N-3 polyunsaturated fatty acid supplementation does not reduce vulnerability to atrial fibrillation in remodeling atria

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BACKGROUND Prophylactic supplementation with omega-3 polyunsaturated fatty acids (PUFAs) reduce vulnerability to atrial fibrillation (AF). The effect of PUFAs given after cardiac injury has occurred is unknown.

OBJECTIVE To investigate using a model of pacing-induced cardiac injury, the time course of development of injury and whether it was altered by postinjury PUFAs.

METHODS Fifty-six dogs were randomized to undergo simultaneous atrial and ventricular pacing (SAVP, 220 beats/min) for 0, 2, 7, or 14 days. Twenty-two dogs received PUFAs (850 mg/d) either prophylactically or after some pacing had occurred (postinjury). Electrophysiologic and echocardiographic measurements were taken at baseline and sacrifice. Atrial tissue samples were collected at sacrifice for histologic and molecular analyses.

RESULTS With no PUFAs, the inducibility of AF increased with pacing duration (P < .001). Postinjury PUFAs (started after 7 days of pacing) did not reduce the inducibility of AF after 14 days of pacing (9.3% ± 8.8% no PUFAs vs 9.7% ± 9.9% postinjury PUFAs; P = .91). Atrial myocyte size and fibrosis increased with pacing duration (P < .05). Postinjury PUFAs did not significantly attenuate the cell size increase after 14 days of pacing (no PUFAs 38% ± 30% vs postinjury PUFAs 19% ± 28%; P = .11). Similarly, postinjury PUFAs did not attenuate the increase in fibrosis after 14 days of pacing (no PUFAs 66% ± 51% vs postinjury PUFAs 63% ± 76%; P = .90).

CONCLUSION PUFAs given after cardiac injury has already occurred does not reduce atrial structural remodeling or vulnerability to AF.

KEYWORDS Atrial fibrillation; Fatty acids; Remodeling; Collagen; PUFAs; Omega-3.

ABBREVIATIONS AF = atrial fibrillation; Akt = protein kinase B; DHA = docosahexaenoic acid; EGF = epidermal growth factor; EPA = eicosapentaenoic acid; LAA = left atrial appendage; PUFAs = n-3 polyunsaturated fatty acids; SAVP = simultaneous atrial and ventricular pacing.

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Introduction

Atrial fibrillation (AF) is the most common arrhythmia seen clinically and is associated with an elevated risk of death, primarily from stroke.1 Current treatments (cardioversion, antiarrhythmic drugs, and ablation) are only moderately effective in preventing AF recurrences.2

Some studies have suggested that the marine derived n-3 polyunsaturated fatty acids (PUFAs)—eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)—could have beneficial effects in the treatment of AF.3,4 The most commonly suggested mechanism for this effect is a direct antiarrhythmic property of PUFAs.5 However, recent studies have not confirmed the antiarrhythmic efficacy of PUFAs.6–9 PUFAs have also been shown to have antiarrhythmic effects.10–15 Cardiac structural remodeling (dilatation, cellular hypertrophy, and fibrosis) is a significant risk factor for AF.1

The simultaneous atrial and ventricular pacing (SAVP) model involves pacing both atria and ventricles rapidly and simultaneously, resulting in substantial structural remodeling of the atria and vulnerability to AF.16 In this model, PUFAs given prior to the start of pacing reduce vulnerability to AF.11 The mechanism of PUFA benefit may be
related to PUFA-mediated changes in the expression of genes related to structural remodeling.\textsuperscript{10} In order to more fully investigate any antistructural remodeling properties of PUFA, we wished to establish more clearly the time course of the development of structural remodeling (and AF vulnerability) in the SAVP model. We also sought to determine the effect of “prophylactic” PUFA on the tissue injury response generated soon after the start of SAVP (48 hours), which may subsequently trigger structural remodeling. We also investigated the effect of PUFA given after some structural remodeling had already been established (“postinjury”).

Methods
The protocol was approved by the Animal Care Committee of St Michael’s Hospital (Toronto, Ontario, Canada). The investigation conformed to the Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996). Detailed methods are available in the online supplement.

Study groups
This study was composed of 9 groups totaling 65 dogs (all mongrels, 1–3 years old, 19–30 kg, of either sex):

1. Unpaced, no PUFA, no sham operation (n = 7)
2. Unpaced, no PUFA, sham pacemaker implantation (n = 4)
3. Unpaced, PUFA 21 days, sham pacemaker implantation (n = 5)
4. Paced 2 days, olive oil 9 days (n = 6)
5. Paced 2 days, PUFA 9 days (prophylactic) (n = 6)
6. Paced 7 days, no PUFA (n = 6)
7. Paced 14 days, no PUFA (n = 12)
8. Paced 14 days, olive oil 21 days (n = 8)
9. Paced 14 days, PUFA 7 days (postinjury) (n = 11)

Groups 2–9 underwent a pacemaker implantation surgery; all groups underwent a sacrifice study including echocardiographic and electrophysiologic measurements. Groups 3, 5, and 9 were supplemented daily with oral PUFA (460 mg of EPA, 380 mg of DHA [Omacor, Solvay Pharmaceuticals GmbH, Hanover, Germany]), and groups 4 and 8 were supplemented daily with oral olive oil (1000 mg [Filippo Berio, San Migliarano, Italy]). Groups 4, 5, and 8 received their supplement beginning 7 days before pacing began and continued until sacrifice (prophylactic treatment). Group 9 received the supplement beginning 7 days after pacing began and continued until sacrifice (postinjury treatment).

