

Long chain n-3 polyunsaturated fatty acids reduce atrial vulnerability in a novel canine pacing model

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Aim Our objective was to assess the effect of omega-3 polyunsaturated fatty acids (n-3 PUFAs) on atrial fibrillation (AF) vulnerability and atrial structure in a new model of atrial cardiomyopathy.

Methods and results Dogs were studied in three groups: seven control dogs (UNPACED) and 24 dogs undergoing simultaneous atrioventricular pacing (for 2 weeks) assigned to placebo treatment (SAVP-PLACEBO, $n = 12$ dogs) or oral n-3 PUFAs (1 g/day) treatment (SAVP-PUFA, $n = 12$ dogs). SAVP-PUFA dogs had less AF inducibility (percentage of burst attempts leading to AF episodes: 5.5 ± 7.4 vs. 20.4 ± 14.2 , $P < 0.001$) and maintenance [median AF duration: 601 s (377–1216) vs. 1598 s (1195–2400), $P < 0.05$] than SAVP-PLACEBO dogs. SAVP-PUFA dogs had significantly less local slowing of conduction and conduction heterogeneity than SAVP-PLACEBO dogs. SAVP-PUFA dogs had a significantly smaller increase in atrial matrix metalloproteinase-9 activity and in collagen type I and III messenger RNA expression (in arbitrary units) than SAVP-PLACEBO dogs (0.62 ± 0.51 vs. 10.80 ± 5.61 , respectively for collagen I, $P < 0.05$; 1.66 ± 0.48 vs. 5.24 ± 1.16 , respectively, for collagen III, $P < 0.05$).

Conclusion n-3 PUFA supplementation can reduce AF vulnerability in a new canine pacing model of atrial cardiomyopathy. The mechanism may be related to attenuation of collagen turnover.

1. Introduction

Atrial fibrillation (AF) is the most common sustained cardiac arrhythmia.¹ AF is noted in ~20% of patients with heart failure; regardless of whether it is the cause or the consequence of heart failure, it is associated with an increased morbidity and risk of mortality.² Antiarrhythmic drug therapies to treat or prevent AF, especially in patients with heart failure, are limited by toxicity and a relative lack of efficacy.³

Dietary and non-dietary intake of n-3 polyunsaturated fatty acids (n-3 PUFAs) may reduce the risk of sudden death after myocardial infarction.⁴ However, population studies testing the hypothesis that n-3 PUFAs can prevent AF have demonstrated conflicting results.^{5–7} Consumption of fish five or more times per week was associated with reduced incidence of new onset AF; other studies, however, have failed to show a relationship between blood level of n-3 PUFAs or fish intake and the risk of developing AF.^{5–7}

Animal models can help to better understand the relationship between atrial structural changes and AF vulnerability. However, current animal models have limitations: rapid ventricular pacing models induce severe heart failure and are associated with high mortality rates (20–30%); rapid atrial pacing and vagal stimulation models may not replicate the most common clinical situation in which the left atrium dilates and becomes fibrosed, exhibiting the conduction slowing.⁸

To address these limitations, we have developed a new simultaneous atrioventricular pacing (SAVP) dog model which induces clinically relevant atrial remodelling and produces reliably inducible sustained AF and less overt congestive heart failure than rapid ventricular pacing models.⁹

The purpose of this study was to test the hypothesis that dietary n-3 PUFAs supplementation can prevent atrial remodelling and AF vulnerability in this new model.

2. Methods

Twenty-four sex-matched adult mongrel dogs (mean weight 24 ± 5 kg) underwent simultaneous atrioventricular pacing (SAVP) for 2

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weeks. Twelve were randomly assigned to oral n-3 PUFAs treatment (SAVP-PUFA group) with eicosapentaenoic+docosahexaenoic acid (EPA+DHA), 1 g/day (Omacor, Solvay Pharmaceuticals GmbH, Hanover, Germany), from 1 week before starting the pacing protocol until the end of the study (3 weeks in total); 12 controls were paced but received no n-3 PUFAs (SAVP-PLACEBO group). A third group of dogs served as unpaced healthy controls (UNPACED group, $n = 7$).

In order to better characterize the mechanical remodelling induced in our new dog model, compared with prior models of AF, an additional eight dogs were subjected to a 'standard' rapid ventricular pacing model (RVP-PLACEBO group).

All the dogs spent 3 weeks in the research facility in the same housing and feeding conditions. The protocol was approved by the Animal Care Committee of St Michael's Hospital, Toronto (ON, Canada). The investigation conforms to the *Guide for the Care and Use of Laboratory Animals*, US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

2.1 Pacemaker implant for simultaneous atrioventricular pacing

Dogs were anaesthetized with sodium pentobarbital (30 mg/kg IV) and maintained with 1–2% isoflurane. Two bipolar screw-in pacing leads (Tendril SDX, St Jude Medical, Inc., St Paul, MN, USA) were fixed in the right atrial appendage and in the right ventricular apex and were connected to a 'Y connector' which was then connected to a single-chamber pacemaker (Verity ADX, St Jude Medical, Inc.), placed in a subcutaneous pocket in the neck. One week later, pacemakers were turned on in bipolar mode to pace the atria and ventricles simultaneously (atrioventricular delay 0 ms) at 220 b.p.m. with 1.0 ms pulse duration and at twice-threshold current.

For the RVP-PLACEBO group, a single active- (screw-in lead) or passive-fixation lead (tined lead) (Medtronic, Inc., Minneapolis, MN, USA) was placed at the apex of the right ventricle and connected to a VVI pacemaker unit (model 8084, Medtronic, Inc.) under the same conditions. Pacemakers were initially programmed at 240 b.p.m. for the first 2 weeks, and for the remaining 3 weeks, the rate was reduced to 220 b.p.m.

