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Establishment of a novel porcine model to study the impact of active stretching on a local Carrageenan-induced inflammation

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Abstract

Objective: Active stretching of the body is integral to complementary mind-body therapies such as yoga, as well as physical therapy, yet the biological mechanisms underlying its therapeutic effects remain largely unknown. A previous study showed the impact of active stretching on inflammatory processes in rats. The present study tested the feasibility of using a porcine model, with a closer resemblance to human anatomy, to study the effects of active stretching in the resolution of localized inflammation.

Design: 12 pigs were trained to stretch before subcutaneous bilateral Carrageenan injection in the back at the L3 vertebrae, 2 cm from the midline. Animals were randomized to No-Stretch (NS) or Stretch (S); 2X/day for 5 min over 48 hours. 48 hours post-injection, animals were euthanized for tissue collection.

Results: The procedure was well tolerated by pigs. On average, lesion area was significantly smaller by 36% in the S group compared to NS ($P = 0.03$).

Conclusion: This porcine model shows promise for studying the impact of active stretching on inflammation-resolution mechanisms. These results are relevant to understanding the stretching-related therapeutic mechanisms of mind-body therapies. Future studies with larger samples are warranted.

Keywords

Active stretching; inflammation resolution; connective tissue; Carrageenan

Active stretching is an integral therapeutic component of many complementary manual and mind-body therapies (*e.g.* massage, yoga, and tai chi), as well as physical therapy. While

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these therapies show clinical promise in reducing pain and enhancing function in multiple musculoskeletal pain conditions[1–11], the biological mechanisms underlying observed therapeutic effects remain largely unknown. A growing body of translational research, largely conducted in rodents and employing *in vitro*, *ex vivo*, and *in vivo* models, has begun to inform mechanisms underlying the biological impact of stretching, especially in processes related to inflammation[12–15]. As a next step, the utility of an animal model that more closely resembles humans anatomically to evaluate the biological impact of stretching was explored. In this pilot study, the feasibility of using a novel active stretching method in a porcine model was established and preliminary findings on the impact of stretching on local and systemic inflammation are reported.

Previous work using rodent models has evaluated the impact of both passive (under anesthesia) and active stretching. In one set of studies, using a Carrageenan-induced localized inflammation of the subcutaneous tissue in rats[16], it was determined that passive and active stretching mitigate the inflammation process and trigger inflammation-resolution mechanisms *i.e.* reduction of lesion size, decreased neutrophil count, and increased Resolvin D1 (RvD1) production[17].

More recently, domestic pigs were used to study subcutaneous tissue inflammation because their skin, connective tissue, and muscle anatomy in their lower back resemble human anatomy; namely, both species lack the subcutaneous *panniculus carnosus* muscle found in rodents in their dorsal trunk[18, 19–25]. It was determined that thoracolumbar fascia injury combined with experimentally-induced movement restriction for two months could trigger a pathology similar to that found in humans with lower back pain[26]. Results showed that the combination of injury plus movement restriction had an additive effect on reducing facia mobility and that daily passive stretching normalized gait speed, but there was no effect on fascia mobility[27]. However, one limitation of this study is that the stretching was passive and thus does not parallel protocols evaluating the effects of active stretching in rodents. Moreover, research evaluating active stretching is more likely to identify the biological mechanisms that typically occur during the physical component mind-body therapies.

The present study extended prior work in pigs by developing and evaluating the impact of an active stretching technique. First, this study adapted and tested the acceptability of a stretching technique in pigs previously used in physical therapy for dogs and cats[28–30]. Second, this study used a Carrageenan inflammation model to collect preliminary data on the impact of active stretching on an array of outcomes. Preliminary data are reported and their relevance in informing the design of future studies are discussed.

METHODS

Animal and Experimental Design

Animal protocols were approved by the Brigham and Women’s Hospital (BWH) Institutional Animal Care and Use Committee (IACUC) and followed BWH institutional guidelines.

Yorkshire female domestic swine (n = 12; 6 – 8 weeks old, 12 – 20 kg) were acquired from Tufts University, Grafton, Massachusetts. Pigs were obtained in batches of 4 animals and were housed in groups of 2 in 8 × 8-ft pens. Animals were kept on a 12-hr light-dark cycle and 1.3 kg of a balance pig diet was provided per day to gain approximately 1 lb/day.