Control groups 1 and 7 have been previously reported.\textsuperscript{10,11} In this study, we have reanalyzed these groups by using more stringent techniques and combined their data with the additional control groups (2 and 8, respectively) for statistical analysis. The new techniques included a stricter definition of AF (modified from MoRillo et al\textsuperscript{17}), a more accurate definition of global atrial conduction time (modified from Kumagai et al\textsuperscript{18}), and a method for calculating fibrosis that used higher magnification images and more accurate collagen identification methods (modified from Verheule et al\textsuperscript{20}).

Surgical procedures
Details of surgical procedures, sample collection, and preparation are available in the online supplement.

Atrial electrophysiology
AF was defined as any irregular atrial tachyarrhythmia (atrial rate > 300 beats/min) with duration > 120 seconds and resistant to pace termination. Global atrial conduction time was calculated as mean conduction time between the superior vena cava and the left atrial appendage (LAA). The atrial effective refractory period was measured via the S1, S2 stimulus technique.

PUFA integration
EPA and DHA levels were measured separately in plasma and atrial tissue samples by gas-liquid chromatography as performed by Stark and Holub.\textsuperscript{21}

Histology
The presence of inflammatory cells was quantified via CD18 staining (method modified from Afanasyeva et al\textsuperscript{22}). Mean atrial myocyte cross-sectional area was calculated via hematoxylin and eosin staining. Atrial fibrosis was quantified via picrosirius red staining. All histological analyses were performed in a blinded fashion.

Molecular analysis of structural remodeling (quantitative real-time polymerase chain reaction)
Quantitative real-time polymerase chain reaction was performed on LAA tissue according to standard techniques. Structural remodeling genes known to be affected by both SAVP and PUFA were measured.\textsuperscript{10,11}

Statistics
Comparisons were performed by using 1-way analysis of variance with a Tukey’s post hoc test, or an unpaired t test; results were significant if P was < .05. Error bars on all charts indicate standard error of the mean. In text and tables, values are presented as mean ± standard deviation.

Results
PUFA integration
Both plasma and tissue showed a significant increase in EPA + DHA levels (as a percentage of total fatty acids in total phospholipids) in dogs supplemented with PUFA-filled capsules (P < .001 for both plasma and tissue; Figure 1). Absolute plasma EPA + DHA levels between baseline and sacrifice were unchanged in unsupplemented dogs (−0.2% ± 0.7%) whereas they increased significantly in dogs supplemented for 7, 9, or 21 days (absolute increase of 1.4% ± 0.7%, 1.4% ± 0.8%, and 1.7% ±
PUFA integration into plasma and tissue. 

A: The relative change in EPA + DHA in plasma phospholipid levels between baseline and sacrifice. B: The level of EPA + DHA in atrial tissue phospholipids at the time of sacrifice relative to the no-PUFAs group. See text for absolute percentages. EPA = eicosapentaenoic acid; DHA = docosahexaenoic acid; PUFAs = n-3 polyunsaturated fatty acids. *P < .05, ***P < .001 compared with the no-PUFAs group.

Time course of the development of SAVP-induced disease

Atrial electrophysiology

The time course of the development of AF inducibility in SAVP dogs between 0 and 14 days is illustrated in Figure 2A. AF inducibility increased with increased pacing duration (P < .001). AF incidents were induced by 0.2% ± 0.8% of bursts in unpaced dogs. This increased to 0.8% ± 1.3% after 2 days of pacing, then to 5.5% ± 5.8% after 7 days of pacing, and finally to 9.3% ± 8.8% after 14 days of SAVP. Similarly, persistent AF could be induced with 0% of bursts in unpaced dogs, with 0.5% ± 1.2% of bursts after 2 days of SAVP, with 1.5% ± 1.7% of bursts after 7 days of SAVP, and with 7.1% ± 9.0% of bursts after 14 days of SAVP (P < .05). Table 1 shows that pacing induced an increase in conduction time that was significant after 14 days (11% increase over unpaced group; P < .05). Pacing did not significantly alter the atrial effective refractory period (Table 1).

Cardiac function

Atrial volume (both systolic and diastolic) was increased by SAVP (P < .05; Figure 2B). No changes in atrial emptying fraction were observed. SAVP induced an increase in left ventricular end systolic and left ventricular end diastolic volume, as well as a decrease in left ventricular ejection fraction (Table 2).

Histology

Figure 2C shows a significant increase in CD18 staining (indicating the presence of inflammatory cells) after 2 days of SAVP that returns toward baseline by 7 days. SAVP increased the LAA myocyte cross-sectional area (P < .05) from 164 ± 44 μm² in the unpaced dogs to 198 ± 60 μm² after 2 days of pacing, to 199 ± 64 μm² after 7 days of pacing, and to 225 ± 45 μm² after 14 days of SAVP (Figure 2D). Collagen area fraction in LAA tissue (Figure 2E) increased with pacing (P < .001). Collagen area fraction of unpaced dogs was 10% ± 5%. It was 8% ± 1%, 10% ± 5%, and 17% ± 5% after 2, 7, and 14 days of SAVP, respectively.

