Effects of Antithrombogenic Agents on Microvenous Anastomoses in a Rat Model

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Objective: To compare venous thrombosis rates among animals treated with aspirin, clopidogrel bisulfate, and ketorolac tromethamine using an anastomotic “tuck” model.

Design: Single-blind randomized animal study.

Setting: An animal laboratory at a tertiary care academic referral center.

Subjects: Forty-two retired Lewis breeder rats divided into 3 equal groups.

Interventions: Before surgical intervention, 1 group received aspirin (10 mg/kg) through gavage; 1 group, clopidogrel bisulfate (5 mg/kg) through gavage; and the final group, ketorolac tromethamine (3 mg/kg) through intramuscular injection. Each rat was then anesthetized, and the femoral veins were prepared bilaterally. A 180° venotomy was made, and the vessels were anastomosed with the tuck model set-up for anastomotic failure. The vessels were checked for patency every 15 minutes for 2 hours after clamp removal.

Main Outcome Measures: The rate of venous thrombosis and the time to thrombosis.

Results: In both the aspirin and clopidogrel groups, 2 of 28 vessels (7%) were thrombosed. Thrombosis occurred in 3 of 28 vessels (11%) in the ketorolac group ($P=.86$). All thromboses in the aspirin and clopidogrel groups took place at 7.5 minutes after clamp removal. In the ketorolac group, the mean time to thrombosis was 7.5 minutes (range, 0-22.5 minutes). There was no difference in time to thrombosis among the 3 groups ($P=.86$).

Conclusion: Using a microvenous tuck model set-up for anastomotic failure, we found no difference in the rate of thrombosis or the time to thrombosis in rats pre-treated with aspirin, clopidogrel, or ketorolac.


INCE THE 1970S, MICROVASCULAR FREE FLAPS HAVE GROWN IN POPULARITY AND NOW REPRESENT THE STANDARD OF CARE FOR THE RECONSTRUCTION OF COMPLEX HEAD AND NECK CANCER ABLATIVE DEFECTS. THE OVERALL SUCCESS RATE OF MICROVASCULAR FREE FLAPS CONTINUES TO BE BETWEEN 94% AND 97%, WITH AN AVERAGE SUCCESS RATE OF 96.4% IN 1 RECENT STUDY.1-3 MOST FREE-FLAP LOSS IS DUE TO THROMBOSIS IN THE VENOUS OUTFLOW OF THE FLAP, TYPICALLY WITHIN THE FIRST 3 AND 5 DAYS POSTOPERATIVELY. TO COMBAT THE CLOTTING CASCADE, AND PARTICULARLY PLATELET PLUG FORMATION, MOST SURGEONS USE THE FREE-FLAP TECHNIQUE INITIATE ANTITHROMBOSCIC MEDICATIONS IN THE PERIOPERATIVE PERIOD. A RECENTLY PUBLISHED SURVEY SHOWED THAT MOST (76.5%) SURGEONS WHO PERFORM THE HEAD AND NECK MICROVASCULAR FREE-FLAP TECHNIQUE PRESCRIBE ASPIRIN, A POTENT PLATELET-AGGREGATION INHIBITOR, FOR THE PERIOPERATIVE PERIOD.1 HOWEVER, THERE IS STILL NO CONSensus ABOUT THE IDEAL ANTITHROMBOTIC REGIMEN. THE STANDARD OF CARE IN OUR INSTITUTION HAS BEEN INTRAVENOUS KETOROLAC TROMETHAMINE TREATMENT IN THE PERIOPERATIVE PERIOD.

Several analyses have been performed in different animals to evaluate the effectiveness of various antithrombotic medications on microvascular thrombosis rates.4-7 However, the current medical literature has some limitations. With several different animals used in these studies, the study results are difficult to compare among each other because the natural thrombogenic nature of each animal is known to differ slightly. Also, several different animals have been used in performing the microvascular anastomoses in a manner that is set up for anastomotic failure.5,6 Thus, it is difficult to compare the studies when 1 study used a “tuck” model and the next study used a “crush” model. In addition, although some studies evaluated microarterial anastomo-
ses, others used microvenous anastomoses.5,8,9 Finally, to our knowledge, no studies directly compare clopidogrel, aspirin, and ketorolac, the 3 most commonly used perioperative antiplatelet medications.

To overcome the shortcomings of the current literature, we designed a study to compare aspirin, clopidogrel, and ketorolac in a randomized manner using a rat microvenous tuck model.10 In this way, we examined 42 rats (and 84 anastomoses).