In this study, active stretching is defined as the use of the strength of agonist muscles and different components of the fascia system to hold a position with human assistance. In previous studies in rats, the active stretching intervention was applied for 10-minutes twice a day for 2 or 12 days[15,17]. For this experiment, the intervention lasted 5 minutes/twice a day over 48 hours. Before Carrageenan injection, pigs were trained for two weeks to perform a wheelbarrow stretch and provided with a food reward of Cheerios™. The stretching procedure was adapted from physical therapy techniques used in dogs and cats[28–30]. This procedure required that the front limbs of pigs remain in motion while maintaining contact with the ground. By allowing the animal to make contact with the floor, the full weight of the animal was not suspended by the hindlimbs. Additionally, the movement of walking created a natural multi-directional stretching of the lower back with the synchronic movement of the legs. A ~45° angle was maintained during this stretching method. When held in this position, pigs spontaneously extend their limbs increasing the distance between the shoulder and hips. Each 5-minutes stretching session began as soon as the animal started walking and stretching its lower back (Fig 1A – B). Animal randomization to either the no-stretch (NS) or the stretch (S) group took place after the training period and before the Carrageenan injection.

Study Interventions

Carrageenan injection—Carrageenan, a linear polysaccharide extracted from seaweed, induces localized inflammation of subcutaneous tissue and it is commonly used in models of musculoskeletal, subcutaneous and intradermal inflammation[16,31]. Animals were anesthetized by intramuscular injection of TKX cocktail (Ketamine 20 mg/kg; Xylazine 0.25 mg/kg; Telazol 0.07 mg/kg) with a dose of 0.5 – 0.7 mL/IM. Once a deep plane of anesthesia was achieved, pigs were moved to a procedure room and placed on a surgical table in a prone position. Each animal was shaved, a line was drawn with a non-removable marker along the spine following the spinous processes from the back of their head until the sacrum and the injection site was localized and marked at lumbar *vertebrae* 3 (L3). A 1% Carrageenan solution was prepared using sterile saline. 1 mL of this solution was injected bilaterally into the subcutaneous space of the lumbar region 1 cm lateral of the spine at L3. Carrageenan lesions did not become ulcerated and animals did not show signs of pain or distress.

Stretching (S)—After Carrageenan injection, pigs randomized to the stretch group were stretched 5 minutes/twice a day for 48 hours and provided with a food reward after each stretching episode. The first stretching session occurred two hours after full recovery from anesthesia. The second session took place 6 hours after the first. The next day two more stretching procedures took place at the same time as on day 1.

Non-stretching (NS)—Animals in the NS group remained in their pen for 48 hours with water *ad libitum* and recommended food intake. Animals received the same daily food rewards as their stretched counterparts.

Sample collection—14 hours following the last stretching session, animals were anesthetized with an intramuscular injection of TKX cocktail (Ketamine 20 mg/kg; Xylazine 0.25 mg/kg; Telazol 0.07 mg/kg) with a dose of 0.8 mL/IM. Before euthanasia with Euthasol (100 mg/kg/IV), blood samples were collected using tubes with and without EDTA for peripheral blood mononuclear cells (PBMCs) isolation by density gradient centrifugation on Ficoll - Paque (GE Healthcare) and serum collection, respectively[32]. PBMCs were slowly frozen in FBS + 10% DMSO and stored in liquid nitrogen until further use. Serum samples were stored at −80°C. Immediately after euthanasia, both Carrageenan-induced lesions were removed by carefully dissecting the skin around the injection site and placed in Petri dishes with PBS on ice. The right lesion was used for flow cytometry, while the left lesion was stored at −80°C for RNA extraction and lipid mediator measurements. Muscle samples were collected from underneath the lesion sites and stored at −80°C for RNA extraction.

Measurement methods

Lesion area and weight measurements—Lesion size and weight were measured after euthanasia. Initially, the length and width of both lesions were measured *in situ* using calipers by a blinded investigator with experience measuring Carrageenan lesions in rat and mouse models (Fig 2A). The area of both lesions was averaged per each animal. After measurement, lesions were excised from the skin and weighed using a Sartorius Practum 213–1S balance and results were averaged per animal.