Molecular analysis of structural remodeling

Quantitative real-time polymerase chain reaction was used to analyze changes in the gene expression (mRNA level) of several structural remodeling-related genes previously shown to be important in this model. Table 3 shows mRNA levels at 0 and 7 days of SAVP. After 7 days of SAVP, there was a significant increase in the expression of Smad7, protein kinase B (Akt), and epidermal growth factor (EGF) compared with these 3 words should be removed unpaced dogs.

Effect of postinjury PUFAs after 14 days of SAVP

Atrial electrophysiology

The effect of postinjury PUFAs (PUFA supplementation beginning after 7 days of pacing) on AF inducibility after 14 days of SAVP is shown in Figure 3A. Postinjury PUFAs were not able to reduce AF inducibility in 14-day SAVP dogs (9.3% ± 8.8% no PUFAs vs 9.7% ± 9.9% postinjury PUFAs [P = .91]). Persistent AF inducibility was also unchanged by postinjury PUFAs (7.1% ± 9.0% no PUFAs vs 3.9% ± 6.8% postinjury PUFAs; P = .33). Postinjury PUFAs did not attenuate the pacing-induced conduction time increase (72 ± 10 ms no PUFAs vs 71 ± 6 ms postinjury PUFAs; P = .72; Table 1). Postinjury PUFAs did not affect the atrial effective refractory period (Table 1).

Cardiac function

No significant effect of postinjury PUFAs was seen on left atrial volumes, left atrial function, left ventricle volumes, or left ventricle function (Figure 3B and Table 2).

Histology

Figure 3C shows that postinjury PUFAs did not attenuate the pacing-induced hypertrophy in dogs paced for 14 days (no PUFAs 38% ± 30% vs postinjury PUFAs 19% ± 28%; P = .11). Figure 3D shows that postinjury PUFAs did not attenuate SAVP-induced fibrosis at the 14-day time point (no PUFAs 66% ± 51% vs postinjury PUFAs 63% ± 76%; P = .90).
Molecular analysis of structural remodeling

After 14 days of SAVP with postinjury PUFAs, collagen I and collagen III levels were significantly greater than those in unpaced dogs. Transforming growth factor-β1, Smad7, EGF, and myosin heavy chain-α levels were significantly less after 14 days of SAVP with postinjury PUFAs than in dogs paced for 7 days with no PUFAs (Table 3).

Table 1  Effect of SAVP and postinjury PUFAs on electrophysiologic parameters

<table>
<thead>
<tr>
<th>Days of SAVP</th>
<th>No PUFAs</th>
<th>Postinjury PUFAs</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GACT (ms)</td>
<td>AERP (ms)</td>
</tr>
<tr>
<td>0</td>
<td>65 ± 4</td>
<td>123 ± 18</td>
</tr>
<tr>
<td>2</td>
<td>65 ± 7</td>
<td>115 ± 3</td>
</tr>
<tr>
<td>7</td>
<td>71 ± 7</td>
<td>115 ± 6</td>
</tr>
<tr>
<td>14</td>
<td>72 ± 10*</td>
<td>118 ± 10</td>
</tr>
<tr>
<td>14</td>
<td>71 ± 6</td>
<td>117 ± 12</td>
</tr>
</tbody>
</table>

GACT was measured in sinus rhythm at 400 ms CL SVC to LAA.
AERP was measured at 200 ms CL.
AERP = atrial effective refractory period; CL = cycle length; GACT = global atrial conduction time; LAA = left atrial appendage; PUFA = n-3 polyunsaturated fatty acids; SAVP = simultaneous atrial and ventricular pacing; SVC = superior vena cava.
*P < .05 compared with unpaced via t test.

Effect of prophylactic PUFAs on the early development of SAVP-induced disease

Atrial electrophysiology and cardiac function

No differences were found between dogs paced for 2 days with PUFAs vs without for any electrophysiologic or echocardiographic parameters (see online supplement Tables 3–6).

Figure 2  Time course of the development of SAVP-induced disease. A: The relation between pacing duration and AF incidence (atrial tachyarrhythmia ≥ 2 min) and AF persistence (AF incidence ≥ 10 min). B: The effect of pacing duration on LASV and LADV. C: The effect of pacing duration on CD18 staining levels in the LAA. D: The effect of pacing duration on myocyte cross-sectional area in the LAA. E: The effect of pacing duration on collagen area fraction in the LAA. F: Representative sections of CD18-stained LAA (left = unpaced-no PUFAs, right = paced 2 d-no PUFAs); scale bar represents 200 μm. G: Representative sections of LAA stained with hematoxylin + eosin (left = unpaced-no PUFAs, right = paced 14 d-no PUFAs); scale bar represents 50 μm. H: Representative sections of LAA stained with picrosirius red (left = unpaced-no PUFAs, right = paced 14 d-no PUFAs); scale bar represents 200 μm. AF = atrial fibrillation; LA = left atrial; LAA = left atrial appendage; LADV = left atrial diastolic volume; LASV = left atrial systolic volume; PUFAs = n-3 polyunsaturated fatty acids; SAVP = simultaneous atrial and ventricular pacing. *P < .05, **P < .01 compared with unpaced. †P < .05, ††P < .01 compared with paced 2 d.
Histology
Figure 4A shows that prophylactic PUFA administration has a strong trend toward reducing the presence of inflammatory cells in the atria of dogs paced for 2 days (P = .055). No differences were found between dogs paced for 2 days with PUFAs vs without in the amount of hypertrophy or fibrosis (see online supplement Tables 7 and 8).

