ORIGINAL RESEARCH

In situ decellularization of a large animal saccular aneurysm model: sustained inflammation and active aneurysm wall remodeling

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ABSTRACT

Objective To investigate in situ decellularization of a large animal model of saccular aneurysm as a strategy for achieving aneurysmal growth and lasting inflammation.

Methods 18 New Zealand White rabbits were randomized 2:1 to receive endoluminal sodium dodecyl sulfate infusion (SDS, 1% solution, 45 min) following elastase or elastase-only treatment (control). All aneurysms were measured by digital subtraction angiography every 2 weeks. Every 2 weeks, three of the rabbits (two elastase + SDS, one control) underwent MRI, followed by contrast injection with myeloperoxidase (MPO)-sensing contrast agent. MRI was repeated 3 hours after contrast injection and the enhancement ratio (ER) was calculated. Following MRI, aneurysms were explanted and subjected to immunohistopathology.

Results During follow-up MRI, the average ER for SDS-treated animals was 1.63±0.20, compared with 1.01±0.06 for controls (p<0.001). The width of SDS-treated aneurysms increased significantly in comparison with the elastase aneurysms (47% vs 20%, p<0.001). Image analysis of thin sections showed infiltration of MPO-positive cells in decellularized aneurysms and surroundings through the 12-week observation period while control tissue had 5–6 times fewer cells present 2 weeks after aneurysm creation. Immunohistochemistry demonstrated the presence of MPO-positive cells surrounding decellularized lesions at early time points. MPO-positive cells were found in the adventitia and in the thrombi adherent to the aneurysm wall at later time points.

Conclusions In situ decellularization of a large animal model of saccular aneurysms reproduces features of unstable aneurysms, such as chronic inflammation (up to 12 weeks) and active aneurysm wall remodeling, leading to continued growth over 8 weeks.

INTRODUCTION

Contemporary large animal models of saccular aneurysms include surgical anastomosis of a vein pouch to an arteriotomy in pigs, rabbits, and dogs or elastinolysis of the common carotid artery in rabbits (reviewed in Marbacher et al 1,2). However, a short time after aneurysm creation,3 these models develop into stable aneurysms that do not demonstrate characteristics of vulnerable aneurysms in humans—namely, inflammation, intramural hemorrhage, and ultimately, rupture (reviewed in Tulamo et al 4), showing only some areas of thin, acellular aneurysm wall.5 To deal with these model limitations, an innovative strategy has been developed to implant decellularized arterial grafts in rodents6 and rabbits,7 the former model showing both growth and rupture of these aneurysms. The objective of our study is to explore the feasibility of inducing in situ decellularization in a common rabbit model of saccular aneurysms8 to recapitulate pathological features of unstable aneurysms and to perform comparative longitudinal non-invasive aneurysms and inflammation-induced MRI signal changes, and pathology of a decellularized saccular aneurysm model as compared with the standard model in a longitudinal study.

METHODS

In vivo feasibility study

All animal research activities were approved by the Institutional Animal Care and Use Committee (IACUC). Initially, an ex vivo study was performed on rabbit carotid arteries to assess feasibility of short exposure to sodium dodecyl sulfate (SDS) to efficiently decellularize the tissue (online supplemental methods). The different stages of the experiment are provided in online supplemental figure 1, which gives details of the variables of each experiment. Eight New Zealand White male rabbits (weight range 3.0–3.5 kg) were used for a pilot study to determine if the animals could tolerate the procedure. All procedures were performed under general anesthesia. Before all surgical procedures, the animals were anesthetized by a subcuticular injection of glycopyrrolate (0.01 mg/kg). Anesthesia was induced by an intramuscular injection of ketamine (35 mg/kg) and xylazine (5 mg/kg) and maintained with mechanical ventilation of 1–3% isoflurane. The physiologic status of the animal was assessed using continuous monitoring of respiration rate, heart rate, oxygen saturation level, end-tidal CO2 level, and temperature. At the conclusion of the experiment, the animals were euthanized by overdose of sodium pentobarbital (150 mg/kg). Similar to elastase incubation, all SDS infusions were...
performed with balloon occlusion of the right common carotid artery origin to prevent systemic administration.

