistochemical examination.

RESULTS

Differences in lumen diameter, lumen area, tunica intima thickness, tunica media area and thickness between all groups were evaluated histochemically. At this point, a significant difference in the thickness of the tunica intima was observed ($p<0.05$). In this context, the intima thickness was greater control group. Considering the obtained data, there was no difference between the groups in the comparisons of lumen diameter, lumen area, tunica media area and thickness ($p=0.251$, $p=0.347$, $p=0.175$, $p=0.754$) (Table 1: Statistics data of luminal diameters, luminal and tunica media areas and tunica intima thickness values).

When the group A was compared with group C, it was shown that TUNEL staining-positive cells increased significantly ($p = 0.019$). When the group A was compared with group B, it was shown that TUNEL staining-positive cells increased significantly in control group ($p = 0.007$). No significant difference was found between

the Apixaban and Rivaroxaban groups (p = 0.176) (figure 4: histological sections of group A-B-C, TUNEL staining).

In addition to TUNEL staining, active caspase-3 staining was performed to identify apoptotic cells. The research data were discussed in 3 groups as control and medicated groups (figure 5: histological sections of group A-B-C, caspase-3 staining). When the control group was compared with the Apixaban group, it was shown that the caspase 3 positive cells increased significantly in the control group (p = 0.023). When the control group was compared with the Rivaroxaban group, it was shown that the caspase 3 positive cells increased significantly in the control group (p = 0.04). No significant difference was found between the Apixaban and Rivaroxaban groups (p = 0.866). Anti-α-SMA antibody staining was performed to determine the α-SMA distribution in the cells. When all groups were compared, no difference was found between the groups in terms of α-SMA distribution (figure 6: histological sections of group A-C, α-SMA staining).

Table 1. Statistics data of luminal diameters, luminal and tunica media areas and tunica intima thickness values. [(Abbreviations: Alpha-smooth muscle actin (α-SMA), Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)]

	LUMEN AREA	LUMEN DIAMATER	TUNICA MEDIA AREA	TUNICA MEDIA THICKNESS	TUNICA INTIMA THICKNESS	TUNEL TEST	CASPASE 3	α-SMA
GROUP A-C	P=0.251	P=0.347	P=0.175	P=0.754	P=0.009	P=0.019	P=0.023	P=0.226
GROUP A-B	P=0.917	P=0.917	P=0.347	P=0.602	P=0.009	P=0.007	P=0.040	P=0.053
GROUP B-C	P=0.465	P=0.117	P=0.754	P=0.465	P=0.117	P=0.176	P=0.866	P=0.592

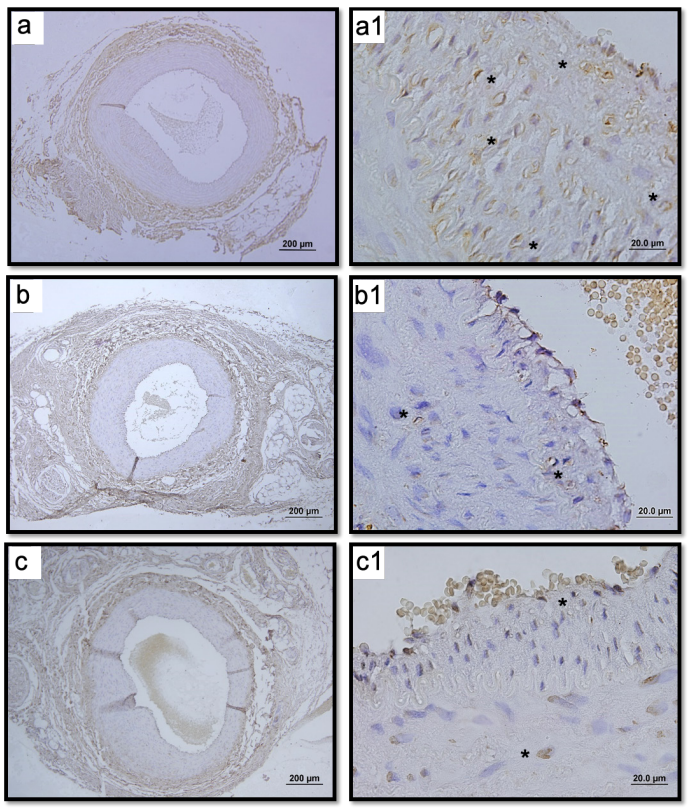

***Figure 4**. Histological sections of groups (TUNEL staining, 10X and 100 X): a (10X) and a1 group A (100X), b (10X) and b1 group B (100X), c (10X) and c1 group C (100X), TUNEL staining. TUNEL positive cells marked with black star.*

