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TITLE:

Creation of Two Saccular Elastase-digested Aneurysms with Different Hemodynamics in One Rabbit

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KEYWORDS:

Aneurysm, Extracranial saccular aneurysm, Bifurcation aneurysm, Stump aneurysm, Animal Model, Rabbit, Elastase

SUMMARY:

This protocol describes the steps for the creation of a rabbit model with two elastase-digested aneurysms with different hemodynamics (stump and bifurcation constellation). This allows the testing of novel endovascular devices in aneurysms with different angioarchitecture and hemodynamic conditions within a single animal.

ABSTRACT:

Preclinical animal models with hemodynamic, morphologic, and histologic characteristics close to human intracranial aneurysms play a key role in the understanding of the pathophysiological processes and the development and testing of new therapeutic strategies. This study aims to describe a new rabbit aneurysm model that allows the creation of two elastase-digested saccular aneurysms with different hemodynamic conditions within the same animal.

Five female New Zealand white rabbits with a mean weight of 4.0 (± 0.3) kg and mean age of 25

(±5) weeks underwent microsurgical stump and bifurcation aneurysm creation. One aneurysm (stump) was created by right common carotid artery (CCA) exposure at its origin at the brachiocephalic trunk. A temporary clip was applied at the CCA origin and another, 2 cm above. This segment was treated with a local injection of 100 U of elastase for 20 min. A second aneurysm (bifurcation) was created by suturing an elastase-treated arterial pouch into the end-to-side anastomosis of the right CCA to left CCA. Patency was controlled by fluorescence angiography immediately after creation.

The average duration of surgery was 221 min. The creation of two aneurysms in the same animal was successful in all rabbits without complication. All aneurysms were patent immediately after surgery except for one bifurcation aneurysm, which showed an extreme tissue reaction due to elastase incubation and an immediate intraluminal thrombosis. No mortality was observed during surgery and up to one-month follow-up. Morbidity was limited to a transient vestibular syndrome (one rabbit), which recovered spontaneously within one day.

Demonstrated here for the first time is the feasibility of creating a two-aneurysm rabbit model with stump and bifurcation hemodynamic characteristics and highly degenerated wall conditions. This model allows the study of the natural course and potential treatment strategies on the basis of aneurysm biology under different flow conditions.

INTRODUCTION:

Intracranial aneurysm is a severe condition with a mortality rate after rupture reaching 50% and long-term disability in 10–20% of patients[1]. The last decade has seen a rapid development of endovascular treatment options but, at the same time, also an increasing rate of recurrence with up to 33% of aneurysm recanalization after coiling[2,3]. To better understand the pathophysiology underlying aneurysm occlusion and recanalization, as well as for the development and testing of new endovascular devices, there is currently a need for reliable preclinical models whose hemodynamic, morphological, and histologic characteristics mimic those of human intracranial aneurysms[4-6]. As of today, there is no defined model as a standard for preclinical trial, and a large range of species and techniques are available to researchers[7,8].

However, the rabbit is a species of particular interest due to the size and hemodynamic similarities between its neck arteries and the human cerebral vessels, as well as its similar coagulation and thrombolysis profiles. Several models with elastase-digested saccular aneurysms on the common carotid arteries (CCAs) have shown qualitative and quantitative similarities with human intracranial aneurysms in terms of flow conditions, geometric features, and wall characteristics[9-12]. This study aims to describe a technique to create a new rabbit aneurysm model with both stump and bifurcation elastase-digested aneurysms in the same animal. The surgical techniques are inspired by those of Hoh et al.[13] and Wanderer et al.[14] with slight modifications to provide a good standardization and reproducibility and to ensure low mortality and morbidity.

PROTOCOL:

NOTE: The experiment was approved by the Local Committee for Animal Care of the Canton Bern, Switzerland (Application Number BE108/16), and all animal care and procedures were performed in accordance with institutional guidelines and 3R principles[15,16]. Data are reported according the ARRIVE guidelines. Peri-operative management was conducted by a board-certified veterinarian anesthesiologist. For the study, female New Zealand white rabbits, with a mean weight of 4.0 (± 0.3) kg and mean age of 25 (±5) weeks, were housed at a room temperature of 22–24 °C with a 12-h light/dark cycle with free access to water, pellets, and hay.

1. Pre-surgical phase and anesthesia

1.1. Perform a clinical examination as recommended by the Association of veterinary Anesthetists and the European and American College of Veterinary Anesthesia and analgesia to confirm that the rabbits are healthy by weighing each animal, evaluating the mucous membrane, documenting the capillary refill time and pulse quality, and performing a pulmonary and cardiac auscultation as well as an abdominal palpation.

1.2. Based on the clinical finding, attribute an American Society of Anesthesiologists (ASA) classification to each rabbit[17]. Perform surgery only on animals with an ASA I score.

1.3. Shave both outer ears, and apply prilocaine-lidocaine cream on auricular arteries and veins. Achieve deep sedation with a combination of ketamine 20 mg/kg, dexmedetomidine 100 mg/kg, and methadone 0.3 mg/kg injected subcutaneously (SC). Leave the animals undisturbed for 15 min. Give supplementary oxygenation (3 L/min) through a loosened face mask, and monitor with a pulse oximeter.

1.4. Place a 22 G cannula in the left auricular central artery as well as in an auricular vein. Induce general anesthesia with propofol 1–2 mg/kg intravenous (IV) until effect (loss of swallowing reflex). Proceed with endo-tracheal intubation via a silicone tube (3 mm internal diameter).

1.5. Shave the forehead to place the pediatric electroencephalographic (EEG) sensors. Shave the surgical field, and inject ropivacaine hydrochloride 0.75 % intradermally.

1.6. Place the rabbit on the operation table in dorsal recumbency, install full monitoring, and connect the endo-tracheal tube to a low-resistance pediatric circle system. Maintain anesthesia with administration of isoflurane in oxygen, targeting a maximal end tidal (Et) concentration of 1.3 %.

1.7. Provide a continuous infusion of ringer lactate 5 mL/kg/h through the venous access. Ensure clinical and instrumental monitoring until extubation by means of pulse oximetry, doppler and invasive blood pressure, 3-lead electrocardiogram, EEG, rectal temperature, and inhaled and exhaled gases.

1.8. Disinfect the surgical field with povidone iodine from the manubrium sterni to jaw angles,

and apply the sterile draping. During surgery, provide analgesia with lidocaine (constant rate infusion (CRI) of 50 μg/kg/min) and fentanyl (CRI of 3–10 μg/kg/h). Perform spontaneous or assisted ventilation. Allow permissive hypercapnia.

1.9. Perform at least one arterial blood gas analysis during surgery. In case of hypotension (mean arterial pressure below 60 mmHg), treat it with noradrenaline, titrated until effect. Use a heating pad or a heating forced-air warming system to prevent hypothermia (aim: rectal temperature 37.5–38.5 °C).

NOTE: As the invasive arterial blood pressure is measured at the left ear artery, the clipping of the left CCA will stop the blood flow and suppress the curve. The blood pressure has then to be measured with Doppler technique until reopening of the vessel.

2. Surgery

2.1. Approach

2.1.1. Make a median skin incision from the hyoid bone until a point 1.5 cm caudal to the manubrium sterni with a scalpel. Prepare the subcutaneous and fat tissue from the medial incision while performing meticulous hemostasis.

2.1.2. Free the sternocephalicus muscle from the adherent connective tissue, and apply lidocaine topically (2–4 mg/kg, prefer lidocaine 1%) to avoid myoclonus. Expose the right CCA medially of the sternocephalicus muscle and keep it wet with wet swabs.