Effect of PUFAs in unpaced dogs
Atrial electrophysiology
AF incidence was not significantly different between unpaced unsupplemented dogs and unpaced dogs given PUFAs for 21 days (P > .05; Figure 5A). However, when all atrial tachyarrhythmias lasting >5 seconds were included in the analysis, arrhythmia incidence was significantly higher in unpaced dogs receiving PUFAs (6.4% ± 5.6% vs 1.0% ± 1.8%; P < .05 for PUFAs vs no PUFAs, respectively; Figure 5A).

Discussion
SAVP for 14 days resulted in progressively increasing susceptibility to AF and structural remodeling. PUFAs supplementation started after 7 days of pacing had already occurred (postinjury) did not significantly attenuate the electrical or structural changes. In addition, SAVP for 2 days induced changes indicating atrial inflammation; prophylactic PUFAs showed a strong anti-inflammatory trend.

Time course of the development of SAVP-induced disease
In combination with our previous work,10,11 these results suggest that SAVP (which imposes mechanical strain on the atria) is associated with inflammation early on, which resolves as pacing continues and is replaced by atrial dilatation, hypertrophy, and then fibrosis. Complementary with these structural changes is an increase in conduction time and vulnerability to AF. After 2 days of SAVP when peak inflammatory changes were observed, there was negligible AF vulnerability. Thus, in this model, AF does not seem to be directly related to inflammation. However, moderate vulnerability appeared after 7 days of SAVP when atrial dilatation and cellular hypertrophy were observed.

Effects of PUFAs on SAVP-induced disease
Prophylactic PUFAs attenuate the development of hypertrophy and fibrosis.10,11 Vulnerability to AF is also significantly reduced by prophylactic PUFA administration.11 Postinjury PUFAs show no ability to attenuate hypertrophy, fibrosis, or AF vulnerability. A potential explanation for the effectiveness of prophylactic PUFAs as compared with postinjury PUFAs may be the suppression of inflammation.
After 2 days of SAVP, prophylactic PUFAs reduced inflammatory changes in the atrium. Although inflammation itself did not cause AF, it can trigger the establishment of structural remodeling, which will create an arrhythmogenic substrate.1,23 Thus, the suppression of early inflammation can lead to a later reduction in AF vulnerability.

**Molecular analysis of structural remodeling**

Molecular data are consistent with our electrophysiological, functional, and histological findings. The differences observed in this study between dogs paced for 7 days and those paced for 14 days with postinjury PUFAs could be due to either the additional pacing time or PUFAs. However, in combination with previous molecular studies, a clearer pattern of the temporal regulation of structural remodeling genes emerges.10,11 After 7 days of pacing, the expression of hypertrophic and fibrotic factors is increased (eg, Smad7, Akt1, and EGF). Genes regulating fibrosis remained upregulated after 14 days of pacing (eg, collagen I and III, transforming growth factor-β1).10,11 Consistent with our hypothesis that extant remodeling reduces the efficacy of PUFAs, prophylactic PUFAs significantly reduced the expression of inflammatory JAM3 and CD99, as well as hypertrophy-related Akt1, EGF, myosin heavy chain-α, and fibrosis-related Smad7 and Timp1,10,11 whereas postinjury PUFAs demonstrated little effect on these same genes.

**Alternative hypotheses of PUFA mechanism**

The PUFA dosage used in this study was lower than that used in others.12,24 It was chosen to resemble commonly used doses in humans (1.0 g/d in 20–30 kg dogs ≈ 2.5–3.0 g/d in humans). Supplementation duration was also shorter than in other studies,21,24 but a significant increase in PUFA incorporation was observed and a similar increase over baseline levels was seen in this study as in others.7,24,25 The significant incorporation of PUFAs into atrial tissue phospholipids after 7 days of supplementation suggests that any acute properties of PUFAs, if present, should be evident in the postinjury PUFA group. The absence of effect in this
Evidence for a PUFA effect on structural remodeling

Although there is inconsistency in published studies on the electrophysiologic effects of PUFAs, there is a general consensus on the antistructural remodeling properties of pro-}

Figure 5  The effect of PUFAs in unpaced dogs. A: The effect of PUFA administration on atrial tachyarrhythmia incidence (atrial tachyarrhythmia ≥ 5 s) and AF incidence (atrial tachyarrhythmia ≥ 2 min) in unpaced dogs. B, C: Representative 1-s atrial electrograms of an AF incident; panel B from a dog paced for 14 d receiving no PUFAs and panel C from an unpaced dog receiving 21 d of PUFAs. Panel B shows rapid, irregular atrial activations characteristic of the AF usually induced in this study, whereas panel C shows rapid, but regular atrial activity characteristic of the AF induced in unpaced dogs treated with PUFAs. AF = atrial fibrillation; ns = not significant; PUFAs = n-3 polyunsaturated fatty acids; *P < .05 compared with unpaced-no PUFAs.