2.2 Echocardiographic studies

Two-dimensional transthoracic echocardiograms were performed with a Sonos 5500 system (Philips Ultrasound, Bothell, WA, USA) with a phased array transducer (S3, Philips Ultrasound). The echocardiographic study results were obtained on standing conscious dogs while in normal sinus rhythm (15–20 min after the pacemaker was turned off). Two-dimensional left ventricular (LV) area was planimetered: diastolic (LVDA) and systolic (LVSA) areas were taken as the maximum and minimum cavity dimensions, respectively, from two-chamber short-axis views. The LV endocardial tracings were drawn to include the papillary muscles inside the outlines. LV shortening fraction area was calculated as follows: $100 \times (LVDA - LVSA) / LVDA$. Left atrial diastolic (LADA) and systolic (LASA) area were obtained at the plane of aortic valve (parasternal short-axis view), incorporating an area defined by both the LA free wall and the appendage. LA-emptying function was defined as the LA fractional shortening area (LAFAS): $100 \times (LADA - LASA) / LADA$.

Echocardiographic parameters were obtained for all dogs at baseline (including the RVP-PLACEBO group) and at the end of the pacing protocol (after 2 weeks of pacing for SAVP-PLACEBO and SAVP-PUFA dogs and after 5 weeks for the RVP-PLACEBO group).

2.2.1 End of study: surgical procedure

After 2 weeks of pacing, dogs were anaesthetized with IV propofol (10 mg/mL, 2.5–3.5 mg/kg) and maintained under isoflurane (1–1.5%). A median sternotomy was performed, and five bipolar stainless steel epicardial electrodes were sewn on the right and left atria as follows: high and low right atrium, right and LA

appendages, and posterior wall of the left atrium. In 15 dogs (five UNPACED, five SAVP-PLACEBO, and five SAVP-PUFA), a clockface electrode (16 peripheral unipolar electrodes, all equidistant from each other and from a central bipolar electrode; radius 7.5 mm) was sutured to the epicardium of the posterior wall of the left atrium (Figure 1). This electrode allows calculation of the atrial conduction velocity in any direction, between the atrioventricular groove and the inferior pulmonary vein ostium. Since conduction time is calculated in all directions, the resultant average value is independent of fibre orientation. Intracardiac electrograms were recorded in bipolar mode (at a filter setting of 30–300 Hz) and unipolar mode (0.05–300 Hz) and stored digitally on a custom acquisition system (AQUI 2, Cartesian Labs, Toronto, ON, Canada).

2.2.2 Electrophysiological study

Atrial effective refractory periods (AERPs) were measured at each of five sites after 30 s of continuous pacing (400, 300, and 200 ms cycle length, CL) (to achieve a steady state), at twice the stimulation threshold; an atrial extra-stimulus was introduced after every eighth drive beat. The initial extra-stimulus coupling interval was set at 120 (for 400 ms CL), 100 (for 300 ms CL), or 80 ms (for 200 ms CL). The coupling interval was increased by 10 ms after every eighth beat until the extra-stimulus resulted in atrial capture. The coupling interval was then reduced by 10 ms and increased in 2 ms steps until the extra-stimulus captured the atria again. AERP was defined as the longest S1S2 coupling interval that failed to result in atrial capture.

Atrial conduction was measured after a stable 30 s period at twice-diastolic threshold and two CLs of stimulation (400 and 200 ms CL). 'Global' conduction times were defined as the time delay between the stimulus artefact at the pacing site and local activation at each of four unipolar recording sites (maximum negative peak of dV/dt), averaged to calculate global conduction. Each conduction time value corresponded to an average of five consecutive beats and was calculated for each of the five pacing sites.

Using the clockface electrode, 'local' atrial conduction properties at the posterior wall of the left atrium were expressed as: the average conduction velocity, conduction time heterogeneity (corresponding to the difference between the fastest and the slowest atrial conduction time), and the 'conduction anisotropic index'. On the basis of prior descriptions of anisotropic conduction properties in canine atria, we derived this new index which is the ratio of the fastest conduction velocity divided by the shortest conduction velocity.¹⁰ Local partial or complete atrial block in this area was also recorded when applying 30 s burst pacing at 150 ms CL.

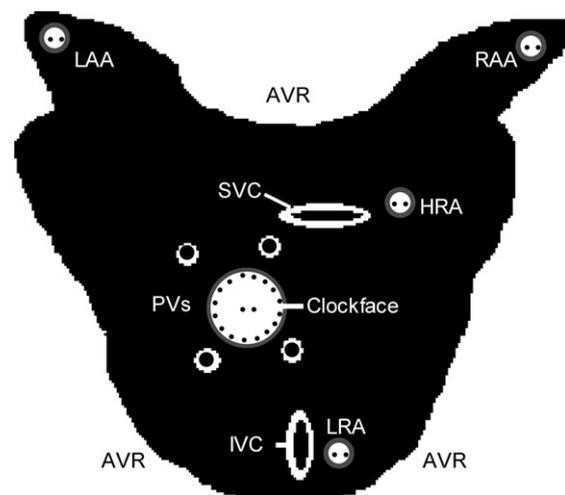


Figure 1 Diagram of clockface and bipolar electrode stimulation site. SVC, superior vena cava; IVC, inferior vena cava; PVs, pulmonary veins; AVR, atrio-ventricular ring; RAA, right atrial appendage; LAA, left atrial appendage; HRA, high right atrium; LRA, low right atrium.

2.2.3 Atrial fibrillation inducibility

An AF episode was defined as an irregular supraventricular arrhythmia with A-A intervals (interval between two consecutive atrial electrograms) <150 ms and lasting >1 min. Bursts of atrial pacing (10 V, 10 ms pulse duration at 10 Hz for 10 s) were applied 10 times at each of the five sites. The tendency to develop and maintain AF in this model was defined in two different ways:

- (1) The ability to induce AF was defined as (a) the percentage of dogs with at least one AF episode during AERP measurements (induced by a single extra-stimulus) and (b) the percentage of burst attempts leading to AF episodes (percentage of attempts leading to AF).
- (2) The ability to maintain AF was defined as (a) the median AF duration per dog (expressed as median and 25th–75th percentile) and (b) the percentage of dogs with at least one AF episode lasting >10 min.