2.1.3. Now prepare the lateral and proximal parts of the sternocephalicus muscle and retract it medially with a vessel loop to expose the CCA. Identify the external jugular vein and protect it with a wet micro swab.

2.1.4. Dissect the connective tissue carefully along the proximal CCA until the bifurcation of the brachiocephalic trunk to expose the artery. In the presence of small branches coming from the artery, coagulate them with the cauterizer.

NOTE: Take care to avoid any nerve damage.

2.2. Stump aneurysm creation and tissue harvesting for the bifurcation aneurysm

2.2.1. Before clipping the right CCA, measure the anti-clotting time (ACT), and give natrium heparin (80 EI/kg) systemically via the ear vein (performed by the anesthesia team) to avoid thromboembolic events.

2.2.2. Now apply 2 temporary clips: the first one at the origin of the CCA and the second one 2 cm distal from it (**Figure 1A**). Place a rubber pad under the vessel and rinse with papaverine HCL (40 mg/ mL; 1:1 dissolved in 0.9% saline) for vasodilatation.

2.2.3. Remove the adventitia carefully using microscissors. Perform an arteriotomy below the distal clip with a 22 G IV-catheter, and insert the catheter caudally up to the proximal clip (**Figure 1A,B**).

2.2.4. Flush the segment intraluminally with heparinized NaCl (500 U/100 mL in 0.9% saline) until there is no blood visible, and finally fix the catheter with a ligature (4-0). Now, through the catheter, inject 0.1–0.2 mL of elastase (100 IU previously dissolved in 5 mL of Tris-Buffer) into the artery segment and incubate for 20 min (**Figure 1B**).

2.2.5. Start with the dissection on the left side to expose the left CCA (see section 2.3). After 20 min of incubation time with elastase, clear the elastase solution, and change the syringe to rinse the artery segment about 10 times with 0.9% NaCl.

2.2.6. Apply 2 ligatures (6-0): the first one 5 mm distal of proximal clip and the second just proximally, under the arteriotomy (**Figure 1C**). Cut the vessel ~3 mm above the first ligature and one more time between the second ligature and the distal clip. Keep this autologous graft in a heparinized solution (500 U/100 mL in 0.9% saline) until the creation of the bifurcation aneurysm (**Figure 1D**). Finally, carefully open the first proximal clip, and measure the aneurysm (length, width, and depth).

2.3. Bifurcation aneurysm creation

2.3.1. Prepare the left side by dissecting the sternocephalicus muscle medially to expose ~2 cm of the left CCA. Apply lidocaine topically on the muscle to avoid myoclonus.

2.3.2. Underlay the carotid artery with a gauze ball and a small swab with a piece of glove. Apply some papaverine.HCl topically (40 mg/mL; 1:1 dissolved in 0.9% saline). Continue to work under microscopic view: prepare the aneurysm pouch and remove the adventitia. Measure the aneurysm pouch (length, width, depth).

2.3.3. Flush the open part of the right CCA with heparinized NaCl and if needed, replace the clip to have ~1 cm to allow free manipulations for the suture. Remove the adventitia carefully, and make a ~2 mm longitudinal incision laterally in the stump of the right CCA.

2.3.4. Now apply two temporary clips on the left CCA to delimit a segment of ~1 cm and remove the adventitia in between. Perform an arteriotomy with a 23 G needle. Flush the segment with heparinized NaCl (500 U/100 mL in 0.9% saline). Enlarge the arteriotomy using microscissors to ~4–5 mm to allow the suturing of the right CCA and the aneurysm pouch (**Figure 1E**). Irrigate the vessels during the whole suturing procedure and protect them with wet micro swaps.

2.3.5. Perform the anastomosis with 9-0 non resorbable suture.

2.3.5.1. Suture the proximal back wall of the right carotid blunt with 5 stitches, starting at the proximal edge of the arteriotomy on the left CCA. Then, suture the backside of the aneurysm pouch with 4–5 stitches, starting at the distal edge of the arteriotomy on the left CCA.

2.3.5.2. Continue with the distal backside at the level of the fish mouth incision to suture with the vertical backside of the aneurysm graft with 3 stitches. Suture the front side of the fish mouth incision with 3 stitches, starting upwards and moving downwards.

2.3.5.3. Finish with the front suture between the left CCA and front side of the aneurysm graft and right CCA with ~6 stitches. Before finishing the anastomosis, rinse the vessels with heparinized 0.9% saline solution intraluminally.

2.3.6. Before removing the clamp, measure the anti-clotting time (ACT) one more time, and administer an adapted dose of heparin systemically (target: 2–3 times baseline ACT).

2.3.7. Remove the clip on the right CCA while putting some pressure on the anastomosis with micro swabs for hemostasis. Then, continue by removing the distal clip from the left CCA. If there is no major bleeding, continue with taking out the proximal clip on the left CCA, to allow blood flow. If there is some bleeding from the anastomose, apply some pressure with the gauze ball and swab; wait for a couple of minutes. If it persists, replace the clips and perform re-stitches.

NOTE: A blood loss of more than 20–30 mL can endanger the recovery phase.

2.4. Patency control and documentation

2.4.1. After opening all the vessels, document the results photographically and measure them (**Figure 1F** and **Figure 2A,B**).

2.4.2. Confirm the restoration of the flow in the distal CCA through the invasive arterial blood pressure curve (measured at the ear artery, a direct branch of the external carotid), which should also return to normal.

2.4.3. Perform fluorescence angiography by administering 1 mL of fluorescein IV, using 2 bandpass filters, a video camera, and a bicycle spotlight. See previous publications for the description of the whole procedure[18,19].

2.5. Closure

2.5.1. Readapt the fat pad on the anastomosis and suture it with a 4-0 resorbable suture. Finally suture subcutis and skin with single stitches using 4-0 resorbable suture.

3. Postsurgical phase

3.1. At the end of the surgery, discontinue isoflurane and systemic analgesia. Ensure that

control of the swallowing reflex has returned before performing tracheal extubation.

3.2. Administer meloxicam 0.5 mg/kg IV to ensure analgesia, aspirin (ASS) 10 mg/kg IV to prevent immediate thrombotic events, vitamin B12 100 μg SC and clamoxyl 20 mg/kg IV as antibiotic prophylaxis.

3.3. Provide supplementary oxygenation and warming until the rabbit spontaneously regains the sternal recumbency. Perform postoperative follow-up and care 4 times a day for the first 3 preoperative days, in accordance with the guidelines for the assessment and management of pain in rodents and rabbits[23,24].

3.4. Ensure postoperative analgesia with fentanyl patch (12 μg/h) applied on the outer ear, meloxicam 1x/ SC for 3 days, and methadone as rescue therapy, along with a score sheet for pain evaluation.

REPRESENTATIVE RESULTS:

The creation of a stump and a bifurcation aneurysm was successful in all 5 New Zealand white rabbits without intraoperative complications. No mortality was observed during surgery or during the follow-up period of 24 ± 2 days. One rabbit experienced postoperative complications with a vestibular syndrome and a blindness of the right side. The animal recovered completely and spontaneously after 24 h. This complication did not interfere with its normal activities (free movements, water and food intake, interactions with other animals) and did not require any specific treatment. There was no spontaneous aneurysm rupture.

The average duration of surgery was 221 min (ranging between 190 and 255 min). All aneurysms were patent immediately after surgery, except for one bifurcation aneurysm that showed an extreme tissue reaction due to elastase incubation and an immediate thrombosis. At follow-up, aneurysm patency was confirmed by magnet resonance angiography (**Figure 3**) and macroscopic inspection after tissue extraction (**Figure 4**). To the exception of the bifurcation aneurysm that already thrombosed during surgery, all aneurysms were still patent at the follow-up endpoint. This resulted in a patency rate of 90% (9 out of 10).