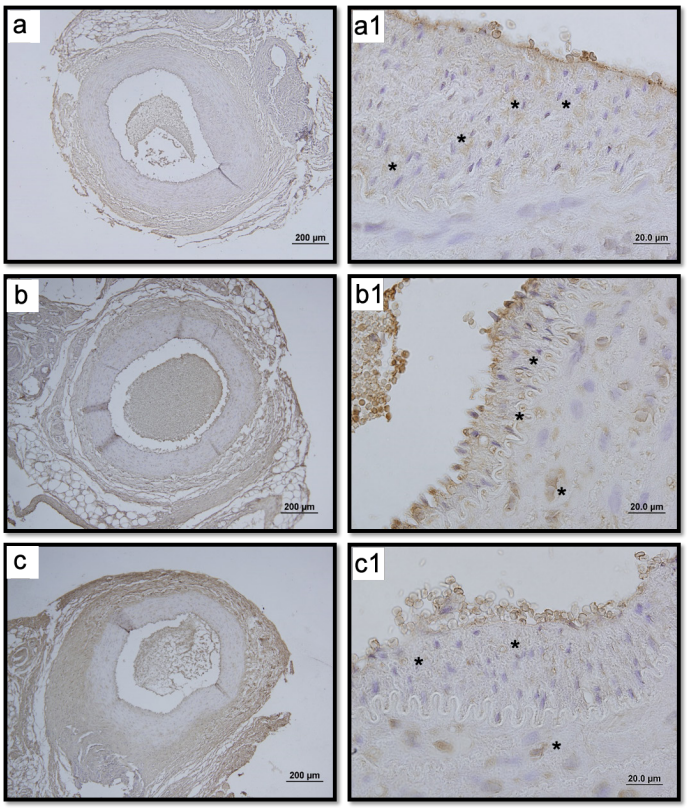

***Figure 5**. Histological sections of groups (Caspase 3 staining, 10X and 100 X): a (10X) and a1 group A (100X), b (10X) and b1 group B (100X), c (10X) and c1 group C (100X), Caspase 3 staining. Caspase 3 positive cells marked with black star.*

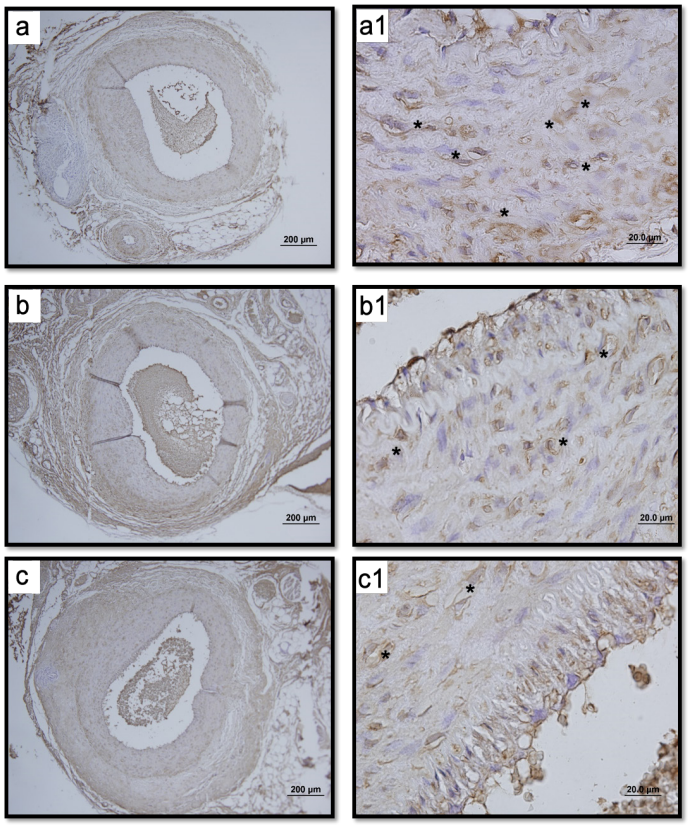

***Figure 6**. Histological sections of groups (a-SMAstaining, 10X and 100 X): a (10X) and a1 group A (100X), b (10X) and b1 group B (100X), c (10X) and c1 group C (100X), a-SMA staining. a-SMA positive cells marked with black star.*