Flow cytometry—The direct effect of active stretching on the immune response at the lesion site was investigated using flow cytometry. Briefly, cells were harvested from the right inflammatory lesion by mincing the tissue with a scalpel in cold PBS, then passing through a 40 μm cell strainer (Corning) and centrifuging at 800 × g for 5 minutes. Cells were counted using an automatic cell counter TC20™ (Bio-Rad, CA) with trypan blue to stain. Cells were incubated in Zombie NIR™ (BioLegend®) to assess viability. For surface receptors, cells at 1×10^6/mL were stained with a mix of mouse monoclonal antibodies, all of which were diluted 1:66 μL of blocking buffer and incubated on ice for 30 minutes: CD45-Alexa Fluor®647 and Granulocytes-FITC. A second mix was used with CD45-Alexa Fluor®647 and CD163-FITC, a macrophage marker (all stains and antibodies from Bio-Rad, Hercules, CA). The gating strategy was adjusted to discriminate doublets (FSC-A versus FSC-H in Dot Plots), and non-viable cells staining with the Zombie NIR were excluded from the analysis. Granulocytes were CD45+GR-1+ and Macrophages were CD45+ CD163+.

ELISA—Lesion supernatant was prepared using ¼ of the left lesion with lysing matrix D MP™ beads in cold PBS using a Fast Prep-24 MP™ homogenizer, for 5 cycles of 20 seconds each at 6.5 mts/second and 5 minutes intervals on ice. After homogenization samples were centrifuged at 800 × g for 5 minutes and supernatant was aliquoted and stored at −80 °C. Lesion levels of Resolvin D1 (RvD1) and Lipoxin A4 (LXA4) and serum levels

of Prostaglandin D2 (PGD_2) and RvD1 were measured using a competitive ELISA kit following the manufacturer's instructions (Cayman Chemical, Ann Arbor, Michigan). Results from the lesion supernatant were normalized to protein content determined using the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA).

Quantitative real-time polymerase chain reaction (PCR)—Sequences of primers used are listed in Supplemental Table 1 and were designed to span an intron-exon border. Total RNA was extracted from PBMCs, muscle, and lesion samples using TRIzol (Invitrogen) and 2 μg of RNA was reverse transcribed into cDNA using SuperScript VILO (Invitrogen). cDNA was amplified by qPCR in a Light Cycler 480 II (Roche) using Light Cycler 480 SYBR Green (Roche). Quantitative real-time PCR was performed as described previously using the delta-delta cycle threshold method, with the geometric mean of beta-actin (ACTB) used to normalize each gene[33].

Statistical analysis

Statistical analyses were performed with GraphPad Prism 7.05 (San Diego, CA) and SAS statistical software version 9.4 (SAS Institute Inc. Cary, NC). Because of the preliminary nature of the study and the small sample size, the analyses were limited to descriptive statistics[34,35]. Data were checked for normality and means and SEMs were presented for normally distributed outcomes. Gene expression data were expressed with geometric mean and confidence intervals (CI). An exploratory analysis comparing NS and S groups, when normally distributed, used a two-sample t-test, both unadjusted and adjusted by batch effect using a randomized complete block design (RCBD). Statistical significance was set at $\alpha = 0.05$.

Cohen's d effect sizes were calculated based on differences between means, which traditionally considered small effect $d = 0.2$; medium effect $d = 0.5$ and large effect $d = 0.8$[36–41]. This approach was used to explore the magnitude of the observed effects in terms of standard deviations to generate new hypotheses for future powered studies.

RESULTS

Establishment of the active stretching porcine model after Carrageenan injection

Overall, pigs tolerated the stretching procedure well. Upon arrival, animals spent the whole day acclimating to their new pens and were given toys, food, and water *ad libitum*. The next day the stretching instructor entered the pen to begin socialization by spending time with the animals and encouraging physical contact. On the third day, the instructor held their posterior limbs and gradually incremented the active stretching training period until reaching 5 minutes. Animals received food rewards after each training session. After randomization and Carrageenan injection, the S group completed four stretching sessions. During stretching, pigs walked around stretching the side of their bodies, increasing the ipsilateral distance between the shoulder and the hip joint by approximately 20 – 30% based on visual observation (Fig. 1A). The NS group remained in their pens. Euthanasia occurred 14 hours after the last stretching session (Fig. 1B).

Effect of stretching on lesion size and weight

Lesion sizes were significantly smaller by 36% in the S group compared to NS (P = 0.03; Fig 2B). Lesion weight was on average reduced by 29% in the S compared to the NS group, although this difference was not statistically significant (P=0.17; Fig 2C).