group on any electrophysiologic or histologic parameters suggests that acute properties are not responsible for the beneficial effects of PUFAs in this study. Some studies have suggested that PUFAs have direct antiarrhythmic effects via sodium, potassium, or calcium channel blockade; however, we did not observe PUFA-mediated changes in refactoriness or slowed conduction, which could indicate altered ion channel function. It has recently been suggested that a direct antiarrhythmic effect of PUFAs may require ≥1 month of treatment with a PUFA dose as high as 6 g/d. We did, however, observe that PUFA supplementation appeared to have a slightly proarrhythmic effect in the absence of SAVP. Although no changes in conduction or refactoriness were observed in the unpaced-PUFAs 21-day group, it is possible that PUFA could be responsible for the increased susceptibility to arrhythmias in the unpaced dogs.

Study limitations

This was an animal study with measurements made at few time points. The development of structural remodeling is a complex process involving multiple signaling cascades and feedback loops. Thus, data presented here represent only a partial picture of SAVP-induced disease and PUFA effects. We used 2 weeks of SAVP to model the AF substrate created by years of hemodynamic overload in humans. Although the substrate produced by this model bears many similarities to humans vulnerable to AF, there are likely important differences. In addition, the reported changes to mRNA levels do not necessarily reflect changes to protein level and activity. However, we feel that all our data including electrophysiology, echocardiography, histology, and molecular are supportive of our conclusion.

Conclusion

In this model, PUFAs given after the establishment of atrial structural remodeling are not effective in reducing vulnerabil-
ity to AF. Clinical trials aiming to study the effect of PUFAs on AF may not be able to detect any beneficial effect of PUFAs if given after remodeling has been established.

Acknowledgments

We gratefully acknowledge the generous donation of PUFA capsules from Solvay Pharmaceuticals, pacemaker equipment from St Jude Medical and Medtronic, Inc, donation of the CD18 antibody from the University of California, pathology assistance from the Centre for Phenogenomics, and assistance with quantitative real-time PCR from Ms Kerri Thai.

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Supplemental Material for N-3 Polyunsaturated Fatty Acid Supplementation Does not Reduce Vulnerability to Atrial Fibrillation in Remodeling Atria

Methods:

Pacemaker implantation

Dogs were fasted overnight except for free access to water. Thirty minutes prior to the procedure dogs were sedated with i.m. acepromazine (Atrovet, 0.05 mg/kg [Ay- erst Veterinary Laboratories, Guelph, ON]), atropine (0.03 mg/kg [Alveda Pharmaceuticals Inc., Bellville, ON]), and buprenorphine (Temgesic, 0.02 mg/kg [Schering-Plough, Kirkland, QC]). Blood samples were drawn from a vein in the left foreleg and stored on ice, in tubes containing 2 mg EDTA, until processed. Anesthesia was induced with i.v. thiopental sodium (5% solution 0.25 mL/kg [Hospira Healthcare Corp., Saint-Laurent, QC]) for pacemaker implant, or i.v. propofol (2.5–3.5 mg/kg [Astra Zeneca, Mississauga, ON]) for sacrifice study. Dogs were then intubated with a 6.0–8.0 French E-T tube and ventilated with a mechanical ventilator (Harvard Apparatus, Inc., Holliston, MA, 16–18 breaths/min, 12–14 mL/kg tidal volume). Anesthesia was maintained with isoflurane (1–2%). Temperature was maintained at 37°C with a heating blanket. Under aseptic conditions, an incision was made in the lateral aspect of the neck to expose the left external jugular vein. Under fluoroscopic guidance, two steroid eluting, bipolar, IS-1 pacing leads (Pacesetter Tendril SDX, St. Jude Medical, Minneapolis, Min) were inserted through the vein and fixed in the RA appendage and RV apex. Leads were connected to a “y-connector” (Lead Adaptor Kit, Medtronic Inc., Minneapolis, Min) which connected the 2 leads to 1 bipolar IS-1 pacemaker (Model 5156 Verity ADx XL SR, St. Jude Medical, Minneapolis, Min). Lead pacing thresholds were verified not to exceed 2.5 V and diaphragmatic stimulation was verified not to occur even with voltage as high as 10 V. Pacemaker, y-connector, and lead function were verified before recovering the dog. Dogs were placed on an antibiotic regime (Baytril 5 mg/kg) for 1 week post implantation surgery.

Pacing

Dogs were allowed to recover from surgery for at least 1 week before the start of the pacing protocol. The pacemaker was turned on to VVI mode at 220 bpm with a 5.0 V pulse amplitude and a 0.5–1.0 ms pulse width. Due to the Y-connector, both leads paced simultaneously resulting in simultaneous AV pacing at 220 bpm. Dual chamber pacing was verified by ECG analysis at the time of pacing commencement and termination.