DISCUSSION

In this study, we compared the effects of two Factor Xa inhibitors, Rivaroxaban and Apixaban, on the neointimal hyperplasia model in rabbit carotid artery, unlike the literature. Both factor Xa inhibitors showed a positive effect on apoptotic death of endothelial cells and prevention of neointimal hyperplasia without being superior to each other.

The studies regarding the anticoagulant effects of NOAC's mostly focused on venous diseases in past decade. Waldemar et al.[15] followed prospectively 750 patients with acute cancer-associated venous thromboembolism. They emphasized that non-major bleeding rate was higher in Rivaroxaban compared to Apixaban when used in standard doses. Also, they found that the recurrence of VTE and major bleeding was same. Following the favorable results with no additional adverse effects, similar studies have been conducted. Dawid et al. [16], underlined that VTE recurrence and bleeding rates for Rivaroxaban, Apixaban and Enoxaparin in atypical locations were not different. Niklas et al. [17], conducted the study of comparative effectiveness and safety of NOACs. They emphasized that there were no statistical differences in risk of stroke, systemic embolism or major bleeding among Apixaban, Dabigatran and Rivaroxaban in non-valvular atrial fibrillation (NVAF) [17]. In these circumstances, such studies highlighting the efficiency and reliability of the NOAC's lead to wider indications. Nevertheless, further studies are required on the comparability of individual NOACs [18].

Cohen et al. [19] conducted a review and indicated that the NOAC's offer clinical benefits over conventional therapy for VTE-related death. Marco et al. [20] found a better safety profile with Apixaban compared to warfarin and NOACs in reducing any thromboembolic events. In addition, they underlined that Rivaroxaban was associated with increased quality-adjusted life years [20]. Although NOAC's have not been investigated in terms of arterial diseases in the early era, further studies focused on this subject. Several studies assessing the risk of cardiovascular death, stroke, and MI (Compass ACS study) reduced the usage of Rivaroxaban [8]. Another study showed that in patients who underwent lower limb revascularization for symptomatic peripheral artery disease (PAH) the frequency of amputation decreased with the usage of Rivaroxaban (Voyager study) [9]. In these circumstances, Rivaroxaban indicated the PAH in recent. In addition, low-dose Rivaroxaban had been reported to reduce heart failure compared to placebo (Commander study).

On the other hand, clinical studies have shown that NOACs are beneficial in reducing VTE recurrence or stroke in AF with a lower risk of intracranial bleeding [18-21]. Marwan Sheikh-Taha [21] evaluated clinical hemostasis in patients presenting with major bleeding using Apixaban or Rivaroxaban. In the study in which 29 patients were included, the intracranial hemorrhage (ICH) rate was found to be 72.4%. We assessed the achievement of clinical hemostasis in rabbits using Apixaban and Rivaroxaban and there was no increased risk of bleeding. The significance of adding NOACs to medical therapy in cardiovascular disease remains controversial.

Apixaban and Rivaroxaban are being increasingly used in routine clinical practice for thromboembolic conditions worldwide [13-21]. However, the protective effect against restenosis is currently unknown between the two drugs. Vascular healing is beyond a simple endothelial proliferation process and ensues following activation of various systems and induction of biochemical agents. Coagulation cascade is one of these components and anticoagulation leads to somewhat blockade this process. Nonetheless, certain underlying mechanism has not been clearly identified. Histopathological and experimental studies may evince this process, thus lead to further randomized human trials in this field. We therefore compared the effectiveness of Apixaban and Rivaroxaban in prevention of neointimal hyperplasia and endothelial cell proliferation in rabbit. For this purpose, TUNEL staining and active caspase-3 staining were performed to identify

