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Human fascia lata ECM scaffold augmented with immobilized hyaluronan: inflammatory response and remodeling in the canine body wall and shoulder implantation sites

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Abstract

We postulate that immobilization of tyramine-substituted hyaluronan (THA) into an extracellular matrix (ECM) scaffold may be a strategy to promote an anti-inflammatory response to the ECM. Further, we posit that the implantation site could influence the inflammatory response and remodeling of an ECM scaffold. Eight beagles underwent implantation of fascia ECM grafts, treated with either immobilized low molecular weight (57 kDa) THA or water only, in both the shoulder injury and body wall sites. Dogs were euthanized at 12 weeks and fascia grafts harvested en bloc for histology. Grafts implanted at the body wall had significantly higher inflammatory cell infiltrate and vascularity, and significantly lower retardance (collagen density), than grafts at the shoulder, suggestive of a more intense, persistent, and perhaps degradative inflammatory and remodeling response at the body wall than shoulder injury site in the canine model. However, the presence of immobilized low MW THA had no effect on the inflammation response or remodeling of fascia ECM compared to water-treated controls. Importantly, these results suggest that the inflammatory response and remodeling of biomaterial implants depends on the location of implantation and therefore our animal models need to be carefully chosen. Further, the potential anti-inflammatory advantages of hyaluronan (HA) in wound healing do not appear to be realized when presenting it to the host as non-degradable hydrogel even if its capacity for binding HA

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Disclosure

Dr. Derwin is an inventor on patents related to reinforced tissue matrices and molecular enhancement of ECM. Dr. Calabro is an inventor on patents related to tyramine-substituted hydrogel biomaterials and molecular enhancement of ECM and receives royalties from Lifecore.

Supplemental data

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binding protein is maintained. Further study treating ECM with uncross-linked (free) HA or immobilized low MW THA as a means to deliver free HA or other biomolecules to a surgical repair site is warranted.

Keywords

tyramine-substituted hyaluronan; biologic graft; graft remodeling; polarized light microscopy; animal model

Introduction

Currently, extracellular matrix (ECM) patches are being investigated for rotator cuff repair augmentation [1] based on the concept that ECM patches may provide a natural and mechanically robust scaffold that reinforces the repair, guides host cell infiltration, and fosters formation of a functional tendon-bone bridge.[2,3] To prevent or limit tendon retear, the ECM must maintain appropriate mechanical properties during the course of its remodeling. Since the host inflammatory response is a critical determinant of the remodeling outcome of an implanted ECM,[4,5] strategies to direct the inflammatory response to an ECM patch toward constructive remodeling and away from inflammation/degradation may enable the ECM patch to maintain adequate mechanical reinforcement of the repair throughout the healing process.

Hyaluronan (HA) is known to possess anti-inflammatory properties [6] leading to cell quiescence and decreased fibrosis/scar formation.[7] Studies have demonstrated that exogenous HA can inhibit the infiltration, proliferation, and cytokine production of various inflammatory cells.[6,8] In addition, HA has been shown to promote the migration of fibroblasts.[9] For this reason, HA has been widely investigated in tissue repair strategies, either as an injectable molecule,[10] as a pure hydrogel [11], or as part of a composite scaffold.[12] Based on the well-appreciated role of HA as an anti-inflammatory agent, we postulate that selective incorporation and immobilization of HA into an ECM scaffold may be a strategy to promote an anti-inflammatory response to the ECM. Specifically, we have utilized tyramine-substituted hyaluronan (THA), which has tyramine adducts coupled to the carboxyl groups of the glucuronic acid residues along 5% of the HA chain.[13] In the presence of hydrogen peroxide and peroxidase, neighboring tyramine adducts cross-link to form a dityramine bridge. The resulting hydrogel is not susceptible to hydrolysis and has a concentration-dependent resistance to hyaluronidase degradation.[13] TS-HA has been shown to be non-cytotoxic and biocompatible with suspended cells remaining metabolically active in the hydrogel.[13] The low tyramine substitution rate (5%) sufficiently maintains the native structure of HA to allow recognition and binding by HA binding proteins (HABP), even after cross-linking into hydrogel form.[13]

We reasoned that immobilized THA could allow for a sustained presentation of the anti-inflammatory properties previously reported for individual HA chains. Hence, we initially immobilized high molecular weight (MW) THA (0.9–1 MDa) into fascia lata ECM at an average concentration of 7.6 µg/mg tissue and investigated inflammatory response in a rat body wall model.[14] In this case, HA augmentation resulted an undesirable increase in

macrophage and giant cell densities, and a lower density of fibroblast-like cells than in water-treated controls,[14] and was characterized by persistent and prominent localizations of THA hydrogel within the fascia ECM matrix.[14] As such, the motivation for the current study was to incorporate low MW THA into fascia ECM to facilitate more uniform diffusion throughout the dense matrix prior to crosslinking, potentially eliminating the prominent localizations of THA and facilitating an anti-inflammatory role for it.