Three rabbits underwent standard elastase incubation, followed by infusion of 1% SDS for 15 min, 60 min, or 2% SDS for 60 min. After 2 weeks, the animals were euthanized. To optimize the SDS infusion protocol, five rabbits underwent bilateral common carotid artery (CCA) exposure followed by placement of a 6F hemostatic introducer, allowing for 10 tests. All arteries were exposed to 50 U of elastase for 15 min. In three of the rabbits, once the elastase incubation was completed, a 1% solution of SDS was infused slowly (30 mL/h) via the microcatheter under balloon protection for 15, 30, or 45 min (figure 1). The SDS was continuously infused via the microcatheter under balloon occlusion of the right CCA origin and circulated distally where it exited the introducer. To assess if the order of elastase and SDS treatment influenced the overall decellularization, the last two animals underwent acute SDS treatment before elastase incubation.

After completion of elastase/SDS incubation, each artery was flushed with 10 mL of saline. After the animals were euthanized, then CCAs were explanted and placed in 10% formalin overnight. The specimens were embedded in paraffin, thin sectioned, stained with hematoxylin and eosin (H&E), and then imaged (Leica DM6 B). Images were analyzed for the density of cell nuclei within the smooth muscle layer. Comparisons were made with the innominate artery as control.

Longitudinal in vivo study
The optimized SDS infusion protocol (1% SDS infusion for 45 min) was used in a longitudinal in vivo study. Eighteen New Zealand White rabbits (sex: either, weight range 3.0–3.5 kg) were randomized 2:1 to receive elastase + SDS and one elastase, were given Gadavist (0.1 mmol Gd/kg, Bayer HealthCare, New Jersey, USA), instead of the MPO-sensitive contrast agent; the same rabbits were then reimaged 4 weeks later with the MPO-sensitive contrast agent. Following MRI, the animals were euthanized, the aneurysm was exposed, flushed with cold saline, filled with optimal cutting temperature (OCT) compound, and snap frozen in liquid nitrogen. Detailed methods for histology and microscopy can be found in the online supplemental data.

Quantification and statistical analysis
For quantification of the MR signal before and after contrast injection, an annular region of interest (ROI) was drawn to include the wall of the aneurysm but exclude the center of the lumen. A second ROI was drawn in the muscle tissue to normalize the signal between scans. Then the enhancement ratio (ER) was calculated by

\[ ER = \frac{Anx_{pre}}{Anx_{post}} / \frac{Mus_{pre}}{Mus_{post}} \]

where ‘Anx’ is the aneurysm ROI, ‘Mus’ is the muscle reference, ‘pre’ is the baseline scan, and ‘post’ is repeat scanning following contrast administration. All ER analysis was performed on the axial MSDE imaging, ranging from one to three slices depending on the size of the aneurysm.

All statistical calculations were performed with R3.5.2 using publicly available packages. For those data that were normally distributed either a Welch’s t-test or an analysis of variance were used to determine if a factor was significant, followed by a Tukey post hoc test. For those that were not normally distributed, a Mann-Whitney U test was used. An alpha of 0.05 was significant for all tests.

RESULTS
In vivo feasibility study
Incubation of 2% SDS for 60 min led to rupture of the CCA (online supplemental figure 2) and the animal died. Incubation of 1% SDS for 15 min failed to achieve decellularization of the CCA, and 60 min led to a decline in animal health that required daily care. Continuous infusion of 1% SDS was well tolerated in all animals. The 45 min continuous infusion of 1% SDS protocol led to optimal decellularization without acute rupture or other clinical sequelae and included histological observations of intramural hemorrhage (figure 2A–D). Measurement of nuclear density showed that continuous infusion of 1% SDS for 45 min resulted in almost complete decellularization of the CCA having less than 10% of the control artery cell (figure 2E) nuclei density

Figure 1  Schematic depiction of isolation of the right common carotid artery from the circulating blood pool. SDS is infused slowly through the microcatheter, into the RCCA, and cleared via the introducer side-port, following the pathway of the blue arrows. LCCA, left common carotid artery; RCCA, right common carotid artery; SDS, sodium dodecyl sulfate. 
Longitudinal in vivo study
Of the 18 rabbits used in the longitude study, 17 reached the designated endpoint. One animal in the elastase + SDS group died during recovery due to rupture of the CCA, leading to an overall morbidity rate of 0% and mortality rate of 8% in the elastase + SDS group, and 0% morbidity and mortality in the elastase only group. The carotid artery diameter in the two groups was not significantly different at baseline (2.4 mm vs 2.2 mm, p=0.23). Addition of the SDS modification led to continuous increase of aneurysm width (figure 3) during the entire observation period. Aneurysm width increase in the control group stabilized after 2 weeks. This led to significant increase in the aneurysm width in the elastase + SDS group at 4, 6, and 8 weeks (p<0.001) with an absolute width difference of 0.92 mm between the two as compared to control.