AF episodes lasting >15 min were cardioverted using an external biphasic defibrillator (LIFEPAK, Medtronic, Inc.).

2.3 Blood samples

Venous blood samples were drawn from conscious dogs before pacemaker implant and on the day of the last electrophysiological study. Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) levels were measured using standard radioimmunoassays. Fatty acid compositions of serum phospholipids were determined by gas-liquid chromatography.¹¹

2.4 Gelatinase activity assays

Gelatin zymography¹² was performed on tissue samples from the left and right atrial appendages of all UNPACED, 10 SAVP-PLACEBO, and 10 SAVP-PUFA dogs, as described previously. The clear gelatinolytic zones were quantified by densitometric analysis using the Molecular Analyst Software (Bio-RAD Laboratories, Inc., Hercules, CA, USA) and were expressed as a percentage of the UNPACED dogs value.

2.5 Histology

Transmural tissue samples from the right and LA appendages of six UNPACED, eight SAVP-PLACEBO, and eight SAVP-PUFA dogs were fixed in 10% neutral buffered formalin. Tissue was processed, embedded in paraffin, and sectioned in 4–5 µm thick sections. Sections were stained with MOVATs pentachrome to differentiate connective tissue and cardiac muscle. Picrosirius red (PSR) was used to stain collagen red while the rest of the tissue remained pale yellow. The images were captured with a CCD camera with a green interference filter in the light path, adding further contrast to the images. The intensity of the staining was then measured using the thresholding function of the analysis program 'Image J' (Rasband, WS, NIH, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij> 1997–2006). This algorithm, which divides the image into objects

and background, was used to select the PSR-stained tissue. Pixels in the selected intensity range and within regions of interest (tissue inner folds) were counted. Connective tissue was expressed as a percentage of the reference tissue area, representing the total collagen area fraction. These analyses were performed by two experienced observers blinded to the treatment groups.

2.6 Collagen I and III and TGF-β1 mRNA measurement using real-time quantitative polymerase chain reaction

Total RNA was extracted using TRIzol (Invitrogen, Corp., Carlsbad, CA, USA) according to the manufacturer's protocol. cDNA was synthesized from DNase-treated total RNA samples by reverse transcription with high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol. PCR was performed by SYBR® Green PCR Master Mix (Applied Biosystems). The increase in fluorescence of the SYBR Green dye was monitored using ABI Prism 7900HT Sequence Detection System (Applied Biosystems). The relative mRNA levels in each sample were normalized to its 18S content. Cycle parameters were 55°C × 5 min, 95°C × 10 min, and then 40 cycles of 95°C × 15 s, 60°C × 60 s. The nucleotide sequences of primers and probes were Collagen I (forward) GTG TGTACA GAA CGG CCT CA, Collagen I (reverse) TCG CAA ATC ACG TCA TCG, Collagen III (forward) ATA GAG GCT TTG ATG GAC GAA, Collagen III (reverse) CCT CGC TCA CCA GGA GC, TGF-β (forward) CAA GGA TCT GGG CTG GAA GTG GA, TGF-β (reverse) CCA GGA CCT TGC TGT ACT GCG TGT.

2.7 Data analysis

Echocardiographic and electrophysiological measurements were analysed using repeated measures of one-way ANOVA for comparisons between groups. Significant differences were analysed with Tukey's *post hoc* test to compare individual group data. Non-normally distributed variables were analysed using the non-parametric Kruskal-Wallis test. Continuous variables were expressed as mean ± SD and compared using the unpaired Student's *t*-test. *P* < 0.05 was considered statistically significant.

3. Results

Relative concentrations of n-3 PUFAs in plasma phospholipids are summarized in *Table 1*. There were no differences between the three groups of dogs at baseline. After 3 weeks of n-3 PUFAs supplementation, n-3 PUFAs and EPA+DHA blood levels in the SAVP-PUFA group increased significantly (38.4% increase in n-3 PUFAs, and 81.4% increase in EPA+DHA, *P* < 0.05).

Table 1 Relative concentrations of n-3 fatty acids in plasma phospholipids

Fatty acids Time	UNPACED (n = 7) BL	SAVP-PLACEBO (n = 12) BL	SAVP-PUFA (n = 12) BL	SAVP-PLACEBO (n = 12) 3W	SAVP-PUFA (n = 12) 3W
n-3 PUFAs (%)	5.7 ± 0.9	5.7 ± 1.1	5.7 ± 0.8	5.7 ± 1.3	7.8 ± 1.0*
n-6 PUFAs (%)	41.6 ± 1.3	41.9 ± 1.3	41.6 ± 1.11	41.1 ± 0.7	39.3 ± 0.9*
EPA+DHA (%)	3.0 ± 0.6	3.0 ± 0.7	3.0 ± 0.7	3.1 ± 0.8	5.3 ± 0.9*
AA:EPA	33.0 ± 5.9	42.2 ± 23.1	42.7 ± 12.5	49.1 ± 16.6	21.4 ± 8.3*

Values are expressed as percentage by weight of total fatty acids at baseline and after 3 weeks of n-3 PUFAs supplementation. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid; PUFA, polyunsaturated fatty acid; BL, baseline; 3W, 3 weeks after n-3 PUFAs supplementation (**P* < 0.001: SAVP-PUFA compared with SAVP-PLACEBO).

3.1 Two-dimensional echocardiographic studies

To characterize the mechanical remodelling induced by pacing the right atrium and ventricle simultaneously, we compared the percentage of changes from baseline in left ventricular systolic function (left ventricular fractional area shortening; LVFAS) and in LA-emptying function (LAFAS) between SAVP-PLACEBO and RVP-PLACEBO dogs. Two weeks of stimulation for SAVP-PLACEBO dogs, compared with 5 weeks for RVP-PLACEBO dogs, produced a greater decrease in LAFAS (-60.8 ± 16.7 vs. $-25.9 \pm 30.4\%$, respectively, $P < 0.05$) but a smaller decrease in LVFAS (-37.9 ± 17.9 vs. $-54.9 \pm 10.2\%$, respectively, $P < 0.05$). No SAVP dog had more than moderate mitral regurgitation.