Further, although body wall sites have been common implant locations to screen the inflammatory response to biomaterials,[15,16] it is likely that the implantation environment could influence inflammatory response and remodeling of an ECM scaffold. In particular, mechanical load [17,18] and the presence of synovial fluid [19] have been shown to profoundly influence tendon-and ligament-to-bone repair tissue and could be crucial in determining the inflammatory response and remodeling of an ECM graft for rotator cuff repair. Hence, investigation of ECM implants in the body wall as well as the shoulder injury site was of particular interest. Since the small size of the rat shoulder limits the accuracy with which one can perform shoulder injury and graft implantation studies, the canine was used in this study to allow investigation in both body wall and shoulder injury models.

Here, we will incorporate and immobilize low MW THA (57 kDa) into fascia ECM at the same approximate final tissue concentration of THA (~7.5 µg/mg) as in our previous study with high MW THA (0.9–1 MDa).[14] Fascia grafts will be implanted at both body wall and shoulder injury sites in the canine model, and the inflammatory response and graft remodeling will be evaluated at 12 weeks. We hypothesize that the immobilization of low MW THA in fascia ECM will reduce the inflammatory cell infiltrate and decrease graft remodeling as compared to water-treated fascia. We further hypothesize that the inflammatory cell infiltrate and graft remodeling will be lower in fascia grafts implanted at the shoulder than at the body wall.

Methods

Study design

Eight female beagles (retired breeders, 4–6 years old) underwent bilateral implantation of fascia ECM grafts, treated with either THA or water, in the body wall and shoulder injury sites. Dogs were euthanized at 12 weeks, and fascia grafts from both sites, including surrounding tissues, were harvested en bloc and processed for histology.

Human fascia ECM grafts

Decellularized, lyophilized human fascia lata from the iliotibial tract of three human donors (Musculoskeletal Transplant Foundation, Edison, NJ, USA) was used. The fascia was processed aseptically and similarly to the antibiotic only treatment method (ABX) described previously.[20] Briefly, fascia pieces were washed in an antibiotic cocktail for 24 h at 37 °C with stirring. The antibiotic cocktail consisted of 1.6 µg/mL Amphotericin B (Sigma-Aldrich, St. Louis, MO), 30 µg/mL Primaxin (McKesson Surgical, San Francisco, CA), and 6 µg/mL Gentamicin sulfate (Alexis, Lausen, Switzerland) in PBS. Fascia were then rinsed twice for 20 min each in PBS at room temperature with constant stirring and lyophilized as

per American Association of Tissue Banks guidelines for soft tissue and stored at room temperature until use. Under aseptic conditions, three 5×5 cm fascia patches (one from each donor) were water treated, and three 5×5 cm fascia patches (one from each donor) were THA treated.

The following THA treatment protocol was designed to achieve the desired ~ 7.5 $\mu\text{g}/\text{mg}$ tissue weight of HA as in our previous study.[14] Specifically, fascia patches were rehydrated in ~ 2 ml of 0.19% low MW THA (57 kDa, Lifecore Biomedical, Chaska, MN) in ultrapure water containing 10 U/ml of horseradish peroxidase (Sigma, St. Louis, MO) for 24 h at 37°C on a shaker. For each sample, all treatment steps were performed in the minimum volume necessary to fully rehydrate the sample. The exact treatment volume used was calculated from the dry weight of the given sample times an 'absorption coefficient' of 2.9 $\mu\text{l}/\text{mg}$, which was determined empirically from pilot studies. Fascia patches were subsequently lyophilized overnight and rehydrated a second time in ~ 2 ml of 0.19% THA. Fascia patches were again lyophilized overnight and then cross-linked by rehydrating in ~ 2 ml of a 0.03% hydrogen peroxide solution (Fisher, Pittsburgh, PA) in ultrapure water for 1 h at room temperature and then at 4°C overnight. Patches were subsequently washed twice over 24 h. Each wash was carried out using 10 ml of ultrapure water at 4°C on a shaker. Control fascia patches were rehydrated once in ~ 2 ml of ultrapure water containing 10 U/ml of horseradish peroxidase for 24 h at 37°C on a shaker, followed by the identical lyophilization, rehydration in 0.03% hydrogen peroxide, and rinsing steps as THA-treated fascia patches.