For the 17 rabbits that underwent MRI after aneurysm creation (figure 4A–D), 11 were treated with SDS while six were control. At all time points the SDS group showed enhancement over the control group with an average ER of 1.63 vs 1.01 (p<0.001, figure 4E). The only time that the control aneurysms showed an enhancement larger than 1.1 was at the 2-week time point, which is known to be within the healing period following elastase incubation.3 8 At the 2-week time point, enhancement for the SDS infused group extended beyond the wall of the aneurysm to the perivascular space. Four weeks following aneurysm creation, enhancement was largely limited to the aneurysm wall. At 8 weeks, the two rabbits that were imaged using Gadavist revealed minimal enhancement (figure 4C); however, at 12 weeks the SDS infused rabbit did show enhancement with the MPO-specific contrast agent (figure 4D).

Immunohistochemistry and fluorescence microscopy
Microscopy of thin sections of rabbit aneurysm samples performed at an early time point (2 weeks after SDS treatment) revealed major differences between the SDS-treated and control (elastase only) groups of rabbits. On aneurysm samples sectioned longitudinally the differences on regular H&E-stained sections were apparent in overall morphology, remnant vessel thickness, the presence of lesions, and the number of infiltrating cells (online supplemental figure 3). We observed more frequent mural thrombus of aneurysms associated with SDS treatment at earlier time points (2–6 weeks), and multiple lymphocyte infiltrations in the area of decellularization, which showed gradual healing with fibroblast proliferation alongside infiltration of MPO-positive cells (see online supplemental histology). Identification

![Figure 2](image2.png)

**Figure 2**  Histology of decellularization. (A) 15 min sodium dodecyl sulfate (SDS) infusion, some nuclei removed, but overall no large areas of decellularization. (B) 30 min SDS infusion, complete removal of nuclei in some areas, with patches of intact cells in other areas. (C) 45 min SDS infusion, almost complete nuclei removal and thinning of the wall. (D) Intramural hemorrhage found after SDS infusion. (E) Control vessel used for quantitative comparison. (F) Number of cell nuclei per mm² left after SDS treatment of rabbit model elastase-induced aneurysms, data shown as mean±SD. Scale bars are 250 μm.

![Figure 3](image3.png)

**Figure 3**  The overall change in aneurysm width comparing the sodium dodecyl sulfate (SDS)-treated aneurysms with the elastase-only control aneurysms. After the 2-week time point, the control aneurysms stopped growing, while the SDS-treated aneurysms continued to grow to the 8-week time point. *p<0.001.

![Figure 4](image4.png)