Echocardiographic parameters from UNPACED, SAVP-PLACEBO, and SAVP-PUFA dogs at baseline and after 2 weeks of pacing (for SAVP-PLACEBO and SAVP-PUFA) are shown in Table 2. At baseline, echocardiographic parameters were similar between the three groups. After 2 weeks of stimulation, the two groups of paced dogs developed a similar extent of left ventricular dysfunction (measured by LVFAS) and a similar decrease in LA-emptying function (measured by LAFAS) (Table 3).

3.2 Clinical outcomes

Six of 12 SAVP-PLACEBO dogs developed clinical ascites (confirmed during the final surgery), whereas eight had mild pericardial effusion. No SAVP-PLACEBO dog showed signs of pulmonary oedema or changes in behaviour or appetite. All RVP-PLACEBO dogs developed signs of pulmonary congestion (crackles, coughing, and increase in respiratory rate), ranging from moderate (six of eight) to severe (two of eight), appetite loss, weakness, decreased activity, and large pericardial effusion. Three RVP-PLACEBO dogs died prematurely (37.5%) prior to or during the final end study (before the completion of the protocol). All SAVP dogs completed the entire planned experiment.

3.3 Atrial natriuretic peptide and brain natriuretic peptide-32 assays

Plasma ANP and BNP-32 concentrations are summarized in Table 4; ANP and BNP levels were similarly increased at 2 weeks vs. baseline in both paced groups.

3.4 Electrophysiological data

Fifty per cent of the SAVP-PLACEBO dogs had AF episodes induced by a single extra-stimulus (during AERP measurements), as opposed to no AF episodes induced in SAVP-PUFA dogs (Table 5). The percentage of burst attempts leading to AF episodes was significantly reduced in SAVP-PUFA dogs ($5.5 \pm 7.4\%$) compared with SAVP-PLACEBO dogs ($20.4 \pm 14.2\%$, $P < 0.001$). SAVP-PUFA dogs also had a shorter AF duration than SAVP-PLACEBO dogs (median: 601 vs. 1598 s, respectively, $P < 0.05$). The percentage of dogs having long-lasting episodes (> 10 min) was significantly reduced in SAVP-PUFA compared with SAVP-PLACEBO dogs (41.7 vs. 83.3%, respectively, $P < 0.05$). Cardioversion was required for persistent AF in eight of 12 SAVP-PLACEBO dogs (two underwent three cardioversions for three different episodes), compared with four of the SAVP-PUFA dogs. All three groups had similar mean AERPs at all paced CLs (Figure 2). SAVP-PLACEBO dogs had a significant increase in mean global atrial conduction times at 200 ms CL stimulation compared with UNPACED dogs (Figure 3). SAVP-PUFA dogs had conduction times intermediate between the UNPACED and the SAVP-PLACEBO, but were not significantly different from either group.

SAVP-PLACEBO dogs had slower local atrial conduction velocity calculated along the 16 equidistant electrodes when pacing at 200 ms CL, compared with UNPACED dogs (395 ± 105 vs. 714 ± 108 mm/s, respectively, $P < 0.05$). SAVP-PLACEBO dogs also had a greater conduction time heterogeneity than UNPACED dogs (22 ± 3.0 vs. 12.5 ± 4.2 ms, respectively, $P < 0.05$) and a lower conduction anisotropic index (2.35 ± 0.22 vs. 3.33 ± 0.56 , respectively, $P < 0.05$).

SAVP-PUFA dogs had less conduction slowing than SAVP-PLACEBO (587 ± 81 vs. 395 ± 105 mm/s, respectively, $P < 0.05$), less conduction time heterogeneity (16.2 ± 2.1 vs. 22 ± 3.0 ms, respectively, $P < 0.05$), and a higher conduction anisotropic index (3.25 ± 0.61 vs. 2.35 ± 0.22 , respectively, $P < 0.05$). We also observed a greater incidence of atrial conduction block at 150 ms CL stimulation along the clockface electrode in SAVP-PLACEBO (5/5) vs. SAVP-PUFA (0/5) dogs.

3.5 Gelatinase (matrix metalloproteinases 2 and 9) activity

Direct observation of the gels showed a reduction in active matrix metalloproteinase-9 (MMP-9) activity in SAVP-PUFA

Table 2 Echocardiographic parameters at baseline and after 2 weeks of pacing

	UNPACED	SAVP-PLACEBO BL	SAVP-PLACEBO 2W	SAVP-PUFA BL	SAVP-PUFA 2W
LASA (cm ²)	$6.2 \pm 1.0\ddagger$	7.2 ± 1.8	$12.9 \pm 2.3^*$	6.3 ± 1.8	$13.7 \pm 2.9\ddagger$
LADA (cm ²)	$9.9 \pm 1.1\ddagger$	10.7 ± 2.3	$14.8 \pm 2.8^*$	9.2 ± 2.5	$15.6 \pm 3.1\ddagger$
LAFAS (%)	$36.5 \pm 10.4\ddagger$	33.3 ± 6.3	$12.6 \pm 4.9^*$	31.7 ± 5.5	$12.7 \pm 3.2\ddagger$
LVDA (cm ²)	$6.7 \pm 1.9\ddagger$	7.5 ± 2.0	$13.9 \pm 3.3^*$	6.7 ± 1.8	$12.7 \pm 1.9\ddagger$
LVSA (cm ²)	$12.4 \pm 1.8\ddagger$	13.1 ± 2.3	$18.8 \pm 3.9^*$	11.8 ± 2.3	$17.8 \pm 2.1\ddagger$
LVFAS (%)	$46.0 \pm 11.2\ddagger$	43.3 ± 8.9	$26.2 \pm 7.4^*$	43.1 ± 8.6	$25.7 \pm 7.0\ddagger$

LASA, left atrial systolic area; LADA, left atrial diastolic area; LAFAS, left atrial fraction area shortening; LVDA, left ventricular diastolic area; LVSA, left ventricular systolic area; LVFAS, left ventricular fraction area shortening. BL, baseline; 2W, 2 weeks after pacing.