After treatment, each fascia patch was lyophilized and cut into ten 10×25 mm samples. Eighteen water- and THA-treated samples were reserved for implantation, and the remaining 12 samples in each group were used to verify with fluorophore-assisted carbohydrate electrophoresis [20] that HA content in the fascia reached our intended level of ~ 7.5 $\mu\text{g}/\text{mg}$. [14] Indeed, the HA content in THA-treated fascia averaged 7.3 ± 1.7 $\mu\text{g}/\text{mg}$ tissue weight and in water-treated control implants averaged 1.7 ± 0.6 $\mu\text{g}/\text{mg}$ tissue weight. To facilitate surgical fixation of the implants, a non-resorbable 2–0 polypropylene fiber (Proxymed Biomedical Ltd, Galway, Ireland) was stitched into each sample in a U-shaped pattern, leaving 8 cm free lengths of fiber extending from one end for fixation (Figure 1(A)). All grafts were implanted with the deep/longitudinal fascicular layer facing the host tissues.

Surgical methods

Surgical procedures were approved by the IACUC at our institution. The dogs were initially anesthetized with an intravenous dose of sodium methohexital (10 mg/kg) to effect and then were intubated orotracheally and maintained on isoflurane in oxygen (3%). Dogs underwent bilateral body wall implantation of fascia grafts. Briefly, 10×25 mm portions of the rectus sheath were resected bilaterally in proximity to the umbilicus and approximately 1 cm on either side of the midline. A water-treated (H₂O-BW) or THA-treated (THA-BW) fascia graft was secured into each defect using 2–0 Prolene® (Ethicon Endo-Surgery) simple sutures on the closed end of the stitched loop caudally and the free ends of the stitched fiber in a simple suture configuration cranially (Figure 1(B)). Dogs also underwent bilateral shoulder surgery to implant water-treated (H₂O-SH) or THA-treated (THA-SH) fascia

grafts. Briefly, in each shoulder the joint capsule under the infraspinatus tendon was resected to model an open intraarticular injury [21] (Figure 1(C)), but the tendon was otherwise not injured.[22] A fascia graft was laid over the bursal surface of the infraspinatus tendon, insertion site and bone, and affixed over the tendon medially and bone laterally (Figure 1(D)). Surgical methods are detailed in Supplemental material.

Analgesia and post-operative care

For pain management, dogs were affixed with a transdermal Fentanyl patch (50 µg/h) starting 18 h prior to surgery and for 72 h in total. Postoperatively, dogs were given 500 mg cephalexin orally twice daily for seven days as a prophylactic antibiotic and allowed immediate, unrestricted cage activity. Dogs were individually housed for the first post-operative week and group housed thereafter.

Euthanasia and tissue harvest

At 12 weeks, dogs were euthanized using a lethal IV injection of barbiturate (1 mL/ 4.5 kg; Beuthanasia-D®; WA Butler, Dublin, OH, USA). At the shoulders, it was noted that the joint capsule injury was partially healed but remained patent to some extent in all animals. The fascia grafts from all sites were harvested en bloc and together with surrounding tissues. All samples were fixed in 10% neutral buffered formalin for 24 h, decalcified in 5% trichloro-acetic acid solution for 8 days, and then routinely processed for paraffin embedding and histologic analysis.

Histologic analysis

Five micron thick longitudinal sections were obtained at five step levels (separated by at least 100 µm) from each tissue block. Sections were stained with hematoxylin and eosin (H&E) or left unstained.

Inflammatory response—One H&E section from three of the five step levels in each tissue block was scored by a board-certified pathologist (CT) for inflammatory cells, fibroblast-like cells, total cellularity and vascularity (blood vessels) within the graft, according to a semi-quantitative scoring system adapted from the International Organization for Standardization (ISO) Standard 10993–6 and used in our previous work.[14] Only the portion of the fascia graft over the tendon insertion site and tendon proper was scored for the shoulder samples because the large amount of suture at the bone end confounded the morphology and scoring of the graft at that location. The entire length of the fascia graft at the body wall location was scored. The scores from the three sections per sample were averaged to represent the group/location scores for a given animal.