Inflammatory cell count

After following the gating strategy to discriminate doublets and non-viable cells, as expected, the acute inflammation exudate was mainly composed of granulocytes. On average the S lesions had 71% fewer granulocytes compared to NS lesions (P=0.22; Fig 3A and 3B). Similarly, the S lesions had 49% fewer macrophages compared to NS lesions (P=0.39; Fig. 3C and 3D). However, the observed decreases were not statistically significant for both cells type.

In vivo active stretching and production of Specialized Pro-Resolving Mediators (SPMs) *in situ* and systemically

The study explored whether active stretching stimulates the production of SPMs that reduce neutrophil recruitment and are necessary for the inflammation resolution. Initially, it was confirmed that the SPMs LXA4 and RvD1were present in this porcine model of inflammation. Within the lesion, stretching did not change RvD1 or LXA4 levels. In serum, on average both LXA4 and RvD1 were 14% higher in the S compared to the NS group (P=0.37 and P=0.38, respectively). Conversely, the pro-inflammatory mediator PDG_2 exhibited a 79% decrease in the S compared to the NS group (P=0.11). However, these differences were not statistically significant (Fig 4A – C). Interestingly, the ratio of LXA4 or RvD1 to PDG2 revealed nearly a two-fold increase in the S compared to the NS group (Fig 4D and 4E).

Gene expression in the lesion, muscle, and PBMCs

The effect of active stretching on the expression of genes associated with inflammation and fibrosis was assessed in three different compartments: directly in the inflammatory lesion, in the muscle underneath the lesion, and in circulating PBMCs. No significant changes were found in expression levels between the NS and S groups in any of the genes evaluated (Table 1).

DISCUSSION

In this preliminary study, a novel stretching technique for pigs, adapted from physical therapy in dogs and cats, was found to be feasible and well-tolerated. Moreover, the results indicated that active stretching significantly decreases the size of a Carrageenan-induced lesion compared to the NS control group. Lesion weight also tended to be reduced in the S group. Likewise, the analysis of inflammatory cell infiltration at the lesion site, the production of pro-resolving and inflammatory lipid mediators locally and systemically, and the gene expression from three different anatomical compartments showed additional interesting trends supporting the need for future studies.

The finding that active stretching decreases lesion area in this porcine model parallels previous results in rats[17]. In contrast to these current results using active stretching, previous studies found that passive stretching did not change lesion area after connective tissue was surgically injured in pigs[26,27]. This suggests that regardless of the source of inflammation (surgical injury versus Carrageenan) active stretching may be more effective at decreasing lesion area.

An interesting secondary outcome was the analysis of the cell populations within the inflammatory exudate of the lesion. Despite a trend towards fewer granulocytes and macrophages, these data did not suggest a significant reduction in the number of neutrophils as observed previously in experiments in rats[17]. It is possible that, in order to trigger significant changes in the inflammatory cell number, pigs may need longer stretching sessions over an extended period due to their lack of the subcutaneous *panniculus carnosus* muscle in their lower back, similar to what is found in humans. This difference leads to their skin being tethered to subcutaneous and perimuscular fascia layers by retinaculae that renders superficial tissues much less mobile relative to deep muscles when compared to rodents. Thus, subsequent studies should not only include more animals but also stretching doses of different intensity and duration to test whether these changes would elicit the therapeutic effect of stretching found in rats regarding the number of infiltrated cells[20,21].

Regarding resolvins and pro-inflammatory lipid mediators, this study found detectable levels of SPMs 48 hours after the injection of Carrageenan, which is 4 days earlier than when SPMs were identified post-skin-wounding in pigs[42]. Intriguingly, these results suggest that active stretching may lead to systemic changes in SPMs[43–45]. This active stretching model in pigs indicates that stretching may activate inflammatory pathways leading to the release of SPMs, and thus might be a key therapeutic component underlying the therapeutic effects of mind-body and physical therapies on inflammatory conditions, including pain.