* $P < 0.05$: SAVP-PLACEBO BL compared with SAVP-PLACEBO 2 weeks; † $P < 0.05$: SAVP-PUFA BL vs. SAVP-PUFA 2 weeks; ‡ $P < 0.05$: UNPACED vs. SAVP-PLACEBO 2 weeks and SAVP-PUFA 2 weeks.

Table 3 Percentage of change in echocardiographic parameters from baseline to after 2 weeks of pacing

Percentage of change	SAVP-PLACEBO (n = 12)	SAVP-PUFA (n = 12)
LASA	88.2 ± 48.3	93.5 ± 34.4
LADA	43.4 ± 36.9	45.0 ± 29.1
LAFAS	-60.8 ± 16.7	-58.9 ± 11.4
LVDA	91.7 ± 49.5	100.2 ± 61.5
LVSA	43.3 ± 16.6	50.6 ± 36.1
LVFAS	-37.9 ± 17.9	-36.9 ± 25

LASA, left atrial systolic area; LADA, left atrial diastolic area; LAFAS, left atrial fraction area shortening; LVDA, left ventricular diastolic area; LVSA, left ventricular systolic area; LVFAS, left ventricular fraction area shortening. There were no significant differences in the extent of changes in atrial or ventricular size or function between the groups.

dogs compared with SAVP-PLACEBO dogs (Figure 4). The corresponding densitometry in both appendages confirmed that SAVP-PUFA dogs had less increase in active MMP-9 activity than SAVP-PLACEBO dogs (220 ± 70 vs. 320 ± 65%, respectively, $P < 0.05$). The separate analysis for right and left appendages did not show any differences between the two groups of dogs, and the results are combined for the sake of clarity.

3.6 Histological analysis

SAVP-PLACEBO dogs had marked cell 'drop out' and more interstitial fibrosis within the atrial appendage than SAVP-PUFA dogs. There was less total collagen area fraction in SAVP-PUFA dogs compared with SAVP-PLACEBO dogs (9.8 ± 2.7 vs. 17.2 ± 3.2%, respectively, $P < 0.05$). There were no significant differences in collagen area fraction between UNPACED and SAVP-PUFA dogs (12.5 ± 1.7 vs. 9.8 ± 2.7%, respectively, $P = \text{NS}$). No differences were observed between right and LA appendages regarding total collagen area fraction in each group of dogs. Representative histological sections from each group are shown in Figure 5.

3.7 TGF-β1 and collagen isoforms analysis

There was significantly less LA collagen type I and III messenger RNA in SAVP-PUFA dogs compared with SAVP-PLACEBO [0.62 ± 0.51 vs. 10.80 ± 5.61, respectively, for collagen I, $P < 0.05$; 1.66 ± 0.48 vs. 5.24 ± 1.16, respectively, for collagen III, $P < 0.05$ (arbitrary units)]. SAVP-PLACEBO dogs had a significant increase in collagen type I and III mRNA

expression compared with UNPACED dogs (1.10 ± 0.49 vs. 1.02 ± 0.23, respectively, for collagen I and III), whereas SAVP-PUFA dogs had similar values compared with UNPACED dogs.

The pro-fibrotic cytokine TGF-β1 was similarly increased in both pacing groups: SAVP-PUFA (1.60 ± 0.41) and SAVP-PLACEBO (1.35 ± 0.25) (arbitrary units) when compared with control dogs (1.01 ± 0.24).

4. Discussion

The main results of this study demonstrate that oral n-3 PUFAs supplementation, in an animal model of atrial remodelling and AF, reduced AF inducibility and maintenance, reduced conduction anisotropy in the left atrium, and prevented pacing-induced increase in collagen turnover and collagen deposition in atrial appendages.

4.1 A novel canine pacing model

This study shows that pacing the right atrium and ventricle simultaneously for 2 weeks induced more atrial and less ventricular mechanical remodelling than pacing the right ventricle only, creating several advantages. First, although our study is not strictly comparable with others, the incidence of induced AF (median AF duration) in SAVP-PLACEBO dogs [1598 (1195–2400)] was more than the mean-induced AF burden usually obtained after 5 weeks of right ventricular pacing only (720 ± 461 s).¹³

Secondly, by inducing profound mechanical atrial remodelling and AF irritability before the onset of severe left ventricular dysfunction, this novel model allowed us to complete the entire planned experiment in all dogs, without premature death (commonly due to overt heart failure and reported in 20–30% of the right ventricular pacing dogs).¹⁴

4.2 Potential mechanism of antiarrhythmic action of n-3 polyunsaturated fatty acids administration

In vitro studies, using cultured neonatal rat cardiomyocytes and extracellular application of EPA and DHA, have shown that n-3 PUFAs have multiple electrophysiological properties in myocardial cell membranes by influencing membrane fluidity and ion transport.¹⁵ Current hypotheses of the possible action of n-3 PUFAs in preventing AF are based on their known capacity to inhibit fast, voltage-dependent sodium currents, L-type calcium currents, the Na⁺/Ca²⁺ exchanger, which might prevent delayed after-depolarizations and triggered activity, as well as their class III antiarrhythmic-like effect on Kv1.5 channel (*I_{KUR}* current present in the atrium).^{16–18} Recent studies have shown that acute

Table 4 Plasma atrial natriuretic peptide and brain natriuretic peptide-32 levels at baseline and after 2 weeks of pacing

Time	UNPACED (n = 7)	SAVP-PLACEBO (n = 12)	SAVP-PLACEBO (n = 12)	SAVP-PUFA (n = 12)	SAVP-PUFA (n = 12)
	BL	BL	2W	BL	2W
ANP (pg/mL)	33.1 ± 6.8*	21.5 ± 3.1	45.7 ± 9.3**	26.3 ± 8.0	55.0 ± 18.2**
BNP-32 (pg/mL)	10.2 ± 6.0	7.4 ± 2.7	24.3 ± 4.5**	9.0 ± 3.8	30.2 ± 9.2**

ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; BL, baseline; 2W, after 2 weeks of pacing.