End study (sacrifice study)

Dogs were fasted overnight except for free access to water. Thirty minutes prior to beginning the procedure, dogs were sedated with i.m. acepromazine (Atrovet, 0.05 mg/kg), atropine (0.03 mg/kg), and buprenorphine (Temgesic, 0.02 mg/kg). Blood samples were drawn from a vein in the left foreleg and stored on ice, in tubes containing 2 mg EDTA, until processed. Anesthesia was induced with i.v. propofol (2.5–3.5 mg/kg as Diprivan [10 mg propofol/mL or 1% soln]). Dogs were then intubated with a 6.0–8.0 French E-T tube and ventilated with a mechanical ventilator (Harvard Apparatus, Inc., Holliston, MA, 16–18 breaths/min, 12–14 mL/kg tidal volume). Anesthesia was maintained with isoflurane (1–2%). Temperature was maintained at 37°C with a heating blanket and ventilatory humidifier system. At the end of the study, with the dog still under deep anesthesia, the heart was quickly excised and tissue samples were taken. Correct placement of the pacing leads was verified by post mortem examination in most dogs.

Electrophysiological study

The pacemaker was turned off at least 30 minutes prior to starting the electrophysiological study. A median sternotomy was performed to expose the heart. Four bipolar, stainless steel, epicardial pacing/recording electrodes were sutured onto the heart at the RA appendage (RAA), LA appendage (LAA), high RA (SVC) and low RA (IVC).

Extrasystole pacing was performed at each pacing electrode (LAA, RAA, SVC, IVC, PVs) with an S1-S2 technique. Electrical stimulation was supplied by a Ventritex HVSO2 (St. Jude Medical, Minneapolis, Min) at twice stimulation threshold at 400 and 200 ms cycle length with a 2 ms pulse width for 30 seconds (to reach steady state). After 30 seconds of continuous pacing, 8 stimuli (S1) were applied followed by an extra stimulus (S2) beginning 100 ms after the last S1 stimulus. The S2 interval was increased in 20 ms intervals until capture was recorded. The S2 interval was then decreased by 10 ms until capture was lost. The S2 interval was then increased in 2 ms increments until capture was recorded again. The atrial effective refractory period (AERP) was determined to be the longest S1-S2 interval which did not result in capture. AERP was calculated as the mean of the AERPs measured at the LAA, RAA, SVC, IVC and PVs at 200 ms cycle length. Conduction time between the LAA and the SVC was calculated at 400 ms cycle length and presented as a measure of global atrial conduction time. Recordings taken just after steady state was reached at the LAA and SVC electrodes were analyzed. The time between the pacing stimulation on the LAA electrogram and the next activation (point of most negative dV/dt) on the SVC electrogram was taken to be the conduction time between the two electrodes (and vice versa SVC to LAA). The times from 3 consecutive stimulations at the LAA to the SVC, and 3 consecutive stimulations at the SVC to the LAA were averaged to produce a mean conduction time for each dog.
Burst pacing was performed at each pacing electrode and recordings of the resulting activation patterns were made at all sites fitted with recording electrodes. Electrical stimulation was supplied by a Ventritex HVSO2 (St. Jude Medical, Minneapolis, Min) at 10 V, 10 Hz (100 ms cycle length), with a 2 ms pulse width for 10 seconds. Any irregular atrial tachyarrhythmia lasting more than 5 seconds was noted; recordings were made if the arrhythmia lasted more than 60 seconds. A protocol to “break” the arrhythmia was employed just after the 60 second mark. A rapid burst of stimulations was applied through the same electrode that initiated the arrhythmia (10 V, 2 ms pulse width, 5–20 Hz, 2–5 sec duration). The protocol was repeated continuously until the 120 second mark, or until the arrhythmia stopped. If the arrhythmia did not stop, it was allowed to continue until self-termination or the 600 second mark at which point the dog was cardioverted with a 30–50 J shock from a Medtronic PhysioControl Lifepak 12 Defibrillator (Medtronic Inc. Minneapolis, Min) in synch mode. If no arrhythmia resulted from the burst, another burst was applied 10–12 seconds later, up to 10 bursts maximum per electrode. If an arrhythmia occurred, a rest period equal to at least half the duration of the arrhythmia was allowed before the next burst was applied. If more than 10 total minutes of arrhythmia were initiated by the same electrode, the protocol was stopped and started again on a new electrode.

AF was defined as any irregular atrial tachyarrhythmia (atrial rate >300bpm) with duration >120 seconds, and resistant to pace termination.

**Echocardiography**

Trans-thoracic and trans-esophageal echo was done preoperatively at the time of pacemaker implantation, and at the end study, with the dog intubated and anesthetized with 1–2% isoflurane, lying on the right side with a Sonos 5500 ultrasound system (Philips Ultrasound, Canada). An effort was made to ensure measurements were taken at the same location and angle in each dog to maximize comparability. At the end study the pacemaker was turned off at least 15 minutes prior to echocardiographic assessment.

**Trans-thoracic Echo**

A 1.8–3.6 MHz phased array transducer (S3, Philips Ultrasound) was used to obtain trans-thoracic recordings. From a parasternal approach, the LV short axis view was recorded at the mid-papillary muscle level for measurement of LV end systolic and diastolic area (LVEDA, LVESA). Apical 4 chamber and 2 chamber views were recorded for measurement of LV end-diastolic and end-systolic volumes, and ejection fraction (EF) according to the Simpson’s biplane method: \((\pi/4) \times \sum_{i=1}^{n} (a_i/b_i) \times (L/n)\) (where \(n\) = the number of cylinders or discs of equal height taken from 2 chamber \((a_i)\) and 4 chamber \((b_i)\) recordings, \(L\) = length from apex to mitral valve annulus). Data from three consecutive beats were averaged in order to calculate each value.