### METHODS

#### GENERAL PROCEDURES

Before study initiation, approval was obtained from the Mayo Clinic Institutional Animal Care and Use Committee. The study was supervised by a Mayo Clinic research veterinarian. In total, 42 retired Lewis breeder rats (weight, 400-500 g) were used for the study and were randomly divided into 3 treatment groups of 14 rats each. To promote microvascular thrombosis, we performed a tuck model on each femoral vein.10 No control group was used because the rate of microvenous thrombosis in this exact rat femoral vein tuck model has been well established as being between 31% and 50% in control subjects.3,7 The study was performed in a single-blind manner such that the observer tasked with recording the venous thrombosis and time to thrombosis was unaware of the group to which each rat was assigned. The groups were alternated in a fashion that minimized the effect of the surgeons not being blinded to the groupings. The 3 groups were as follows:

- **Aspirin group**: These animals were briefly anesthetized with 1% isoflurane 90 minutes before their surgical intervention. When under anesthesia, the animals were given aspirin (10 mg/kg) through gavage feeds in 0.2 mL of sterile water (concentration, 25 mg/mL) and then allowed to wake up. A 90-minute period was then allowed to enable the aspirin to circulate, as previously established in the medical literature.4

- **Clopidogrel group**: These animals were briefly anesthetized 120 minutes before their surgical intervention. When under anesthesia, the animals were given clopidogrel bisulfate (5 mg/kg) through gavage feeds in 0.2 mL of sterile water (concentration, 12.5 mg/mL) and then allowed to wake up. Next, 120 minutes were allowed to pass to enable the clopidogrel to circulate, as previously established in the literature.5

- **Ketorolac group**: These animals were briefly anesthetized 1% isoflurane 30 minutes before their surgical intervention. When under anesthesia, the animals received ketorolac tromethamine (3 mg/kg) in an intramuscular injection in their hind quarters and were allowed to wake up. Thirty minutes were allowed to pass to enable the ketorolac to circulate, as previously established in the literature.6

#### SURGICAL PROCEDURE

After the study medication had time to circulate, each animal was anesthetized with 1% isoflurane and intraperitoneal pentobarbital (35-50 mg/kg). The surgical microvenous procedure was then performed, and the anastomoses were observed for 2 hours. The animals were then humanely killed.

After induction of general anesthesia, each rat was placed in the supine position and the surgical site was shaved and prepared with povidone iodine solution. A standard groin incision was made, and the femoral vessels were exposed. The femoral vein was isolated and vascular cross clamps were placed. A 180° venotomy was made in the femoral vein, and the vein was irrigated. Aided by a standard operating microscope, we used a 10-0 nylon monofilament suture to repair the veinotomy and create a tuck of adventitia in the vessel lumen by passing the suture through the proximal side of the vessel, bringing it out through the same side of the vein wall, then passing it through the veinotomy and out of the vein wall on the second side (Figure).10 This technique creates a telescoping of the proximal side of the anastomosis into the distal side, bringing adventitia into the lumen and setting up a prothrombogenic environment. The cross clamps were then released and the flow reestablished. The same procedure was repeated on the contralateral side. In this manner, we placed 3 or 4 interrupted sutures in each venotomy.

#### MONITORING

After the femoral vein tuck procedures were performed bilaterally, the vessels were maintained in a bath of normal saline to avoid vessel-wall drying and vasospasm. Every 15 minutes for the next 2 hours, each vein was checked for patency, or thrombus. Patency was evaluated using the "strip test," with gentle pinching of the vein with 2 microvascular instruments just proximal to the veinotomy and milking the blood out of the vein in an antegrade fashion. The second, more distal instrument was then released, and refill across the venotomy was observed. Two points of data were recorded for each vessel every 15 minutes: whether a clot existed and the time to thrombosis. When a clot was detected, the time to thrombosis was calculated as being half-way between the last observed patency and the current observation in which thrombosis was found. For example, if the right femoral vein was patent at 15 minutes after surgery and was clotted at 30 minutes after surgery, the time to thrombosis would be calculated at 22.5 minutes. Thus, each group had available for calculation the total vessel patency rate and the mean time to thrombosis.
Table. Rate of Vessel Thrombosis Within 2 Hours of Undergoing the Microvascular “Tuck” Technique

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Venous Outcome, No. (%)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Patent</td>
<td>Thrombosed</td>
</tr>
<tr>
<td>Aspirin</td>
<td>26 (93)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Clopidogrel bisulfate</td>
<td>26 (93)</td>
<td>2 (7)</td>
</tr>
<tr>
<td>Ketorolac tromethamine</td>
<td>25 (89)</td>
<td>3 (11)</td>
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</tbody>
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The difference among the 3 groups was not statistically significant through the $\chi^2$ test ($P = .86$).

Each treatment group contained 28 vessels.