Macroscopic inspection and measurement of the aneurysms after sampling show a growth of all aneurysms with an average size of 5.4 mm x 2.4 mm x 2.3 mm ± 1 mm x 0.6 mm x 0.3 mm at creation and 4.5 mm x 3.1 mm x 2.5 mm ± 1.5 mm x 0.9 mm x 0 mm at harvesting for the stump aneurysm; and 3.4 mm x 2 mm x 2.1 mm ± 0.6 mm x 1 mm x 0.4 mm at creation and 3.8 mm x 2.8 mm x 2.6 mm ± 1.2 mm x 0.3 mm x 0.6 mm at harvesting for the bifurcation aneurysms. Interestingly, bifurcation aneurysms grew more than stump aneurysms with a mean volume of 14.4 mm^3 ± 3.5 mm^3 at creation and 28.6 mm^3 ± 16.4 mm^3 at extraction (ratio 1.9) versus a volume at creation of 30.8 mm^3 ± 15 mm^3 and 34.9 mm^3 ± 24.1 mm^3 at extraction (ratio 1.1) for the stump version.

FIGURE AND TABLE LEGENDS:

Figure 1: Steps of the surgery. (**A**) Application of the 2 temporary clips on the right CCA: the first one at its origin from the brachiocephalic trunk and the second one ~2 cm distal to the first. The asterisk indicates the localization of the arteriotomy with a 22 G intravenous catheter (IV-catheter). (**B**) After insertion and fixation of the IV-catheter with a 4-0 ligature, flush the segment with heparinized NaCl (500 U/100 mL of 0.9% saline), and inject 0.1–0.2 mL of elastase (100 U previously dissolved in 5 mL of TRIS buffer). Incubate for 20 min. (**C**) Apply 2 non-resorbable ligatures (6-0): the first one 5 mm distal to the proximal clip and the second just proximally under the arteriotomy. (**D**) Cut the vessel ~3 mm above the ligatures to create the stump aneurysm and the autologous graft for the bifurcation aneurysm. (**E**) Anastomosis of the right CCA and the autologous graft on the left CCA to create the bifurcation aneurysm. (**F**) Final result with a stump aneurysm on the right side and a bifurcation aneurysm on the left side. Abbreviations: CCA = common carotid artery; IV = intravenous.

Figure 2: Intraoperative photo documentation of the results. The yellow dotted line represents the midline with indication for cranial and caudal directions. (**A**) View of the stump aneurysm on the right side of the neck. The SCEM is retracted medially by the mean of a vessel loop (in blue). (**B**) View of the bifurcation aneurysm on the left side of the neck. Abbreviations: SCEM = Sternocephalicus muscle; SA = Stump aneurysm; JV = jugular vein; rCCA: right common carotid artery; lCCA= left common carotid artery; Tr = Trachea; * = Recurrent or laryngeal branch; BA = Bifurcation aneurysm.

Figure 3: Magnetic resonance angiography results at follow-up. Images from three-dimensional TOF sequences acquired with a 3 Tesla MRI, focused on the neck arteries. (**A**) Stump aneurysm (yellow arrow) on the right subclavian artery. (**B**) Bifurcation aneurysm (yellow arrow) on the bifurcation created by anastomosing the right CCA on the left one. Abbreviations: TOF = time-of-Flight; MRI = magnetic resonance imaging; CCA = common carotid artery.

Figure 4: Macroscopic photo documentation after tissue extraction. Major grooves (2 divisions) on the clip indicate 1 mm and minor grooves in between (one division) indicate 0.5 mm. (**A**) Stump aneurysm on the brachiocephalic trunk and right subclavian artery. (**B**) Bifurcation aneurysm on the bifurcation created by anastomosing the right CCA on the left one. Abbreviations: SA = Stump aneurysm; BCT = brachiocephalic trunk; rSC = right subclavian artery; BA = bifurcation aneurysm; CCA = common carotid artery; rCCA = right CCA; lCCA = left CCA.

Figure 5: Histological findings of stump and bifurcation aneurysms. Specimen stained with hematoxylin-eosin (2-fold magnification). (**A**) Microscopic overview of a stump aneurysm (a) with the brachiocephalic trunk (b) and the right subclavian artery (c). (*) indicates the direction of the blood flow. (**B**) Microscopic overview of a bifurcation aneurysm (a) with the proximal left CCA (b), the distal left CCA (c), and the distal right CCA (d). (*) indicates the direction of the blood flow. In the insets in (**A**) and (**B**), I) represents the tunica intima of the aneurysm wall, II) the tunica media, and III) the tunica externa (20-fold magnification). Abbreviations: CCA = common carotid artery.

DISCUSSION:

The most common technique for aneurysm creation involves the creation of a stump aneurysm at the origin of the right CCA, either through an open or an endovascular method. The model has been validated to be a stable non-growing aneurysm that remains open with time[20,21]. The second possible technique involves the microsurgical creation of an arterial bifurcation aneurysm by anastomosing the right CCA on the left one and suturing an aneurysm pouch on the bifurcation[14,22,23]. Although both methods have shown suitability for the testing of endovascular devices and studying pathophysiology, the aneurysm morphologies and thus, the hemodynamical forces and flow characteristics involved are substantially different. Given that existing models allow the creation of only one aneurysm type per animal, a direct comparison between the natural course of aneurysms from the bifurcation type with those from the stump type is currently difficult.

Indeed, physiological differences between animals (such as blood pressure or exact collagen content of the vessel wall) cannot always be fully controlled in an experimental setting and can influence the aneurysm biology and natural course. This study demonstrates the feasibility of creating a rabbit model with both stump and bifurcation hemodynamic and degenerated wall conditions in the same animal (or in a single animal). This technique yielded reproducible aneurysms with low morbidity and mortality and a high patency rate (90%). The main drawback of this method remains the same as for the creation of the classical stump or the bifurcation models themselves—the need for sophisticated laboratory equipment and specific microsurgical skills.

Especially two steps were identified to be critical during this surgery: the first is the dissection and exposure of the right CCA until its origin at the brachiocephalic trunk. The following vital structures may be particularly at risk during this approach: the trachea, the jugular vein, and the laryngeal nerve. As trachea manipulation can impair respiration, the previous intubation ensures the patency of the airways. Furthermore, the surgery being long and in the vicinity of vital structures, full monitoring is helpful to promptly recognize any physiological deviations. The surgeon should also pay attention to avoid direct pressure or extreme traction on the trachea itself. The jugular vein runs directly next to the carotid and, in certain cases, is adherent to it. Extreme care is needed to avoid any lesion. We recommend protecting the vein and keeping it wet by the application of a wet swab.

Lastly, previous studies have already described the importance of preserving the laryngeal nerves. Any lesion on these nerves would postoperatively lead to the appearance of a stridor with consecutively impaired breathing and high probability of death of the animal. To prevent iatrogenic lesion of the nerves, CCA dissection should avoid traction of the tissues rounding the artery. We recommend the use of scissors to cut the adhering tissues instead of distracting them. The nerves also have to be identified as soon as possible after retraction of the musculature to keep them under visual control during the surgery. The second critical step is the creation of a tensionless micro-anastomosis with the elastase-digested aneurysm. This aneurysm presents a high degeneration of its wall structure, hindering the manipulation of the tissues. It requires good microsurgical skills, and a learning curve is to be expected.