**Trans-esophageal Echo**

A 5–7 MHz phased array multiplane trans-esophageal transducer (Philips Ultrasound) was used to obtain trans-esophageal recordings. LA area and length were measured from apical 4 chamber and 2 chamber views during the systolic and diastolic phases. LA systolic and diastolic volume (LAVS, LADV) were calculated as follows: \((8/(3 \times \pi)) \times ((4 \text{ Ch LA area})(2 \text{ Ch LA area}/(\text{LA length}))\) (where LA length is the distance from LA back wall to mitral valve annulus). Data from three consecutive beats were averaged in order to calculate each value.

**Calculations**

Changes in echo parameters were calculated as percentage changes between pacemaker implant and end study. If data from either timepoint were missing, the mean value from the group for that parameter at that timepoint was substituted so that percentage change could be calculated. Values “interpolated” in this manner were excluded from the final analysis if they fell outside the range of those values calculated from non-interpolated data.

**Sample collection and preparation**

As much as possible, care was taken to make sure corresponding samples were taken from approximately the same location in every dog.

**Plasma**

Whole blood was centrifuged at 2,200 rpm for 15–20 minutes at 5–10°C. Plasma was separated and frozen at −80°C. The remainder was discarded.

**Liquid N2**

Samples of LAA were snap frozen in liquid N2 and stored at −80°C.

**Formalin**

Samples of LAA were preserved in 10% neutral buffered formalin (10% formaldehyde, 2% anhydrous sodium acetate, 88% water). One sample was fixed for at least 14 days at room temperature (14 day formalin). A second sample was put on a shaker at room temperature for 24–36 hours, then put into 70% ethanol (70% ethanol, 30% saline) (1 day formalin). Samples were prepared for histology by the pathology department at St. Michael’s Hospital (Toronto, ON) or The Centre for Phenogenomics (TCP, Toronto, ON).

**PUFA phospholipid integration**

Total lipid extracts were prepared from frozen plasma or frozen atrial tissue samples according to the method of Bligh and Dyer (1). Briefly, approximately 1 mL of plasma, or 200 mg of tissue, was washed repeatedly with a mixture of chloroform and methanol (1:2 by volume) in order to separate phospholipids from the rest of the mixture. A known amount of internal standard as diheptadecanoyl phosphatidylcholine was added to the mixture. After vortexing repeatedly and centrifuging, the chloroform (lipid) phase was recovered, concentrated, and applied to thin-layer chromatographic plates for the isolation of the phospholipid fraction followed by transmethylation and fatty acid analyses by gas-liquid chromatography on a Varian 3800 gas-liquid chromatograph (Palo Alto, CA) with a 60-m DB-23 capillary column (0.32 mm internal diameter). See Stark et
al. (2) and Madden et al. (3) for details of gas-liquid chromatography.

**Histology**

Histological analysis was performed via light microscopy using a Nikon Eclipse E800 microscope (Nikon, Inc.) with an attached Nikon DXM 1200 digital camera. Images were captured with Nikon ACT-1 software v2.70. Any image manipulation and analysis was performed with ImageJ software (Rasband, W.S., ImageJ, National Institutes of Health, Bethesda, MD, [http://rsb.info.nih.gov/ij/]).

**Atrial inflammation**

CD18 staining was performed on 1 day formalin fixed, paraffin embedded sections of LAA tissue by The Centre for Phenogenomics (TCP, Toronto, ON) with the CA16.3C10 antibody (University of California, Davis, CA).

CD18 stained sections of 1 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 10X magnification. The entire specimen was photographed. Cells were considered positively stained if they had heavily stained (dark brown) membranes. Each picture was assigned a grade between 0 and 4 indicating the volume of positive stain present in the image (0 no visible staining, 4 pervasive positive staining). Grading was done in a blinded fashion and was repeated 3 times to ensure accuracy.

**Cellular hypertrophy**

Hematoxylin and eosin (H+E) staining was performed on sections of 14 day formalin fixed, paraffin embedded LAA tissue by the pathology department at St. Michael’s Hospital (Toronto, ON).

H+E stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 40X magnification. Approximately 25 images were taken per animal. Images presenting large collections of myocytes cut in cross section were selected for further analysis. Myocytes were judged to be cut in cross section if they appeared circular in shape, with a well-defined membrane and large, circular, darkly staining nuclei appearing near the center of the cell. Myocyte outlines were traced and analyzed with the “circularity” function. A circularity of at least 0.8 (out of 1.0) was considered circular. Cross sectional area was measured by the “area” function. Approximately 150 cells were measured from each animal. All analysis was done in a blinded fashion and measurements were repeated 3 times to ensure accuracy.

**Atrial fibrosis**

Picrosirius red (PSR) staining was performed on sections of 14 day formalin fixed, paraffin embedded LAA tissue by the pathology department at St. Michael’s Hospital (Toronto, ON) or by The Centre for Phenogenomics (TCP, Toronto, ON).