**RESULTS**

In the 42 rats, 84 anastomoses were performed. One vessel showed no flow after the vascular clamps were released. The other 83 vessels were patent after the anastomoses. The 1 vessel that did not show flow was counted on an intention-to-treat basis and was considered as thrombosed at 0 minutes.

In both the aspirin and clopidogrel groups, 2 of 28 vessels (7%) had thrombosis (Table). Thrombosis occurred in 3 of 28 vessels (11%) in the ketorolac group. The difference between the 3 groups was not statistically significant through $\chi^2$ analysis ($P = .86$). Because vessel failure in the same rat may be correlated, we ran a nonlinear random-effects model. In this model, the estimated odds ratio of thrombosis for aspirin vs ketorolac was 0.64 (95% confidence interval [CI], 0.10-4.18) and for clopidogrel vs ketorolac was 0.64 (95% CI, 0.10-4.18). An odds ratio of 1 represents an equal likelihood of thrombosis; an odds ratio of less than 1 represents a lesser likelihood of thrombosis. The difference between groups was not statistically significant ($P = .65$ for either comparison).

The mean time to thrombosis in the aspirin and clopidogrel groups was 7.5 minutes after clamp removal (all thromboses in these 2 groups occurred at 7.5 minutes). In the ketorolac group, the mean time to thrombosis was also 7.5 minutes (range, 0-22.5 minutes). With the Kruskal-Wallis test, no difference was found across groups in the time to thrombosis ($P > .99$). In addition, with the Wilcoxon rank sum test, comparing time to thrombosis for aspirin vs ketorolac, clopidogrel vs ketorolac, and aspirin vs clopidogrel, there was no difference ($P > .99$ for all comparisons). Finally, with the Cox proportional hazards regression model for comparing time to thrombosis across groups, the differences across groups were not significant ($P = .85$); the hazard ratios comparing aspirin with ketorolac and comparing clopidogrel with ketorolac were both at 0.65 (95% CI, 0.11-3.89).

**COMMENT**

Although microvascular free-tissue transfer has become a reliable method for reconstruction of head and neck cancer defects, the treatment failure rate due primarily to venous thrombosis is reported to be 3% to 7%. Treatment failures may be disastrous and may lead to considerable morbidity. Even though technique is the most important factor in determining anastomotic patency, pharmacologic therapies are often used to improve success. Despite the many options in prophylactic antithrombotic agents, surgeons have no standard therapy regimen. Numerous studies have examined the efficacy of individual antithrombotic agents. However, across the studies, differences are common in the animals used, the type of vessel evaluated (artery vs vein), and the model used to promote thrombosis (eg, tuck, crush). The goal of this study was to compare the rate of thrombosis among aspirin, clopidogrel, and ketorolac with a microvenous rat tuck model.

Aspirin is a salicylate that irreversibly acetylates cyclooxygenase and ultimately blocks the formation of thromboxane $A_2$, a vasoconstrictor. Thromboxane $A_2$ stimulates activation of platelets and increases their aggregation. Aspirin has been shown to be effective in decreasing graft occlusion in various vascular procedures. Peter et al found that low-dose aspirin administered preoperatively decreased venous anastomotic thrombosis and increased distal microcirculatory perfusion significantly. Li and Cooley demonstrated an increased venous patency rate with administration of aspirin and dipyridamole (10 mg/kg for each drug). This study did not address the time to thrombosis as we did in the present study. In addition, aspirin use with free-flap reconstructive surgical procedures has produced minimal complications, the greatest of which were gastrointestinal upset and hemorrhage. Specifically, Chien et al found that patients who received a combination of aspirin and heparin prophylactically had hematoma rates similar to those reported for patients who did not receive anticoagulation regimens. We chose to use the dose of aspirin that Li and Cooley used for their study.

Clopidogrel irreversibly binds to the platelet adenosine diphosphate receptor. This binding blocks the activation and initiation of platelet aggregation. It has been shown to reduce the rates of thrombosis in injured vessels. Previous studies have shown a dose-dependent reduction in the rate of thrombosis, with the dose of 5 mg/kg giving maximal results. Using the venous tuck model, Moore and Deschler found a significant decrease in rat venous thrombosis with preoperative administration of clopidogrel, 5 mg/kg, vs a saline control (7.9% vs 31.4%). The mean (SD) time to thrombosis in the study by Moore and Deschler was 2.00 (1.00) hours in the clopidogrel group and 1.27 (0.47) hours in the saline control rats. This rate of thrombosis with the use of clopidogrel is similar to our rate of 7%. In addition, Nayak and Deschler reported a decreased rate of thrombosis in a rat model for arterial anastomosis. The 2 most common adverse effects of clopidogrel are gastrointestinal upset and hemorrhage.