* $P < 0.05$: UNPACED vs. SAVP-PLACEBO at BL; ** $P < 0.001$: SAVP-PLACEBO and SAVP-PUFA had significant increases in ANP and BNP levels at 2 weeks (** $P < 0.001$) compared with BL, with no significant differences between treatment groups.

Table 5 Atrial fibrillation inducibility and maintenance

AF		UNPACED (n = 7)	SAVP-PLACEBO (n = 12)	SAVP-PUFA (n = 12)
Inducibility	AERP-induced AF (%)	0.0	50.0	0.0*
	Percentage of attempts leading to AF	1.0 ± 1.7	20.4 ± 14.2	5.5 ± 7.4**
Maintenance	Median AF duration (s)	0 (0-0)	1598 (1195-2400)	601 (377-1216)*
	Percentage of dogs with AF > 10 min	0.0	83.3	41.7*

Data are presented as percentages with or without standard deviation, and median (25th–75th percentile).

AF inducibility: AERP-induced AF percentage—percentage of dogs with at least one AF episode lasting >1 min after a single extra-stimulus; percentage of attempts leading to AF—percentage of burst attempts leading to AF episodes lasting >1 min.

AF maintenance: percentage of dogs with AF > 10 min—percentage of dogs which developed at least one AF episode lasting >10 min. SAVP-PUFA dogs had less AF inducibility (**P* < 0.05, ***P* < 0.001) and less AF maintenance (**P* < 0.05) than SAVP-PLACEBO dogs.

administration of fish oils prevents the shortening of refractoriness in response to rapid pacing in dogs,¹⁹ and after supplementation for 12 weeks, in response to stretch-induced electrical remodelling in a rabbit model.²⁰

However, n-3 PUFAs administration in our study did not result in slowing of conduction or prolongation of refractoriness as may be expected on the basis of *in vitro* or *in vivo* actions. We did not measure n-3 PUFAs effects on individual ion currents, and rapid pacing is known to alter Ca²⁺, K⁺, and Na⁺/Ca²⁺ exchange currents.²¹ Although we cannot rule out n-3 PUFAs effects on ionic currents in this study, the integrated effects of n-3 PUFAs do not seem to be primarily mediated by a conventional ‘antiarrhythmic’ mechanism.

A previous animal study, published in abstract form, has suggested that n-3 PUFAs might help to reduce AF vulnerability by preventing the atrial mechanical remodelling induced by rapid ventricular pacing (significant reduction in right and LA pressures).²² The absence of n-3 PUFAs effect on echocardiographic parameters or ANP and BNP levels suggests that, in our study, n-3 PUFAs supplementation did not influence the mechanical remodelling (systolic dysfunction or dilation) of the atria or the ventricles caused by SAVP. In the SAVP model, there is marked slowing, and local heterogeneity in atrial conduction (at the posterior wall of the LA), which are well-known requisites for re-entry mechanisms.²³ The SAVP model is also characterized by a decrease in local conduction anisotropy, accounting for more slowing in the fastest segments than in the slowest segments (recorded along the clockface electrode).

In this study, we found that in n-3 PUFAs-supplemented dogs, local conduction parameters tend to return towards normal values. In SAVP-PUFA dogs, average conduction velocity was faster, conduction time heterogeneity was less, and conduction anisotropy was higher than in SAVP-PLACEBO dogs. This may in part explain the lower AF vulnerability of n-3 PUFAs compared with no n-3 PUFAs dogs. Atrial conduction velocity is dependent on membrane excitability (availability of I_{Na}) and passive tissue resistivity (changes in extracellular matrix and degree of anisotropy of conduction).²⁴ An Na⁺ blocking effect of n-3 PUFAs¹⁵ would be expected to slow rather than prevent slowing of conduction velocity.

Structural remodelling, including changes in extracellular matrix (collagen turnover, amount, composition, and distribution of collagen fibres), and gap junction dysfunction have been shown to play an important role in atrial conduction velocity reduction and genesis of atrial arrhythmia by re-entry mechanisms in dogs.^{10,16} The increase in collagen turnover (reflected by increased atrial gelatinase activity) in paced dogs was significantly attenuated in SAVP-PUFA dogs, resulting in a lower total collagen area fraction compared with SAVP-PLACEBO dogs. A more detailed analysis of the shift in the collagen isoforms has shown that LA collagen type I and III mRNA was lower after PUFA supplementation compared with non-PUFA-treated paced dogs, in whom there was significant increase in collagen mRNA signal. Transforming growth factor-β1, a potent pro-sclerotic cytokine, has been implicated in the pathogenesis of an arrhythmogenic substrate including conduction heterogeneity and atrial vulnerability.^{25,26} In the present study,

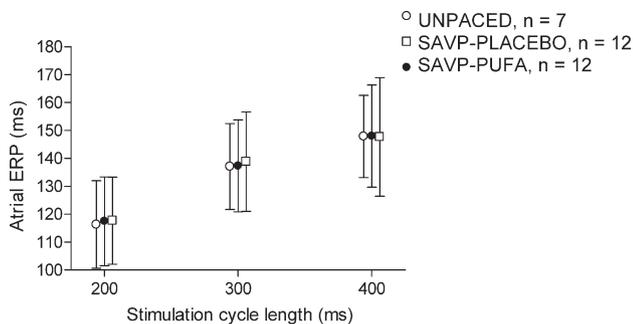
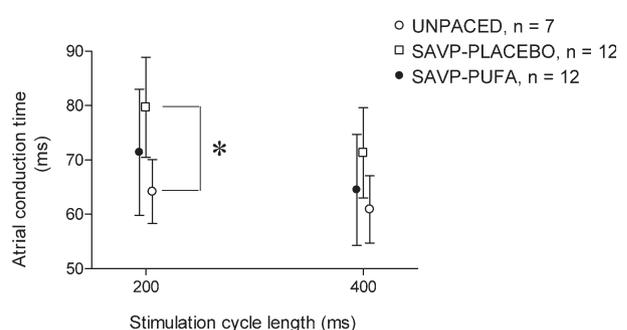