The last exploratory outcome in this pig model of active stretching evaluated gene expression at the injection site (lesion and nearby muscle) and at the systemic level using PBMCs. Interestingly, gene expression in muscle samples showed no change in the muscle stem cell marker *PAX7*, or fibro-adipogenic progenitor cell marker *PDGFRA*, suggesting that there is no increase in these cell types in response to subcutaneous inflammation[46]. These results further imply that both the inflammatory reaction and the stretching procedure had minimal effects on adjacent muscle tissue. However, it cannot rule out that after 48 hours a delayed inflammatory effect might develop in the adjacent muscle[47–49]. Furthermore, in this Pig model of active stretching Carrageenan behaves as a foreign body which should lead to a granulomatous inflammatory reaction. Indeed, the S group had 3- to 4-fold higher expression compared to the NS group of *CTGF/CCN2* and *COL1A1*, genes involved in fibrosis and collagen deposition, respectively. These gene expression results suggest that stretching might be accelerating the process of containment of a foreign substance such as Carrageenan.

The present study had challenges and limitations. First, a significant challenge was the logistics and expense of working with a large animal model such as pigs as opposed to smaller animal models such as rodents. Second, this study was not adequately powered.

However, the goal of this pilot study was to test the feasibility of a new active stretching method for pigs before embarking on a larger trial. Third, the dose, frequency, and intensity of stretching were not varied in this study, and are important factors to consider for future studies. Fourth, the present active stretching model is complex, involving not only the stretching of the connective tissue but also stretching of other body parts such as muscle, joints, and bones. Fifth, despite pretraining, there is some inherent stress involved in the stretching procedure which is not accounted for in the control group[50]. However, studies in rodents have found that passive anesthetized stretching also reduces the inflammatory process[17]. While, activity of any kind involves an inherent degree of physical stress[51], potential stress in this study was reduced by training each animal for 2 weeks before the Carrageenan injection. Finally, this study did not evaluate the NS and S groups without Carrageenan injection, which would have assessed how active stretching affects the SPMs clusters found in the connective tissue.

CONCLUSIONS

In conclusion, the preliminary results from a porcine model combining subcutaneous induced inflammation with active stretching support the feasibility of this model and suggest that stretching may decrease lesion area. Future studies with increased power will allow us to understand the therapeutic benefits of active stretching in both inflammatory and non-inflammatory settings, and contribute to understanding the complex biological mechanisms underlying the physical component of mind-body therapies and their potential therapeutic effects on pain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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What is Known:

Active stretching of a Carrageenan subcutaneous inflammation model in rodents reduces inflammation area, exudative infiltration and increased tissue Resolvin D1 concentrations. These results provide evidence of a direct mechanical impact of stretching on the mechanisms involved in the resolution of inflammation within the connective tissue.

What is New:

This translational model tested the feasibility of an active stretching paradigm in pigs, a species that anatomically resembles humans, to study the mechanisms behind inflammation-resolution in active stretching, a known component of several physical and mind-body therapies.

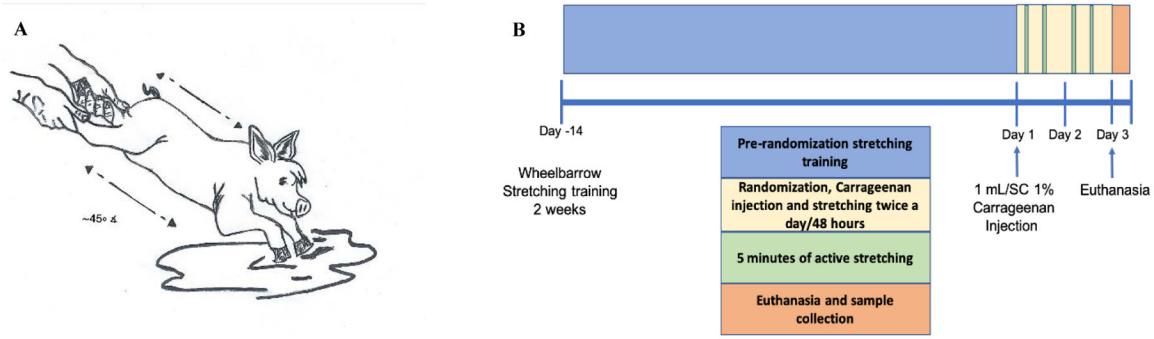

Fig 1.