Ketorolac reversibly inhibits cyclooxygenase, thus blocking the formation of thromboxane $A_2$. However, the inhibitory effects on vasoconstriction and platelet aggregation normalize within 24 hours. Ketorolac may be administered either intravenously or intramuscularly, unlike many other nonsteroidal anti-inflammatory drugs and aspirin. Buckley et al showed that in a rat arterial model, single-dose ketorolac provided a significant increase in
vessel patency for 24 hours compared with a control group. He also showed that the bleeding rates were significantly prolonged with the administration of a single dose of both 1 mg/kg and 3 mg/kg. There was no measure of the mean time to thrombosis in the study by Buckley et al.6 Shufflebarger et al11 compared a rabbit arterial model and a control group with 2 groups that had been given ketorolac. One group received a dose of 1.72 mg/kg/d, and the other group, 3.44 mg/kg/d. Both the low-dose group and high-dose group had a significantly greater rate of patency (70% and 86%, respectively) compared with the control group (52%). Thus, we chose to use a dose at the upper end of the spectrum used in the literature (3 mg/kg).

In our single-blinded, randomized study, the difference in rates of thrombosis between aspirin, clopidogrel, and ketorolac treatments was not statistically significant. No control group was created because studies have supported the efficacy of both aspirin and clopidogrel in preventing venous thrombosis.4,5,9,16,18,19 In addition, the rate of venous thrombosis in control rats using the same microvenous tuck model has been well established at 31% to 50%.37 Given this foundation, if ketorolac showed rates of thrombosis similar to aspirin and clopidogrel, it could be considered efficacious in the prevention of thrombosis. In addition, the main goal of the study was to determine whether 1 antiplatelet drug has a distinct advantage regarding efficacy.

The anastomoses were monitored for 2 hours in our study. This length was derived from the study by Nayak and Deschler,9 which found that, among all the vessels that had thrombosed, the thrombosis had occurred within the first 60 minutes. In addition, the mean time to thrombosis in both the studies by Moore and Deschler9 and Emerick and Deschler7 was 2 hours or less.

According to 1 survey, most otolaryngologists use aspirin in the perioperative period of free-tissue transfers.1 A possible reason for this choice is that much of the literature supports the efficacy of both aspirin and clopidogrel in preventing venous thrombosis.4,5,9,16,18,19 In addition, the rate of venous thrombosis in control rats using the same microvenous tuck model has been well established at 31% to 50%.37 Given this foundation, if ketorolac showed rates of thrombosis similar to aspirin and clopidogrel, it could be considered efficacious in the prevention of thrombosis. In addition, the main goal of the study was to determine whether 1 antiplatelet drug has a distinct advantage regarding efficacy.

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According to 1 survey, most otolaryngologists use aspirin in the perioperative period of free-tissue transfers.1 A possible reason for this choice is that much of the research about antiplatelet medications in regard to free flaps focuses on aspirin. Furthermore, aspirin offers many advantages, such as few adverse effects and wide availability. However, clopidogrel and ketorolac also offer these advantages. In addition, ketorolac offers the advantage of administration either intravenously or intramuscularly—options that can be especially helpful during intraoperative treatment.

Caution must be exercised when translating animal research to humans, since the rat is known to have different inherent clotting properties than humans. It should be mentioned that the senior author (R.E.H.) has been using intravenous ketorolac in his microvascular cases since 2002 with a greater than 98% flap survival rate (unpublished data, 2008). Thus, we believe that the results of the present study may allow the surgeon more versatility when choosing an antiplatelet medication for a free-flap procedure.

This study has several small shortcomings that warrant brief discussion. Only the monitor was blinded to the group into which each rat was assigned. This could have introduced some bias into the surgeons performing the surgery. However, we believe that this bias was negligible given that the 3 groups had equivalent thrombosis rates. In addition, we randomly alternated the groups to minimize the effects of the surgeons being aware of the groupings and the effects of fatigue. We did not perform coagulation studies such as bleeding times to prove that the medications had effectively inhibited platelet function. However, we used the same doses that had been shown to be efficacious in increasing the bleeding times in previous animal studies, and we also used the same time for absorption and activation of the medications that have been previously published.4,5,9,16,18,19 The biggest shortcoming of the study was the lack of a saline control group. Since both aspirin and clopidogrel had been previously found to be superior to saline control in preventing microvenous thrombosis, we did not think that saline controls were needed in this study. Rather, we used the aspirin and clopidogrel groups as the controls and the ketorolac group as the treatment group.

In conclusion, our study shows that the efficacy of preventing venous thrombosis does not differ among aspirin, clopidogrel, and ketorolac in the rat venous tuck model. Therefore, decisions on the use of these agents in the perioperative period can be based on route of administration, availability, and patient comorbidities.

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REFERENCES