Furthermore, we recommend selecting rabbits weighing at least 4.0 kg (mean age of 25 (±5) weeks) to guarantee a correct size of the neck vessels. In the classical single-stump aneurysm model, the main reported complication in the literature was the tracheal necrosis following the application of elastase due to tracheoesophageal arteries arising from the right CCA. Several modifications of the techniques have already been suggested to avoid the problem[13,24-26]. This approach allows the easy identification of these branches and their coagulation prior to elastase application to avoid any outflow of the elastase solution and similar complications.

The anticoagulation regime applied during the surgery consists of heparin application prior to the first clip application at the right CCA and before removing the clip as well as restoring circulation to the left CCA. This could effectively prevent thrombus formation due to temporary flow interruption and vessel manipulation. In addition, a unique dose of aspirin (10 mg/kg IV) is given immediately after the end of surgery to prevent thrombus formation due to the thrombogenic effect of suture material and elastase. This protocol allows the control of thrombogenic events and ensuring aneurysm patency without increasing bleeding complications.

The stump model is the most common saccular aneurysm rabbit model and has already been used several times for translational studies of endovascular therapies. The bifurcation model is also well described in the literature and suitable for the study of aneurysm pathophysiology and testing of novel therapeutic strategies. However, both models show distinct morphologies, which indicates distinct hemodynamic characteristics. It is known that aneurysms preferentially appear at bifurcation and that growth is dependent on wall shear stress[27,28]. Previous publications also showed higher spontaneous thrombosis in surgically created sidewall aneurysms compared with bifurcation ones[29] and a higher occlusion rate of stump aneurysm after flow diversion in comparison with other more complex models[8]; however, the comparison was always between two different animals.

In the present study, standard aneurysms of 2–4 mm diameter were created, as previously described [14,22,29-36]. We aimed at creating a stump aneurysm with a similar size as the bifurcation aneurysms for comparison. Thus, the current volume is somewhat smaller as has been reported[5,8,10,11,13,21]. Both aneurysms however showed a tendency to grow at 1 month follow-up. Thus, a longer follow-up period might induce aneurysm formation with greater volumes, which would allow better long-term comparison with aneurysms in humans. Additionally, these histological findings, based on hematoxylin-eosin staining, show a cellular aneurysm wall and the presence of smooth muscle cells in a linear or a disorganized pattern, as well as a disorganization of the elastic fibers (**Figure 5**). These results correlate with current findings showing histological similarities between the rabbit elastase-induced aneurysms and intracranial aneurysms in humans[11,32,37-41].

The results show the technical feasibility of creating both stump and bifurcation aneurysms using the same surgical approach. The limitation of this study is the small sample size, which does not allow for statistical analysis or a real comparison of the histological differences between stump and bifurcation aneurysms. Nevertheless, this model offers the possibility to investigate the differences between both aneurysms in term of growth, rupture, spontaneous occlusion, and

histological changes in future experiments with increased sample sizes and different follow-up time, to precisely determine the advantages and the characteristics of both types of aneurysms. Additionally, this new surgical model allows the application of endovascular devices in two distinct configurations and flow conditions in one animal, as well as during a unique procedure. This reduces the number of animals needed and potentially increases the efficiency of preclinical trials.

To conclude, this study describes a reproducible method to create 2 aneurysms with distinct flow conditions and highly degenerated walls within one single animal. The proposed model allows for a direct comparison of the natural course and effects of endovascular therapies of saccular aneurysms with respect to the role of hemodynamics . Lastly, it provides an efficient model that contributes to the reduction of animals used and overall experimental costs.

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DISCLOSURES:

The authors declare no conflict of interest.

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Figure 1

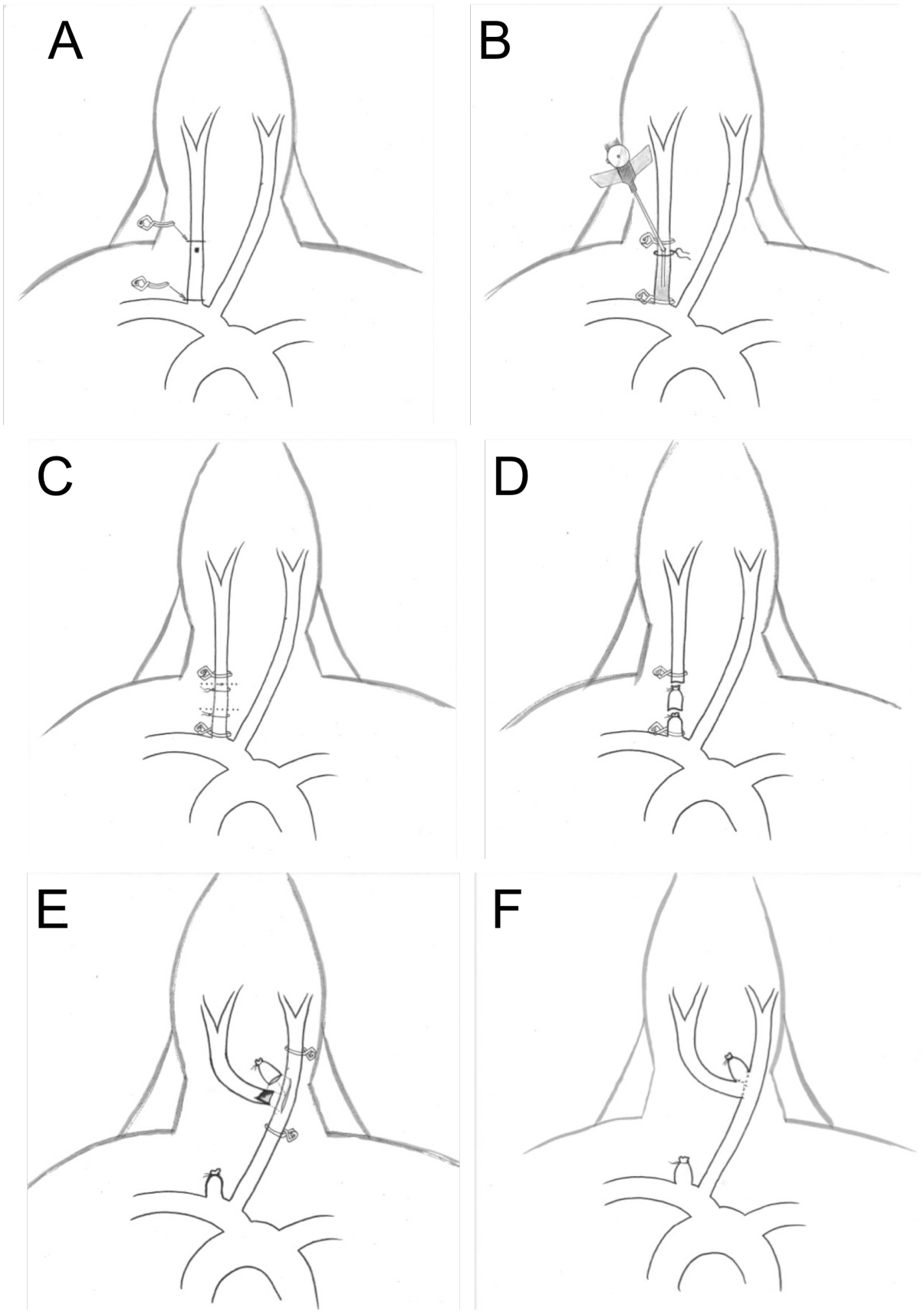
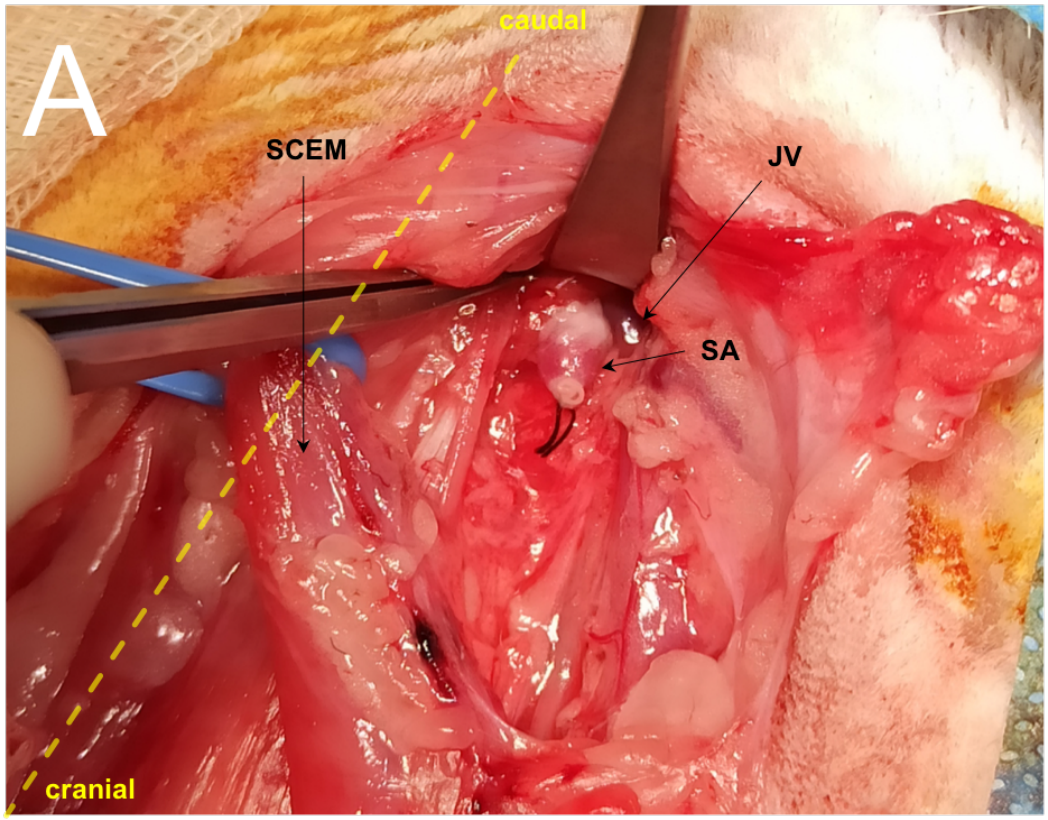