Azimuth angle and retardance—Slow axis azimuth angle and retardance are measures of collagen orientation and density, respectively,[22,23] and changes in these parameters reflect alterations in the collagen network.[24] To measure these parameters, one unstained section from the third step level in each tissue block was analyzed by circular polarized microscopy (Abrio Birefringence Microscopy Imaging System, CRI Inc, Woburn, MA) for azimuth angle and retardance. Eight to twelve 20× images were analyzed along the length of the fascia graft for each section. Data from all images for a given section (animal) were

combined to generate one azimuth angle histogram with two degree bins and one retardance histogram with 2 nm bins for each animal. The mode of each histogram (i.e. the bin with maximum frequency) was determined. The full width at half maximum (FWHM), defined as the width of each histogram at a frequency equal to half the frequency at the mode, was also determined.

Statistical analysis

To account for the dog-to-dog variability, a two-factor analysis of variance (ANOVA) was used with dog and treatment as factors to test for differences between treatment (H2O and THA) and implantation sites (BW and SH) for all outcomes. Where ANOVA showed significant differences (i.e. $p < 0.05$), a Tukey HSD *post hoc* test was used to identify significant differences between experimental groups ($p = 0.05$). The statistical analyses were performed with JMP Pro 9.

Results

Qualitative histologic observations

Representative low-magnification histologic sections from the body wall (Figure 2(A)) and shoulder (Figure 2(B)) are provided for perspective of graft location with respect to host tissues. At the body wall, cellular infiltration by inflammatory cells and spindle-shaped fibroblast-like cells (white arrows) was seen throughout the fascia grafts in both treatment groups (Figure 3(A) and (B)). In both groups, the inflammatory cell infiltrates were a mixture of lymphocytes (Figure 3(A) and (B), (circles)), plasma cells (Figure 3(A) and (B), (+)), neutrophils (Figure 3(A) and (B), (#)), and macrophages (Figure 3(A) and (B), (*)). At the shoulder, cellular infiltration was by predominantly spindle-shaped fibroblast-like cells in both groups (Figure 3(C) and (D), (white arrows)). Only sparse inflammatory cell infiltrates, typically around suture knots, were observed in grafts from either group implanted at the shoulder. The persistence of hydrogel in THA-treated grafts was noted by the ubiquitous presence of wispy extracellular blue material (Figure 3(B) and (D), (black arrows)).

Inflammatory response

The mean scores for the inflammatory and non-inflammatory cell infiltrates as well as vascularity (blood vessels) within fascia grafts at 12 weeks are presented in Table 1. Eosinophils were not present. Neutrophils counts were low in all groups but were significantly higher in THA- than water-treated grafts at the body wall, which were both significantly higher than either water- or THA-treated fascia grafts at the shoulder. For all other inflammatory cell types (lymphocytes, plasma cells, macrophages, and giant cells), there was no difference between treatment groups at either implantation site. However, all of these cell types were significantly higher in the fascia grafts at the body wall than in the fascia grafts at the shoulder. Vascularity was also significantly higher in the fascia grafts at the body wall than in the fascia grafts at the shoulder. No difference was found in the number of spindle-shaped fibroblast-like cells as function of treatment or implantation site.

Azimuth angle (collagen orientation)

Histograms of the azimuth angle for fascia grafts from individual animals as a function of treatment and implantation site are plotted in Figure 4 (A)-(D). It can be noted that the histograms were generally unimodal in shape. No significant differences were found in the mode (Figure 4(E)) or FWHM (Figure 4(F)) of azimuth angle as a function of treatment or implantation site.

Retardance (collagen density)

Histograms of retardance for fascia grafts from individual animals as a function of treatment and implantation site are plotted in Figure 5 (A)-(D). Again, it can be noted that the histograms were generally unimodal in shape; however, in some instances, the data appear to be modestly dispersed from the mode. No significant differences were found in mode (Figure 5(E)) or FWHM (Figure 5(F)) of retardance between the water and THA-treated fascia grafts at either the body wall or shoulder sites. However, the mode and FWHM of retardance for the water-treated fascia grafts at the shoulder were significantly higher than for either the water- or THA-treated fascia grafts at the body wall, and the mode for the THA-treated fascia grafts at the shoulder trended higher ($p < 0.1$) than either the water- or THA-treated fascia grafts at the body wall.