apoptotic cells. TUNEL staining was performed to determine the DNA fragmentation in the cells. TUNEL and active caspase-3-positive cells in the control group increased significantly. This significant difference indicates increased endothelial cell death in the control group. There is increasing interest in research to inhibit endothelial cell death to reduce endothelial damage. Caspase 3 and TUNEL are markers of apoptosis in intimal hyperplasia studies, and a decrease in Caspase 3 level is beneficial [24,25]. When evaluated together, the low Caspase 3 and TUNEL levels in both drug groups in our study may provide endothelial protective benefit with antiapoptotic effect. In the study of Akkaya et al. [2], Rivaroxaban showed a protective effect against intimal hyperplasia. Our study, unlike that study, contains two Factor Xa inhibitors and reveals a protective effect without creating superiority to each other, suggesting the effect that can be attributed to the Factor Xa class. Moreover, Akkaya et al. [2], focused on histological results without examining possible mechanisms of action. In our study, apoptosis markers were used as a possible mechanism of action. With the significance of the results, it was thought that these drugs provided protection with their antiapoptotic effect.

Intimal hyperplasia is characterized by an increase in the tunica intima after vascular injury and can progress to vascular occlusion over time. Moreover, IH emerges as an important problem after open surgery or endovascular intervention. Introducing the targets in the pathophysiology and treatment options will provide more successful long-term results [23]. In our study, the intima thickness was higher in the control group. Therefore, comparable intimal efficacy in preventing restenosis due to injury was observed between Apixaban and Rivaroxaban. Rivaroxaban possessed improved anti-inflammatory and vascular tone modulatory effects with lipopolysaccharide (LPS)-induced acute vascular inflammatory response in experimental models [22]. We interpreted these protective effects of the drugs in favor of their inflammation-reducing effects.

A study comparing bemiparin and Dabigatran, which is not a factor Xa inhibitor but one of the direct oral anticoagulants, may show that although Dabigatran prevents thrombosis angiogenesis, it cannot prevent intimal hyperplasia [26]. In addition, rivaroxaban's inhibitory effects on angiogenesis were also observed, as well as neovascularization inducing effects [27-28]. On the other hand, Bivalirudin, another direct thrombin inhibitor used intravenously, has a protective effect against intimal hyperplasia in rabbit carotid artery [29]. When evaluated together with our study, the preventive effect of intimal hyperplasia observed in Rivaroxaban and Apixaban may be related to the factor Xa inhibitor drug group rather than the Direct Oral Anticoagulant Group. Apoptosis marker results may indicate one of the mechanisms of action of rivaroxaban, which has beneficial effects in peripheral arterial disease. At the same time, similar results obtained in the study may give an idea that apixaban may be useful in this disease. An extensive study that would include other Factor Xa inhibitors could better reveal the linkage of drug class. Herein, we have demonstrated improved clinical benefits with Rivaroxaban vs. Apixaban in this experimental study. This study has also revealed that apoptotic cell death was significantly increased in the control group than in NOAC-treated groups.

Conclusion

Our hypothesis is consistent with the new findings of recent clinical trials. Rivaroxaban and Apixaban are the most commonly used FXa inhibitors anticoagulants because of their favorable pharmacological profiles.

Highlights

These drugs proved themselves in PAH and major cardiovascular events. In the light of further histological evaluations, Apixaban and Rivaroxaban were found to be protective against intimal damage either.

Primary limitation

Oral use has low bioavailability and rapid clearance in rabbits. It turns into more than one metabolite, such as m1 m2 m14, and it is not clear which of them creates how much activity. In the lack of sufficient budget, we were not able to attach and evaluate the oxidative stress parameters in this study. Further investigations are therefore needed to confirm the clinical significance.

Ethics Committee Approval: A randomized experimental study was conducted at the animal laboratory of the Dokuz Eylül University, with the approval of the ethics committee for animal research on June 10, 2020; protocol number: 20/2020. The study was conducted in accordance with the principles of the Declaration of Helsinki.

Patient Consent for Publication: Individual informed consent was waived due to retrospective design.

Data Sharing Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Author Contributions: All authors contributed equally to the article.

Conflict of Interest: The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

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