Figure 2 Mean atrial effective refractory periods. Average atrial effective refractory periods at five different locations (high right atrium, low right atrium, right appendage, left appendage, and left atrium posterior wall) as a function of cycle length (400, 300, and 200 ms CL stimulation) for seven control dogs (UNPACED), 12 paced placebo dogs (SAVP-PLACEBO), and 12 paced dogs supplemented with n-3 PUFAs (SAVP-PUFA).



**P* < 0.05 between SAVP-PLACEBO and UNPACED dogs.

Figure 3 Mean global atrial conduction time. Average of mean conduction times on all five atrial sites as a function of cycle length (400 and 200 ms CL stimulation). SAVP-PUFA dogs had longer atrial conduction time than UNPACED dogs (**P* < 0.05).

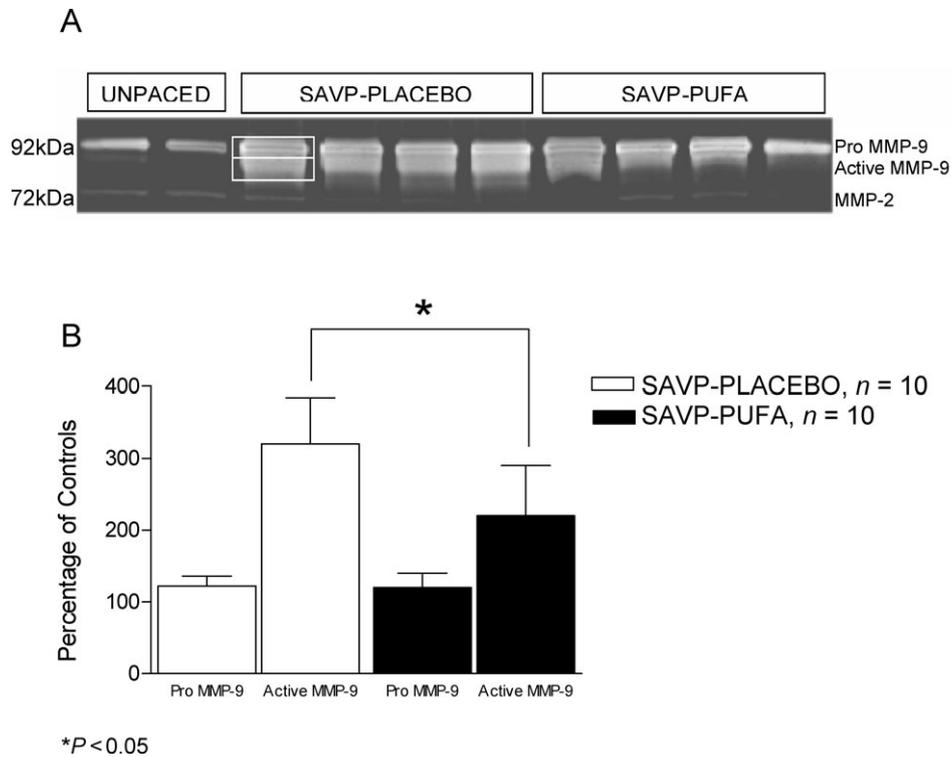


Figure 4 Gelatinase activity assays. (A) The two first upper bands represent pro- and active MMP-9 activity (white boxes), and the lower band represents MMP-2 activity. (B) Mean values of pro- and active MMP-9 activity for 10 SAVP-PLACEBO and 10 SAVP-PUFA dogs were calculated as a percentage of the value in UNPACED dogs. SAVP-PUFA dogs had less active MMP-9 activity than SAVP-PLACEBO (*P < 0.05).

both paced groups were associated with a similar increase in mRNA expression of TGF-β1 compared with UNPACED dogs; however, only paced dogs not supplemented with PUFA experienced an increased fibrillar collagen deposition.

The differences demonstrated between SAVP-PUFA and SAVP-PLACEBO dogs suggest that the roles of TGF-β1 and MMPs in cardiac remodelling are not completely elucidated and that other cytokines are probably involved in the pathogenesis of atrial fibrosis and atrial arrhythmogenesis. Total

matrix collagen content is a function of both synthesis and degradation. Degraded products of matrix proteins serve as a stimulus for collagen synthesis; this in turn results in increased deposition of type I collagen in the atria of patients with AF and cardiomyopathy.²⁷ Atrial collagen composition (shift in the ratio of collagen types III/I) may also be predictive for AF by increasing atrial conduction heterogeneity.²⁷ Thus, possibly, in part, by attenuating the increase in MMP-9 activity, n-3 PUFAs supplementation may prevent