**Figure 4**  MRI scan of rabbit aneurysm models. (A) Standard elastase aneurysm, arrow indicates the wall of the artery. No enhancement was observed following administration of the myeloperoxidase (MPO)-sensitive contrast agent. (B) Sodium dodecyl sulfate (SDS) treated rabbit at the same time point as the control showing clear enhancement of the aneurysm after administration of the MPO-sensitive contrast agent. (C) Eight-week imaging of the SDS rabbit after Gadavist injection; almost no enhancement was seen. There was slight thickening of the wall (arrow). (D) Twelve-week MRI scan of the same rabbit (see panel C), this time with the MPO-sensitive MR contrast agent. Clear enhancement of MR images was noted. Arrows indicate the aneurysm. (E) Enhancement ratio of SDS-treated versus control (elastase treatment only). Data was averaged over all time points, with the elastase + SDS a showing significantly higher enhancement ratio compared with control (1.63 vs 1.01, **p<0.001).
of MPO-positive cells using a red fluorescent substrate (figure 5) showed that both control (elastase-only, figure 5A) and elastase + SDS treated aneurysms (figure 5B) experienced an influx of red fluorescent MPO-positive inflammatory cells. However, in control aneurysm sections MPO-positive cells were less numerous and this difference was more pronounced at the later observation time points (4–12 weeks). To track the cell numbers and their MPO-positive area on sections we performed artificial intelligence (AI)-assisted segmentation and quantification of cell-associated versus background fluorescence (online supplemental figure 4) using AI-generated probability maps that reflected the distribution of MPO activity (online supplemental figure 4C and D). On fluorescence images, two types of MPO-positive cells could be roughly classified as ‘large’ cells (area >45 µm²), which were morphologically similar to monocytes/macrophages, and ‘small’ cells (area <45 µm²), morphologically identical to neutrophils. The proportion of macrophages in the total population present in SDS-treated aneurysms remained high throughout the experiment. The numbers of large cells decreased in the control aneurysms group together with the total cell number per section area, as shown in figure 6A,B. Both types of cells were present as MPO-antigen positive cells (figure 6A), some of which were monocytes/macrophages, while many more cells showed strong staining with anti-calprotectin (S100A8/A9 complex) antibodies, which react with both monocytes/macrophages and granulocytes on frozen sections (online supplemental figure 4). The decellularized regions of the vessel wall showed an extensive network of collagen fibrils that had strong autofluorescence under exciting blue light. These areas were surrounded by “swarms” of MPO-positive neutrophils (figure 6C, online supplemental figure 5A). Some of the neutrophils were observed infiltrating decellularized collagen and forming MPO-positive neutrophil extracellular traps (figure 5D,E).

**DISCUSSION**

Treatment of rabbit carotid arteries with elastase results in characteristics resembling those of human saccular cerebral aneurysms. The rabbit model of saccular aneurysms created through elastinolysis is well-suited for neurointerventional device testing. Recent literature has suggested that the intact nature of the wall of aneurysm models might lead to increased rates of healing in response to endovascular treatment. This could explain the differences seen between meta-analysis of human aneurysm coiling data showing occlusion rates of less than 60% in 14–15 and recurrence rates as high as 28% compared with a more limited dataset of coil treated rabbit aneurysms with recurrence rates between 0% and 17%. The ability of the smooth muscle cells within the lining of the rabbit aneurysm wall to affect and increase the rate of coiled aneurysm healing could explain this disparity, and an aneurysm model that more closely mimics that of the more dangerous acellular type D would allow for a closer comparison with healing rates in humans. Interestingly, there does not appear to be a difference in the healing rate for flow diverting stents; 60%–80% occlusion rate in humans compared with 74% for rabbits, suggesting that the rate is due to healing of the intact parent artery and not of the aneurysm wall. Overall, SDS treatment of an elastase-induced aneurysm model could allow for better understanding of the biological response to closing and other intrasaccular devices.

The standard elastase-induced saccular aneurysm model has been recently shown to recapitulate several characteristics of human aneurysms pathology. Over several weeks the aneurysm models may develop multiple human-like pathology phenotypes and some of them bear signatures of potential instability. However, an aneurysm model with predictable growth patterns and instability-linked molecular marker expression is still lacking. We hypothesized that in situ decellularization of a commonly used large animal model of saccular aneurysms may lead to active wall remodeling. Decellularization primarily leaves the collagen and elastin matrix as an available basement for potential new growth (figure 2 and online supplemental figure 3). While the aneurysm is healing, there appears to be active remodeling and continued migration of cells containing myeloperoxidase—that is, neutrophils and macrophages, into the vessel wall (figure 5). Consistent with inflammatory cell presence, as determined by cells positive for MPO activity (fluorescent substrate) and MPO

**Figure 5** Immunofluorescence (IF) and immunohistochemistry (IHC) of rabbit aneurysms at 2 weeks after creation. (A) IF image of control rabbit aneurysm; (B) IF image of sodium dodecyl sulfate (SDS)-treated rabbit aneurysm. Red → myeloperoxidase (MPO) substrate (5HT-Cy3), green → autofluorescence of collagen, blue → 4',6-diamidino-2-phenylindole (nuclei); (C) IHC of SDS-treated rabbit aneurysm. Blue → DIG-labeled anti-MPO mAb/anti-DIG-AP conjugate. (D) IHC of neutrophils and neutrophil extracellular traps in decellularized area, (E) Control IHC image (no anti-MPO mAb). L, lumen. *Decellularized area of the vessel wall.