A. Illustration of the wheelbarrow active stretching method. B – Schematic timeline of the experiment.

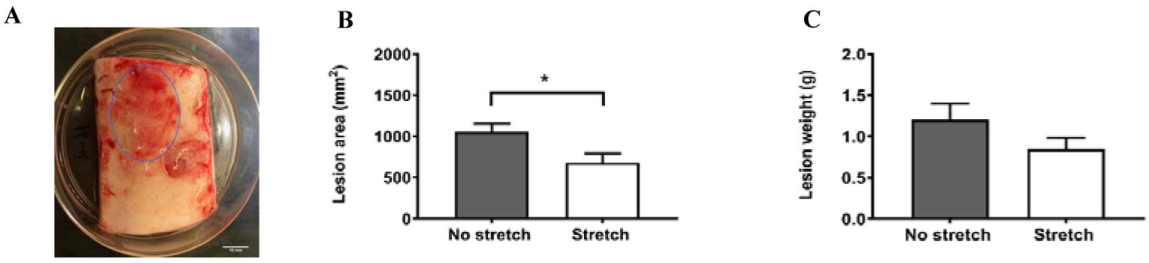

Fig. 2.

Effect of a pig active stretching model on Carrageenan-induced inflammation: A – Macroscopic view of subcutaneous Carrageenan lesion, B – Lesion area, and C – Lesion weight 48 hours after subcutaneous Carrageenan 1% injection, in the stretched compared to the non-stretched group (*P=0.029 lesion area; P=0.16 lesion weight; N = 6 pigs per group). Mean and SEM are shown.

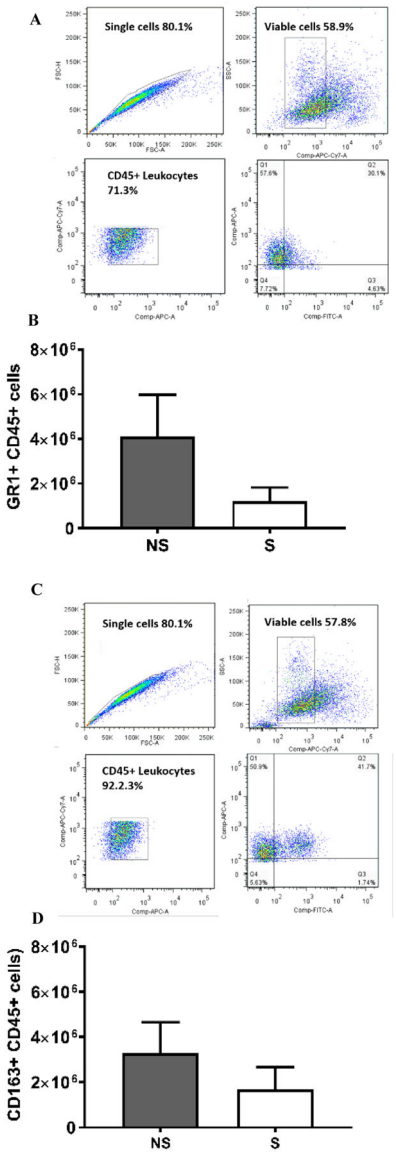

Fig. 3.

Effect of *in vivo* stretching on Carrageenan-induced inflammation: A – Gaiting strategy for granulocytes (GR1+). B – Relative number of GR1+ Granulocyte cells (P=0.2 for Granulocyte cells). C – Gaiting strategy for Macrophages (CD163+). D – Relative number of CD163+ Macrophage cells (P= 0.4 for macrophages). 48 hours after subcutaneous 1% Carrageenan injection in the stretched and non-stretched groups. N = 5 pigs NS and N= 4 pigs S group. Mean and SEM are shown.