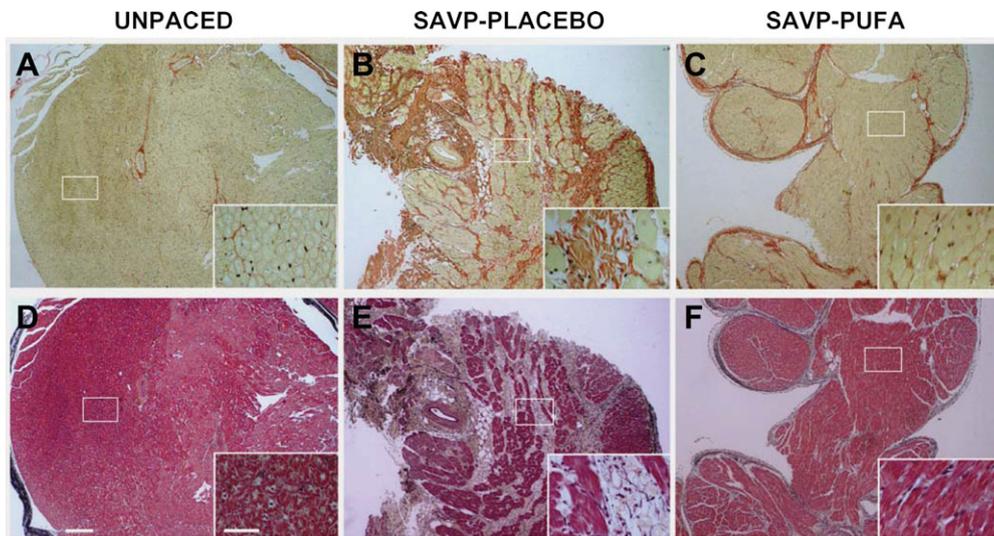


Figure 5 Tissue samples from left atrial appendages stained for collagen. (A–C) Picosirius-stained sections: cellular areas appear red and collagen-rich areas stain yellow. (D–F) MOVAT-stained sections: cellular areas appear red and fibrotic, collagen-rich areas stain light (yellow) colour. As evident in this figure, there was marked interstitial fibrosis accompanied by cell 'drop-out' in SAVP-PLACEBO left atrial appendage compared with UNPACED and SAVP-PUFA dogs. Scale bar: 200 μm (4× objective) and 50 μm (20× objective).

changes in both the content and the quality of collagen in atrial appendages and result in less atrial fibrosis, with the corresponding electrophysiological consequences.

Although mechanical stretch and strain induced by haemodynamic load in the atria may contribute to the transcriptional regulation of MMPs, it is unlikely that these factors played a direct role in this study since there were no differences in mechanical remodelling between the two groups of paced dogs.²⁸ Sympathetic activation in patients with cardiac heart failure has also been demonstrated to influence extracellular matrix turnover via MMP induction.²⁹ However, we observed no differences in mean heart rate and blood pressure between the dogs. Stimulation of the rennin-angiotensin-aldosterone system, oxidative stress imbalance, and cytokine release, which are additional recognized pathways of MMP induction in heart failure, may have been modulated by the administration of n-3 PUFAs. Oxidative stress is known to mediate the early electrophysiological remodelling associated with AF, which suggests that antioxidant treatments may provide significant benefit in preventing AF.³⁰ The efficacy of n-3 PUFAs may also be related to the antagonism of oxidative pathways or to an anti-inflammatory effect.³¹ Further studies of the effects of n-3 PUFAs on these pathways are needed to further define the mode of efficacy.

Unrelated to fibrosis *per se*, a modulation in the expression of connexins 40 and 43 could also be involved in the mechanism by which n-3 PUFAs attenuate local changes in atrial conduction and anisotropy, as recently proposed in an AF-induced vagal stimulation model.³²

Cardiac protection from PUFAs against arrhythmia might depend on the type, route of administration, and dosage. A retrospective analysis of the Physicians Health Study³³ has shown that baseline blood levels of long-chain n-3 fatty acids were inversely related to the risk of sudden death. For patients with documented coronary heart disease, on the basis of the 45% reduction in sudden death from the GISSI trial,³⁴ the American Heart Association recommends ~1 g of EPA and DHA (combined) per day.³⁵

In our study, with a supplementation of one capsule of PUFAs (1 g daily) corresponding to EPA+DHA of 840 mg, doses of PUFAs by weight were two to three-fold higher than doses in human studies of omega-3 supplementation. SAVP-PUFA dogs reached a relative concentration of EPA+DHA (expressed as a percentage by weight of total fatty acids in the blood) of $5.3 \pm 0.9\%$. In comparison, the relative concentrations range of EPA+DHA in plasma phospholipids was 4.6% in the cardiovascular health study population protected against AF while consuming fatty fish regularly.³⁶

5. Limitations

Although the animal model in this study induces clinically relevant atrial remodelling, it remains a surrogate for human AF. Similar to other animal models, it can only help to better understand the relationship between n-3 PUFAs supplementation and atrial remodelling. Unlike spontaneous human AF, but similar to other animal models, pacing is required to induce AF.

It would have been interesting to investigate whether any n-3 PUFA effect on connexins could contribute to the anti-arrhythmic effect. However, measuring the protein levels of

Cx40 and Cx43 by western blot and analysing the distribution of Cx43 between SAVP-PLACEBO and SAVP-PUFA dogs by immunochemistry may not be sufficient to assess potential relationship between n-3 PUFA, connexins, and conduction anisotropy.³⁷ Prior experiments studying acute electrophysiological effects of PUFAs used high doses *in vitro*. We attempted to use clinically relevant doses (1 g per day) and long-term oral administration. It is possible that higher doses of PUFAs may have induced effects on AERP.

N-3 PUFAs supplementation was started 1 week before starting the atrioventricular pacing. This sequence does not replicate the two most common clinical situations: fatty fish consumption for years in observational studies and n-3 PUFAs supplementation in patients with prior recurrent, symptomatic AF. An ongoing clinical trial will test the efficacy of n-3 PUFAs in preventing AF recurrence (<http://clinicaltrials.gov/ct/show/NCT00402363?order=1>).

Based on our results, n-3 PUFAs supplementation might be more useful in the prevention of atrial structural remodelling in patients subject to atrial stretch such as mitral regurgitation or hypertension, than in the treatment of patients with AF and established heart disease.

6. Conclusion

Dietary n-3 PUFAs supplementation attenuates the development of atrial fibrosis and inducibility of AF in an animal model of pacing-induced structural atrial remodelling. Ongoing clinical trials with n-3 PUFAs supplementation will improve our understanding of the potential therapeutic benefits of fish oils for this common condition.

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