Figure 2



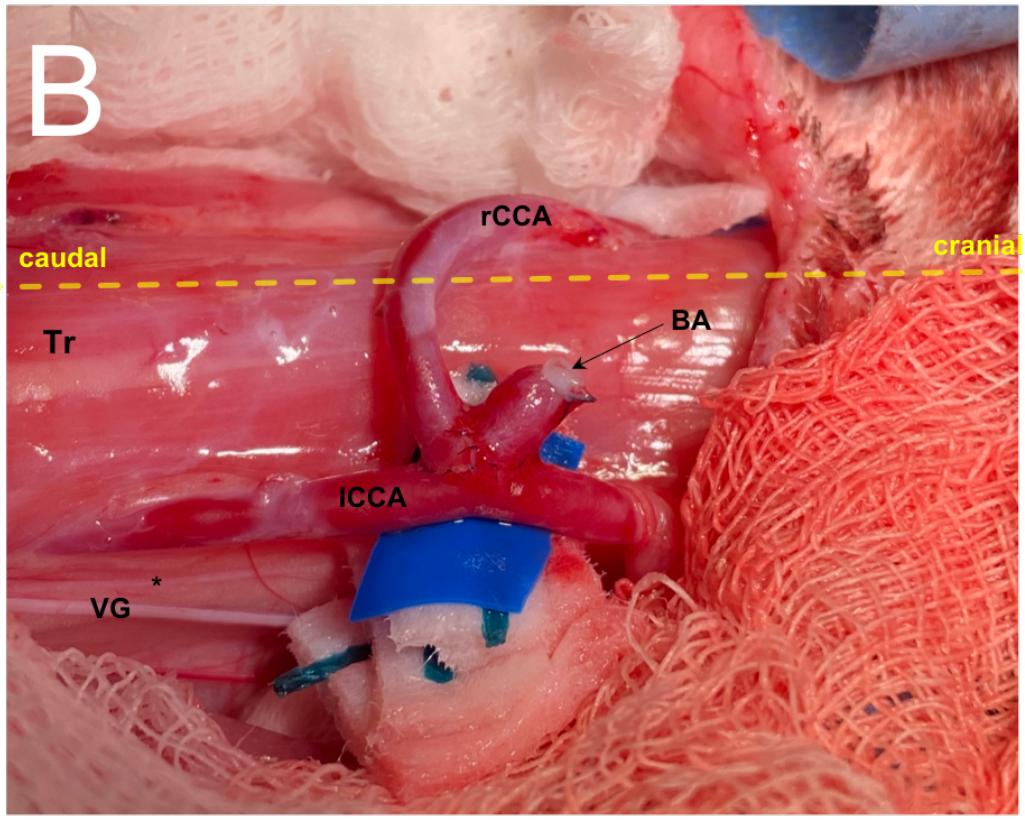


Figure 3

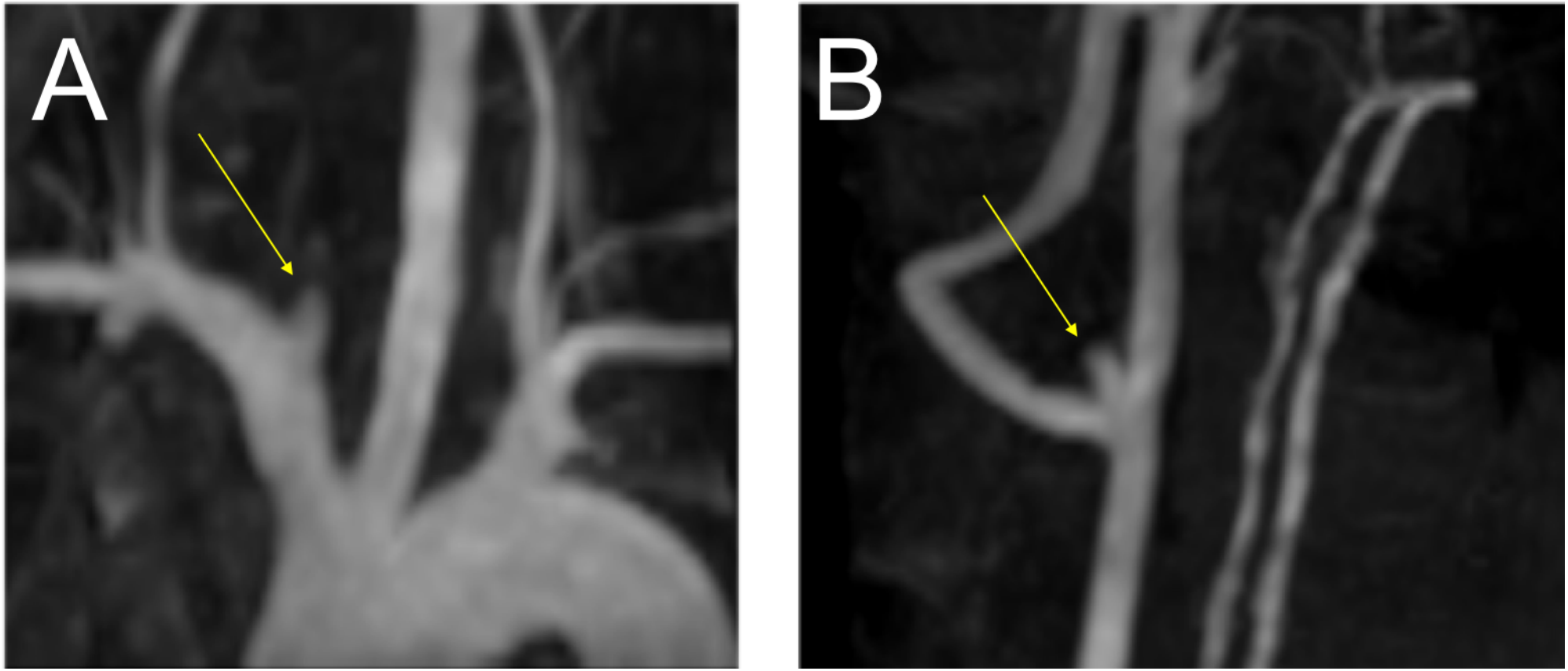


Figure 4

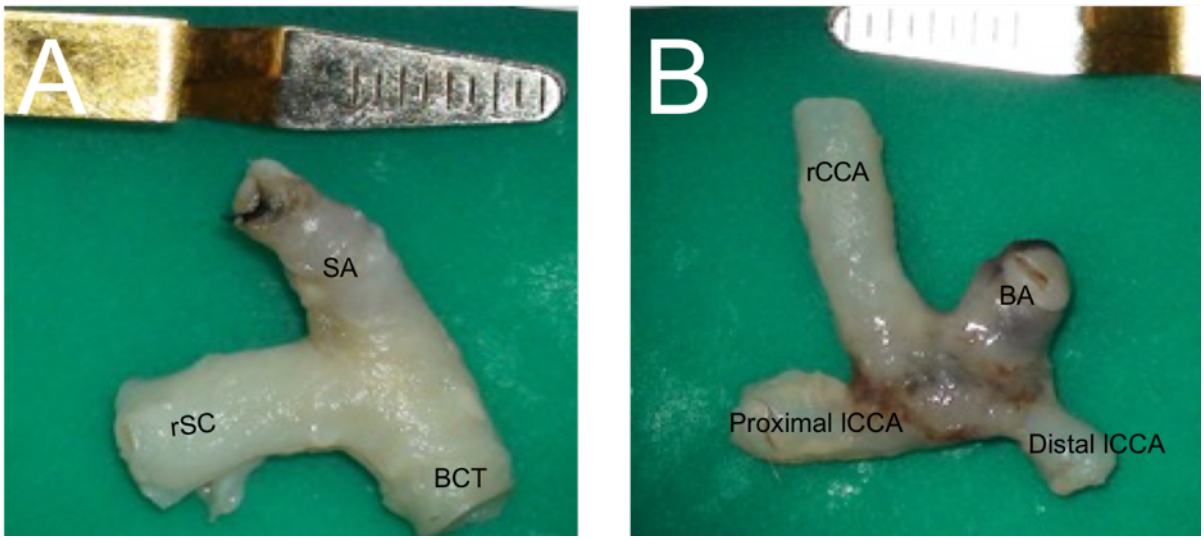
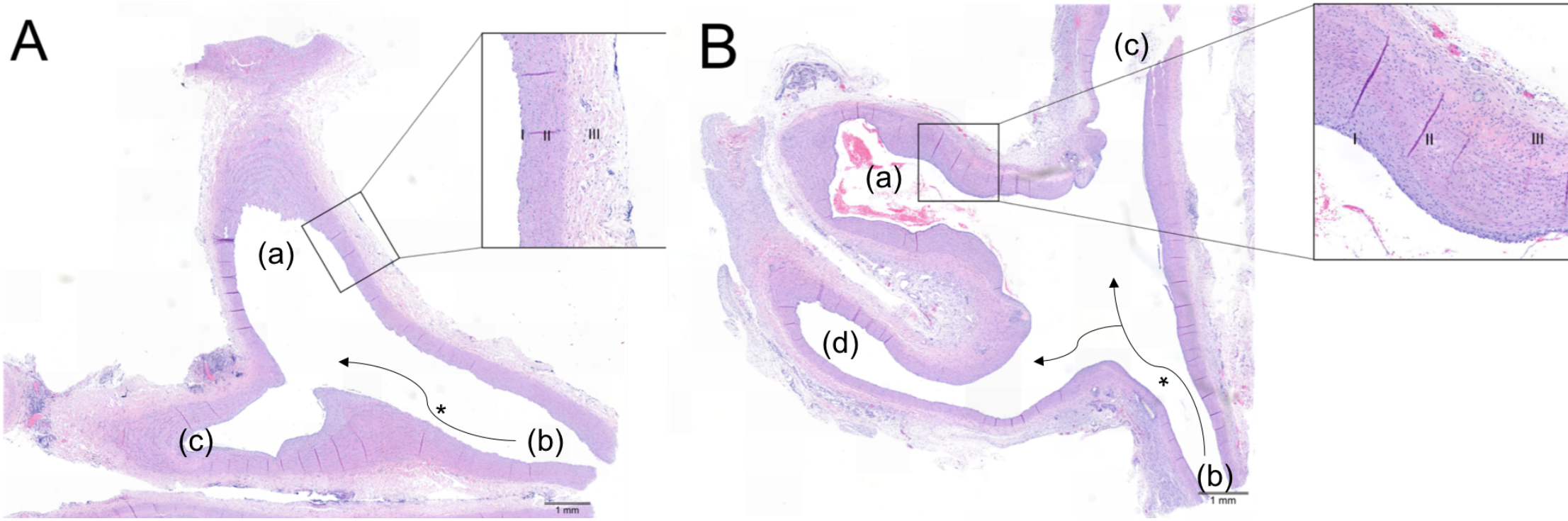


Figure 5



Name of Material/ Equipment	Company	Catalog Number
4-0 resorbable suture	Ethicon Inc., USA	VCP292ZH
4-0 resorbable suture	Ethicon Inc., USA	VCP304H
6-0 non absorbable suture	B. Braun, Germany	C0766070
9-0 non absorbable suture	B. Braun, Germany	G1111140
Adrenaline	Amino AG	1445419
Amiodarone	Helvepharm AG	5078567
Anesthesia machine	Dräger	
Aspirin	Sanofi-Aventis (Suisse) SA	622693
Atropine	Labatec Pharma SA	6577083
Bandpass filter blue	Thorlabs	FD1B
Bandpass filter green	Thorlabs	FGV9
Biemer vessel clip (2x)	B. Braun Medical AG, Aesculap, Switzerla	FD560R
Bipolar forceps		
Bispectral index (neonatal)		
Blood pressure cuff (neonatal)		
Bycilces spotlight		
Clamoxyl	GlaxoSmithKline AG	758808
Dexmedetomidine	Ever Pharma	136740-1
Elastase	Sigma Aldrich	**E7885**
Electrocardiogram electrodes		
Ephedrine	Amino AG	1435734
Esmolol	OrPha Swiss GmbH	3284044
Fentanyl (intravenous use)	Janssen-Cilag AG	98683
Fentanyl (transdermal)	Mepha Pharma AG	4008286
Fluoresceine	Curatis AG	5030376
Fragmin	Pfizer PFE Switzerland GmbH	1906725
Heating pad or heating forced-air warming system		
Isotonic sodium chloride solution (0.9%)	Fresenius KABI	336769
Ketamine	Pfizer PFE Switzerland GmbH	342261
lid retractor		
Lidocaine	Streuli Pharma AG	747466