**Figure 6** Distribution of myeloperoxidase (MPO) positive cell numbers and cell areas per section of sodium dodecyl sulfate (SDS)/elastase-treated rabbit aneurysm and control elastase-only models and the measurements of time-dependent changes in MPO-positive cell numbers. (A) Distribution of MPO-positive cells derived from 95% probability maps obtained by trainable wekasegmentation (TWS) processing of fluorescent images of rabbit sections obtained at different time points. Insets show representative enlarged (250x) color fluorescent images of ‘large’ cells (area >45 µm²) and ‘small’ cells (area <45 µm²); (B) cumulative MPO-positive cell number change per section area over time as measured in animals at the indicated time points. The data are shown as mean±SD, (n=2–3 sections/time point).
antigen (anti-MPO antibody staining), was in vivo MPO activity imaging performed during the period of up to 12 weeks following SDS-modified aneurysm creation. This is a long-term increase in comparison with the standard elastase model, which showed only elevated inflammatory infiltrates within the first 3 weeks of creation. It has been shown that progressive amounts of cell loss, inflammation, and mural thrombus are associated with rupture status, suggesting cellular mechanisms of aneurysm instability. Moreover, the standard rabbit elastase model can demonstrate all four of the human aneurysm wall states. Thrombus formation in the standard elastase model was seen only in the apex of the aneurysm, where over time the previously patent carotid artery undergoes thrombosis. Inflammation was observed only at 2 weeks after elastase incubation. The use of decellularization in surgical graft aneurysms has been recently reported in rabbits, with similar finding of increased inflammation in the decellularized aneurysms. However, unlike the surgical model, this endovascular model does not involve vascular response to suturing at the neck. The addition of SDS in the aneurysm creation process showed evidence of all three characteristics of unstable human aneurysms—namely, hypocellular walls, continued inflammation over 12 weeks, and mural thrombus within the aneurysm lumen at numerous locations.

The prevalence of the use of MR vessel wall imaging for the screening and follow-up of aneurysms has increased. The goal is to identify at-risk patients who show enhancement within the wall of the aneurysm after a contrast injection; however, the exact mechanism and interpretation of enhancement is under debate. It is known that ruptured aneurysms show higher levels of inflammation, neutrophil and macrophage presence, and secreted enzymes such as MPO, and the signatures of inflammation could be collectively or individually considered as biomarkers of at-risk aneurysms. The development of small-molecule MR contrast agents that selectively accumulate in the area of MPO, and thus inflammation, helped to clarify this subject; with the ability to filter out many of the associated artifacts. Thus it provided assurance that the accumulation of inflammation-specific low molecular weight imaging agent in the aneurysm and the MR signal enhancement seen is not simply a flow artifact, or extensive vasavasorum. Here we clearly demonstrated that an aneurysm that is undergoing active inflammation; 2 weeks for elastase only, or 12 weeks for elastase + SDS, shows selective enhancement with an MPO-specific MRI contrast agent. Furthermore, we have presented evidence that the standard clinical MR contrast agent (gadobutrol) does not show the same level of continued enhancement.

The model of in situ decellularization is still limited by some of the aspects common to all extracranial models. Because the perivascular tissues surrounding carotid arteries are nothing like those of the brain, the source of the neutrophils and, consequently, MPO, are not that same as human brain aneurysms. There is a lymph node in the proximity of the CCA, which is an additional potential source of macrophages and neutrophils, which are migrating into the area caused by SDS damage to the vessel wall. However, our goal was to create aneurysms that harbor ongoing inflammation, and though the traffic of neutrophils and macrophages may be dissimilar to that of a human, the outcomes may be comparable. Furthermore, we were not able to observe the aneurysm rupture during a relatively short observation period, other than in the reported acute case.

CONCLUSIONS
We describe a method of in situ decellularization in a large animal model of saccular aneurysms. This model included active remodeling and aneurysm growth, persistent inflammation over at least a 12-week period, and intramural thrombus. Although no ruptures occurred during follow-up, this model exhibits characteristics of unstable aneurysms in humans.

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Contributors All authors: designed and performed the experiments, analyzed the data, drafted the manuscript, approved the final manuscript.

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