Discussion

The purpose of the current study was to evaluate the effect of low MW THA incorporation on the inflammatory response and remodeling of human fascia lata ECM grafts at both the body wall and shoulder injury sites. Grafts infiltrated with immobilized THA or water, were implanted bilaterally in shoulder and body wall sites in the canine model. Implantation site did significantly influence inflammatory response and graft remodeling. However, treatment with low MW THA had no effect on the inflammation or remodeling parameters measured.

We previously immobilized high MW THA (0.9–1 MDa) into fascia ECM at 7.6 µg/mg tissue weight. In the rat body wall model at 12 weeks we noted increased macrophage and giant cell densities and less fibroblast-like cells than in water-treated controls.[14] Furthermore, the density of lymphocytes and plasma cells (as well as macrophage and giant cells) was modest to heavy in THA implants. In this study, we immobilized low MW THA (57 kDa) into fascia ECM at 7.3 µg/mg tissue weight. In the canine model (body wall or shoulder site), we noted essentially no differences in inflammatory cell infiltrate in THA fascia compared to water controls, and the inflammatory cell counts in THA fascia were low, especially at the shoulder, but also at the body wall site excepting lymphocytes (Table 1). Since the inflammatory response to *water-treated* fascia at the *body wall* was similar between the rat and dog models, the differences in absolute inflammatory response to THA-treated fascia ECM between the two studies are likely related to differences in THA presentation within the fascia ECM.

Because THA is cross-linked to form a hydrogel after infiltration into the ECM matrix, molecular weight should not directly influence the presentation of HA to the host. However, low MW THA should diffuse more efficiently into the ECM than high MW THA, possibly

resulting in a less aggregated and more uniform distribution of hydrogel. This conclusion is supported by the observation of only 'wispy' localizations of non-degradable hydrogel and scarce giant cells in the current study, which were much less conspicuous than the more prominent localizations of THA hydrogel observed together with a prevalence of giant cells in the previous study.[14] Hence, the use of low MW THA in the current study may well be responsible for mitigating the increased inflammatory response to THA-treated fascia ECM observed previously. Yet, low MW THA provided no particular advantage in inflammatory response or remodeling outcomes compared to water-treated fascia. This finding suggests that the potential advantages of HA in wound healing, in particular its role as an anti-inflammatory agent,[6] are abrogated by presenting it as non-degradable hydrogel even though its HABP binding capacity is maintained.[13] Further study treating ECM with uncross-linked HA and investigating the effect on inflammatory response is warranted. Furthermore, it may be possible to use the immunotolerant low MW THA treatment as described here, as a means to localize free HA or other biomolecules within a dense ECM graft for slow release from the hydrogel after implantation.

This study did show that implantation site significantly influenced inflammatory response and graft remodeling in the canine model. Grafts implanted at the body wall - regardless of treatment group- had significantly higher inflammatory cell infiltrate and vascularity, and significantly lower retardance (collagen density) than grafts at the shoulder. Grafts at the shoulder were associated with scarce inflammation at 12 weeks. These findings suggest that grafts at the body wall are undergoing a more intense, persistent, and perhaps degradative inflammatory and remodeling process, whereas grafts at the shoulder are undergoing a more gradual process of cell infiltration, engraftment, and constructive remodeling. There were no obvious differences in bleeding, pain, or implant tolerance between the implantation sites. However, differences in inflammatory response and remodeling between the two sites in the canine may be attributable to the following factors: grafts at the body wall are associated with dissimilar surrounding tissues (muscle and subcutaneous fat), surgical trauma to the highly vascular underlying muscle tissue at implantation, and modest mechanical loading. In contrast, grafts at the shoulder are associated with relatively avascular underlying tendon tissue that is similar to fascia,[20] as well as the presence of synovial fluid, bony fixation, and robust mechanical loading. To date, there is very limited data on inflammatory response to ECM implants in human patients to which our results can be compared.[24,25] Therefore, the extent to which the inflammatory response to ECM grafts implanted in the canine shoulder injury site represents the cell and remodeling course of ECM grafts implanted in human rotator cuff repair patients would require further study and validation.

Our study is not without limitations. First, only a 12-week time point was evaluated so as to observe chronic inflammatory response and remodeling outcomes, which should be indicative of graft fate after long-term implantation. However, early inflammatory response mechanisms as a function of THA treatment or implantation site could not be appreciated. Secondly, while it has been shown that the inflammatory response is a critical determinant of successful and constructive remodeling of an implanted ECM,[4,5] we could not characterize the macrophage phenotypes because the macrophage response was largely resolved at 12 weeks. Finally, we did not perform a functional test of ECM remodeling because histology and mechanical testing could not be performed on the same samples.