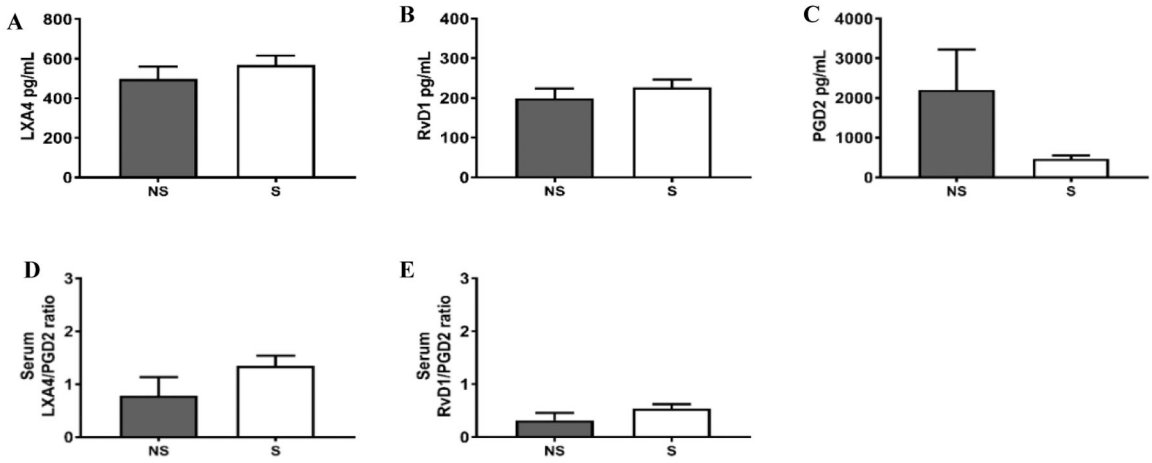

Fig. 4.

Effect of *in vivo* stretching on Carrageenan-induced inflammation: A – Lipoxin A4 in serum (P=0.3). B – RvD1 in serum (P=0.4). C – Prostaglandin D2 in serum (P=0.1). D – Ratio of serum Lipoxin A4 and Prostaglandin D2 (P=0.2). E – Ratio of serum Resolvin D1 and Prostaglandin D2 (P=0.2). N = 6 pigs per group. Mean and SEM are shown.

Table 1.

Gene expression analysis in a carrageenan subcutaneous porcine inflammation model.

		N/group	No Stretch[#]	95% CI	Stretch	95% CI	Cohen's d[†]	P-value
Lesion	*CD86*	6	1	0.49 – 2.05	0.58	0.31 – 1.07	0.7	0.2
	TNF-α	6	1	0.53 – 1.87	0.52	0.29 – 0.92	1.0	0.1
	NF-κB1	6	1	0.65 – 1.53	1.04	0.81 – 1.35	0.0	0.9
	NF-κB (RELA)	6	1	0.62 – 1.62	1.47	0.93 – 2.31	0.9	0.1
	ANXA 1	6	1	0.46 – 2.18	1.62	1.09 – 2.40	0.4	0.5
	CCL2/MCP1	6	1	0.63 – 1.60	0.79	0.35 – 1.74	0.1	0.9
	CCL5	6	1	0.49 – 2.01	0.69	0.23 – 2.08	0.1	0.9
	CxCL10	6	1	0.23 – 4.21	3.34	0.72 – 15.6	0.7	0.3
	CTGF/CCN2	6	1	0.25 – 3.95	4.56	1.35 – 15.5	1.5	0.06
	COL1A1	6	1	0.19 – 5.38	3.13	0.65 – 15.3	0.6	0.4
	ALOX5	6	1	0.25 – 3.93	1.63	1.06 – 2.52	0.0	0.9
Muscle	*PAX7*	6	1	0.32 – 3.10	1.07	0.43 – 2.61	0.1	0.8
	PDGFRα	6	1	0.33 – 3.04	1.18	0.41 – 3.42	0.2	0.7
	CCL2/MCP1	6	1	0.54 – 1.82	1.42	0.65 – 3.09	0.5	0.4
	CCL5	6	1	0.70 – 1.41	1.17	0.70 – 1.97	0.5	0.4
	CxCL10	6	1	0.31 – 3.16	1.27	0.63 – 2.56	0.1	0.8
	CxCL8/IL-8	6	1	0.50 – 1.98	1.02	0.29 – 3.48	0.3	0.6
PBMCs	*ANXA1*	6	1	0.80 – 1.24	1.13	0.90 – 1.41	0.5	0.4
	ALOX5	6	1	0.58 – 1.73	1.09	0.69 – 1.72	0.2	0.8
	ALOX15	6	1	0.59 – 1.66	1.04	0.62 – 1.78	0.1	0.9
	COX2	6	1	0.52 – 1.91	1.37	0.72 – 2.63	0.4	0.5
	CCL2/MCP1	6	1	0.55 – 1.82	0.70	0.23 – 2.18	0.1	0.9
	CCL5	6	1	0.61 – 1.65	1.47	0.98 – 2.21	1.0	0.1

[#]Geometric mean ± 95% Confident intervals

[†]Cohen's d effect size classification: 0.2 small effect; 0.5 medium effect; 0.8 large effect