Longuettes		
Metacam	Boehringer Ingelheim	P7626406
Methadone	Streuli Pharma AG	1084546
Micro-forceps curved	Ulrich Swiss, Switzerland	U52-015-15
Micro-forceps straight 2x	Ulrich Swiss, Switzerland	U52-010-15
Microscissors	Ulrich Swiss , Switzerland	U52-327-15
Midazolam	Accord Healthcare AG	7752484
Needle 23 G		
Needle holder		
O_2-Face mask		
Operation microscope	Wild Heerbrugg	
Papaverin	Bichsel	
Povidone iodine	Mundipharma Medical Company	
Prilocaine-lidocaine creme	Emla	
Propofol	B. Braun Medical AG, Switzerland	
Pulse oxymeter		
Rectal temperature probe (neonatal)		
Ringer Lactate	Bioren Sintetica SA	
Ropivacain	Aspen Pharma Schweiz GmbH	1882249
Scalpell	Swann-Morton	210
Small animal shaver		
Soft tissue forceps		
Soft tissue spreader		
Stainless steel sponge bowls		
Sterile micro swabs		
Stethoscope		
Surgery drape		
Surgical scissors		
Syringes 1 mL, 2 mL, and 5 mL		
Tris-Buffer	Sigma Aldrich	93302
Vascular clip applicator	B. Braun, Germany	FT495T
Vein and arterial catheter 22 G		

vessel loop		
video camera or smartphone		
Vitarubin	Streuli Pharma AG	6847559
Yasargil titan standard clip (2x)	B. Braun Medical AG, Aesculap, Switzerla	FT242T

Comments/Alternative
any generic
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any generic
any generic
any other
any other
any other
any other
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any other
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any generic
any generic
Approach

Medication
Sedaton
arteriotomy
topical application
any generic
General anesthesia
Infusion
Local anesthesia
Elastase solution

Approach

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Arau, 15.03.2021

Dear Editorial Board Members and Reviewers,

We want to thank you very much for the time you took to review our manuscript and for your pertinent questions and suggestions about this new animal model.

We took care to integrate and answer your comments, please find our revised manuscript and a supplementary figure attached.

We hope that our manuscript might now be considered for publication in JoVE.

Sincerely yours,

Gwendoline Boillat
Department of Neurosurgery, KSA

Authors' Response to Editorial Board Comments

Editorial board:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. E.g. lines 97, 228-229, 253, etc.

The manuscript has now been carefully proofread by a native English teacher.

2. Do not use parentheses for in-text reference citations. E.g. "…patients1" instead of "…patients(1)"

All parentheses have been deleted from the reference citations. The reference style has been reviewed in the whole document using Endnote with the JoVE EndNote style file.

3. Please provide details about the animals used – breed, gender, age etc. at the start of the protocol.

We added the following precision at the beginning of the protocol (lines 85-87):

"The animals were female New Zealand white rabbits with a mean weight of 4.0 (± 0.3) kg and mean age of 25 (±5) weeks and were housed at a room temperature of 22-24°C with a twelve-hour light/dark cycle with free access to water, pellets and hay".

4. Line 89-90: Please add some details about these steps. Alternatively, cite references for these.

This step describe the pre-anesthesia clinical exam, which is the routine fundamental step prior each anesthesia. This follow the recommendations of the AVA (Association of veterinary Anesthetists), as well as the ECVAA and ACVAA (European and American College of Veterinary Anesthesia and analgesia, respectively). It permits to evaluate the an aesthesia risk for every animal undergoing anesthesia and surgical procedure. In our specific case, it allowed to confirm that the rabbits are clinically unremarkable.

The references have been added into the text.

5. Use standard symbols and abbreviations for units: "µg" instead of "mcg", "oC", "mL" instead of "ml",etc. Include a single space between the quantity and its unit: "2 mm" instead of "2mm", etc. Follow this convention for the figure legends as well.

The whole manuscript (figure legends included) has been carefully checked and rectified with standard symbols and units.

6. Line 211: What is CAVE?

It is a Latin word which means "to pay attention". We changed it into "NOTE" (line 213).

7. Lines 258-263: Use decimal points instead of commas in the numbers. Also, for dimensions repeat the unit to remove any ambiguity. E.g.: 35 mm x 48 mm, 30 cm x 50 cm x 10 cm

The whole manuscript has been carefully checked and rectified with full stops instead of commas in the numbers, and the units have been repeated to remove ambiguity (now lines 260-266).

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Shorter steps could be combined.

We reviewed the manuscript and adapted the protocol in order to provide shorter steps and smaller paragraphs. We hope these changes meet your expectations.

9. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. E.g. ropivacaine, Draeger Primus, venflon, Yasargil, Dafilon, Vicryl, Silkam etc.

All the trademark symbols and company names have been removed from the manuscript and the table of materials has been checked.

10. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names.

The reference style has been reviewed in the whole document using Endnote with the JoVE EndNote style file.

Authors' Response to Reviewers Comments

Reviewer #1:

Manuscript Summary:
The reviewer appreciates the authors to provide the detailed information regarding the aneurysm model in rabbit, being especially useful for the development of endovascular devices.

The reviewer would like to ask the authors to provide some histopathological data of induced lesions to clarify whether the lesions share some histopathological features in human intracranial aneurysms.

Dear reviewer, thank you for your pertinent and precious comments.

We provided a new figure (figure 5) in order to offer a better overview of the histological findings with histological specimen (2 fold magnification) of a stump (A) and a bifurcation (B) aneurysms, stained with hematoxylin-eosin (lines 298-304). A 20-fold zoom of each aneurysm wall is included to better identify the tunica intima (I), media (II) and externa (III). The legend appears now at lines 298-304. Furthermore, in the discussion, we specified that our results correlate with the ones of the current literature concerning other elastase-induced aneurysms and human intracranial aneurysms (lines 383-387).

We hope this supplement will meet your expectations.

Reviewer #2:

Manuscript Summary:
In this paper, the authors describe a novel technique by which to create both a stump aneurysm as well as a bifurcation aneurysm in the same animal. 5 animals were used in these experiments, and aneurysms were successfully developed in all animals.

Major Concerns:
- The authors describe the purpose behind this study as creating two aneurysms distinct in hemodynamic conditions in the same animal. Furthermore, they state "The model offers assuredly the possibility to conduct further studies to investigate the differences between both models with a long-term follow-up in order to better characterize them. It also allows to test endovascular material in two distinct configurations and flow conditions in the same animal, and during a unique procedure. This reduces the number of animals needed and potentially increases the efficiency of preclinical trials." I would have liked to see a more complete discussion on how two different aneurysms could be used in the same animal. Is this simply a technique to provide two different conformations of aneurysms in the same animal, or is there more we can learn about aneurysm pathogenesis by further studying this model. If so, how would the authors propose to study aneurysms using this model? More details are required to better justify the aims of this study.

Dear reviewer, thank you very much for your review and the very complete comments.

This model offers now the possibility to investigate the differences between both aneurysms in term of growth, rupture, spontaneous occlusion and histological changes. Further studies are needed, with greater sample size and with different short and long term follow-up, to precisely determine the advantages and the characteristics of both types of aneurysms and their potential roles in the preclinical studies. Additionally, previous studies mentioned some differences in term of occlusion rate and reperfusion after endovascular treatment of stump or bifurcation aneurysms[1]. Our model allows to apply endovascular devices in two distinct configurations and flow conditions but in the same animal. This will allow a strict comparison of both configurations. These points appear now in our discussion (lines 393-399).