In conclusion, we have shown that ECM grafts implanted at the body wall had significantly higher inflammatory cell infiltrate and vascularity, and significantly lower matrix density than grafts at the shoulder, suggesting that the inflammatory response and remodeling of biomaterial implants depends on the location of implantation and therefore our animal models should be carefully chosen. Further immobilization of low MW THA into fascia ECM did not influence the inflammatory and remodeling response to the fascia graft at either the shoulder injury or body wall sites in the canine. This finding suggests that the potential anti-inflammatory advantages of HA in wound healing are not realized by presenting it to the host as non-degradable hydrogel even though its HABP binding capacity is maintained.[13] Further study treating ECM with uncross-linked (free) HA or immobilized low MW THA as a means to deliver free HA or other biomolecules to a surgical repair site is warranted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Surgical implantation. (A) Polypropylene reinforced fascia implant. (B) Fascia grafts implanted at abdominal wall site. (C) Resection of joint capsule under infraspinatus tendon in shoulder injury model. (D) Fascia graft implanted at shoulder site.

Figure 2. Photomicrographs of representative H&E sections of graft explants. (A) Body wall and (B) Shoulder. Scale bar represents 1 mm; original magnification, 4 $\times$. In each section, the graft is delineated by black dashed lines.

Figure 3.

Photomicrographs of representative H&E sections of fascia grafts. (A) Water and (B) THA treated at body wall, (C) water and (D) THA treated at shoulder. Scale bar 500 μm ; original magnifications, 400 $\times$. White arrows - fibroblast-like cells; circles - lymphocytes; + plasma cells; # neutrophils; * macrophages; black arrows - 'wispy' localizations of THA hydrogel.

Figure 4. Slow axis azimuth angle (collagen orientation). (A-D) Histograms of azimuth angle for fascia grafts from individual animals ($n = 8$): (A) water treated at body wall (H2O-BW), (B) THA treated at body wall (THA-BW), (C) water treated at shoulder (H2O-SH), (D) THA treated at shoulder (THA-SH). Mean (standard deviation) of (E) mode and (F) FWHM of azimuth angle by group.

Figure 5. Retardance (collagen density). (A–D) Histograms of retardance for fascia grafts from individual animals ($n = 8$): (A) water treated at body wall (H2O-BW), (B) THA treated at body wall (THA-BW), (C) water treated at shoulder (H2O-SH), (D) THA treated at shoulder (THA-SH). Mean (standard deviation) of (E) mode and (F) FWHM of retardance by group, where horizontal lines denote significance differences and like letters indicate a trend ($p < 0.1$).

Table 1.

Mean (range) of histologic scores.

Location	Group	Inflammatory cells					Non-inflammatory	
		Eosinophils	Neutrophils	Lymphocytes	Plasma cells	Macrophages	Giant cells	Fibroblasts
Body wall	H2O	0.1 (0–1) ^a	0.6 (0–3) ^b	2.0 (0–4) ^a	0.8 (0–3) ^a	1.3 (0–4) ^a	0.6 (0–3) ^a	1.0 (1) ^a
	THA	0.0 (0) ^a	1.1 (0–3) ^a	2.3 (0–4) ^a	1.0 (0–3) ^a	1.2 (0–3) ^a	0.4 (0–3) ^a	1.0 (0–1) ^a
Shoulder	H2O	0.0 (0) ^a	0.0 (0) ^c	0.2 (0–3) ^b	0.0 (0) ^b	0.0 (0–1) ^b	0.0 (0–1) ^b	1.0 (1) ^a
	THA	0.0 (0) ^a	0.2 (0–4) ^c	0.4 (0–3) ^b	0.2 (0–4) ^b	0.2 (0–2) ^b	0.1 (0–2) ^b	1.0 (0–1) ^a
ANOVA <i>p</i> -value		NA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	NA

Notes: Like letters indicate no significant differences for an outcome across group and location.

Histologic scoring was adapted from ISO 10993–6,[14] Inflammatory cells and vascularity: 1 = 1–5/hpf; 2 = 5–10/hpf; 3 = heavy infiltrate; 4 = packed. Fibroblasts: 1 = rare – 100/hpf; 2 = 100–1000/hpf; 3 = heavy infiltrate; 4 = packed. hpf = high powered field (40×).

Mean = average of mean repeated measures from eight animals.