[1]Fahed, R. *et al.* Testing flow diversion in animal models: a systematic review. *Neuroradiology.* **58** (4), 375-382, (2016).

Minor Concerns:
1. With only 5 animals reported in this study, the sample size is small.

The main limitation of this work is the small number of animals used, which does not allow us any statistical analysis or a real comparison of the histological differences between stump and bifurcation aneurysm. However, we aimed here to provide a feasibility study about the technique and to introduce the surgical procedure. Further studies are now planned to better describe the difference between the stump and bifurcation aneurysm. We added this limitation to the discussion (lines 389-392).

2. The bifurcation anastomosis aneurysms as well as the stump aneurysms have reasonable shapes, but they are very small. In fact, these aneurysms were generally quite a bit smaller than typically treated human aneurysms (minimum 5mm in diameter on average). Furthermore, rabbit stump aneurysm volumes are generally reported to be on the order of 100 mm3 or more, and the ones in this manuscript were 35 mm3 and 29 mm3 for stump and bifurcation aneurysms, respectively. How would the authors suggest improving the size of aneurysms resulting from this model to produce more clinically relevant sizes? At the very least, the authors should comment on the size of the resulting aneurysms in the discussion, and perhaps list this as a limitation (as well as consider and describe other limitations of this model).

In this study, we created aneurysms with standardized dimensions between 2 and 4 mm based on our experience and our previous publications[1-4]. Our aim was to create a stump aneurysm with a similar size as the bifurcation's one to allow a strict and contingent comparison. The mean volume of our stump aneurysms is thus slight under the one sometimes described in the literature. However, it remains possible to adapt this size at the time of the aneurysm creation. Alternatively, both aneurysms showed a tendency to grow after 1 month follow up and longer follow up timings should offer the possibility to obtain aneurysms with greater volumes. This comment has been added to our discussion (lines 375-382).

[1]Sherif, C., Marbacher, S., Erhardt, S. & Fandino, J. Improved microsurgical creation of venous pouch arterial bifurcation aneurysms in rabbits. *AJNR Am J Neuroradiol.* 32 (1), 165-169, (2011).
[2]Marbacher, S. *et al.* Complex bilobular, bisaccular, and broad-neck microsurgical aneurysm formation in the rabbit bifurcation model for the study of upcoming endovascular techniques. *AJNR Am J Neuroradiol.* 32 (4), 772-777, (2011).
[3]Gruter, B. E. *et al.* Comparison of Aneurysm Patency and Mural Inflammation in an Arterial Rabbit Sidewall and Bifurcation Aneurysm Model under Consideration of Different Wall Conditions. *Brain Sci.* 10 (4), (2020).

[4]Wanderer, S. *et al.* Arterial Pouch Microsurgical Bifurcation Aneurysm Model in the Rabbit. *J Vis Exp.* 10.3791/61157 (159), (2020).
[5]Wanderer, S. e. a. Aspirin treatment prevents inflammation in experimental bifurcation aneurysms in New Zealand White rabbits. *Journal of NeuroInterventional Surgery.* Doi: 10.1136/neurointsurg-2020-017261, (2021).

3. It would have been very useful to provide histological data on the resulting aneurysms (including any possible histological differences between the stump and bifurcation aneurysms present at the time of extraction.

We provided a new figure (figure 5) in order to offer a better overview of the histological findings with histological specimen (2 fold magnification) of a stump (A) and a bifurcation (B) aneurysms, stained with hematoxylin-eosin (lines 298-304). A 20-fold zoom of each aneurysm wall is included to better identify the tunica intima (I), media (II) and externa (III). This appears now at lines 298-304. Furthermore, in the discussion, we specified that our results correlate with the ones of the current literature concerning other elastase-induced aneurysms and human intracranial aneurysms (lines 383-387).

We hope these supplements will answer your questions and meet your expectations.

Reviewer #3:

Manuscript Summary:
This is an interesting study in which the authors created both a sidewall and bifurcation saccular aneurysms in the same animal. This is a new and interesting technique which combines two already well established saccular aneurysm models. This study is important because it allows for the investigation of aneurysm treatment strategies under different flow conditions while eliminating between animal physiological differences.

Major Concerns:
- The major criticism of this study is that the authors did not investigate the histological changes of the sidewall and bifurcation aneurysm tissue. This data would greatly improve the impact of this study and would allow for a better understanding of the aneurysm biology in this model versus the currently used sidewall and bifurcation models.

Dear Reviewer,

Thank you very much for your positive feedback and your interesting comments.

We provided a new figure (figure 5) in order to offer a better overview of the histological findings with histological specimen (2 fold magnification) of a stump (A) and a bifurcation (B) aneurysms, stained with hematoxylin-eosin (lines 298-304). A 20-fold zoom of each aneurysm wall is included to better identify the tunica intima (I), media (II) and externa (III). This appears now at lines 298-304. Furthermore, in the discussion, we specified that our results correlate with the ones of the current literature concerning other elastase-induced aneurysms and human intracranial aneurysms (lines 383-387).

The main limitation of this work is the small number of animals used, which does not allow us any statistical analysis or a real comparison of the histological differences between stump and bifurcation aneurysm. However, we aimed here to provide a feasibility study about the

technique and to introduce the surgical procedure. Further studies are now planned to better describe the difference between the stump and bifurcation aneurysm. We added this limitation into the discussion (lines 389-392)

We hope this supplement will meet your expectations.

Minor Concerns:

1. In the discussion section, please discuss the relative difference in the aneurysm size, volume, and growth observed in this study compared to the established sidewall and bifurcation models.

In this study, we created aneurysms with standardized dimensions between 2 and 4 mm based on our experience and our previous publications[1-4]. Our aim was to create a stump aneurysm with a similar size as the bifurcation's one to allow a strict and contingent comparison. The mean volume of our stump aneurysms is thus slight under the one typically described in the literature. However, it remains possible to adapt this size at the time of the aneurysm creation. Alternatively, both aneurysms showed a tendency to grow after 1 month follow up and longer follow up timings should offer the possibility to obtain aneurysms with greater volumes. This comment has been added to our discussion (lines 375-382).

[1]Sherif, C., Marbacher, S., Erhardt, S. & Fandino, J. Improved microsurgical creation of venous pouch arterial bifurcation aneurysms in rabbits. *AJNR Am J Neuroradiol.* 32 (1), 165-169, (2011).
[2]Marbacher, S. *et al.* Complex bilobular, bisaccular, and broad-neck microsurgical aneurysm formation in the rabbit bifurcation model for the study of upcoming endovascular techniques. *AJNR Am J Neuroradiol.* 32 (4), 772-777, (2011).
[3]Gruter, B. E. *et al.* Comparison of Aneurysm Patency and Mural Inflammation in an Arterial Rabbit Sidewall and Bifurcation Aneurysm Model under Consideration of Different Wall Conditions. *Brain Sci.* 10 (4), (2020).
[4]Wanderer, S. *et al.* Arterial Pouch Microsurgical Bifurcation Aneurysm Model in the Rabbit. *J Vis Exp.* 10.3791/61157 (159), (2020).
[5]Wanderer, S. e. a. Aspirin treatment prevents inflammation in experimental bifurcation aneurysms in New Zealand White rabbits. *Journal of NeuroInterventional Surgery.* Doi: 10.1136/neurointsurg-2020-017261, (2021).

2. Although easy to read the manuscript needs to be edited by a native English speaking colleague or by an editing service.

The manuscript was now carefully proofread by a native English teacher.