PSR stained sections of 14 day formalin fixed, paraffin embedded LAA tissue were analyzed by light microscopy at 10X magnification. Approximately 7 images were taken per animal in black and white with a green interference filter to maximize contrast. Threshold was manually adjusted until only collagen containing pixels were highlighted (compared each picture to unedited original to ensure correctness).

Area fraction was calculated based on largest area selected to exclude vessels and include mainly midmyocardium (avoiding edges). Analysis was done in a blinded fashion and measurements were repeated 3 times to ensure accuracy.

**QT-RT-PCR**

RNA was extracted from snap frozen LAA tissue samples as follows. Approximately 50 mg of tissue were crushed with a mortar and pestle filled with liquid N₂. Powder was homogenized with 1 mL of TRIzol reagent (Invitrogen, Carlsbad, CA), and then incubated for 2-3 minutes at 25°C. 0.2 mL chloroform were added, the solution was shaken, incubated 2-3 minutes at 25°C, and then centrifuged (12,000 g, 15 min, 4°C). RNA remains in the upper (non-chloroform) phase. The supernatant was recovered and mixed with 0.5 mL of isopropyl alcohol to precipitate RNA. The mixture was centrifuged (12,000 g, 10 min) and the pellet was recovered; RNA remains in the pellet. The pellet was washed in 1 mL of 75% ethanol, vortexed and centrifuged (7,500 g, 5 min, 4°C). The supernatant was discarded and the pellet was air-dried for 5 min, and then dissolved in 50 μL of RNAase free water. Purity was verified by spectrophotometry (NanoDrop ND-100, NanoDrop Technologies, Rockland, DE). An absorbance ratio 260 nm/230 nm of 1.8-2.1 was considered acceptable.

Quantitative real time PCR (QT-RT-PCR) was performed as follows. cDNA was created from 10 μL of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Briefly, 10 μL of sample RNA were combined with kit contents and placed in a thermal cycler (MJ Mini, Bio-Rad, Hercules, CA). A single cycle (10 min @ 25°C, 120 min @ 37°C, 5 min @ 85°C, end @ 4°C) was run to create cDNA. Real time PCR reaction was performed with the Universal Master Mix kit (Applied Biosystems, Foster City, CA) according to manufacturer’s instructions. Briefly, primers were prepared as 1X dilutions and loaded with kit contents and cDNA onto a 384 well plate. All reactions were done in triplicate. The plate was loaded into a 7900 HT Fast Real-Time PCR System machine with SDS v2.3 software for the real time reaction. The reaction consisted of 1 cycle (50°C, 2 min) then 1 cycle (95°C, 10 min) followed by 40 cycles (95°C, 15 sec, 60°C, 1 min) completed in approximately 90–100 minutes.

Primers were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA) or ACGT Corp (ACGT Corp, Toronto, ON).

Primers for the following genes of interest were obtained from Applied Biosystems (Foster City, CA). JAM3 (Cf02650628_m1), TIMP1 (Cf02621937_g1), CD99 (Cf02657856_m1), Smad7 (Cf00998193_m1), EGF (Cf02622123_m1), Akt1 (Cf02654390_m1), MHC-α (Cf02624757_m1). Primers for the following genes of interest were obtained from ACGT Corp (Toronto, ON), Collagen I (forward) GTGTGTGACGCGGCTCA (reverse) TCGCAAATCGTACGGTCA, Collagen III (forward) ATAGAGCCTTTGATGACGAA (reverse)
CCT CGC TCA CCA GGA GC, TGF-β (forward) CAA GGA TCT GGG CTG GAA GTG GA (reverse) CCA GGA CCT TGC TGT ACT OCG TGT. 18S or rpl13a were used as the housekeeping genes.

Statistical analysis
Comparisons were performed using one-way analysis of variance with a Tukeys post-hoc test, or an unpaired t-test; results were significant if P<0.05. Error bars on all charts indicate SEM. In text and tables values are presented as mean±SD.

Results:

PUFA integration

Table 1  Effect of olive oil supplementation on plasma PUFA composition: change in absolute % of EPA+DHA in plasma phospholipids between baseline and sacrifice in dogs not receiving PUFAs

<table>
<thead>
<tr>
<th></th>
<th>No supplement</th>
<th>Olive oil (≥9 days)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA+DHA (Δ%)</td>
<td>−0.11 ± 0.65</td>
<td>−0.35 ± 0.79</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 2  Effect of olive oil supplementation on tissue PUFA composition: % of EPA+DHA in tissue phospholipids at sacrifice in dogs not receiving PUFAs

<table>
<thead>
<tr>
<th></th>
<th>No supplement</th>
<th>Olive oil (≥9 days)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPA+DHA (%)</td>
<td>2.55 ± 0.40</td>
<td>3.02 ± 0.78</td>
<td>NS</td>
</tr>
</tbody>
</table>

Electrophysiology
AF inducibility

Table 3  Effect of PUFA supplementation on arrhythmia inducibility in dogs paced for 2 days: % of burst pacing attempts resulting in atrial arrhythmia

<table>
<thead>
<tr>
<th></th>
<th>Paced 2 days, Olive oil 9 days</th>
<th>Paced 2 days, PUFAs 9 days</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A Tachyarrhythmia (%)</td>
<td>5.48 ± 8.90</td>
<td>12.78 ± 9.09</td>
<td>NS</td>
</tr>
<tr>
<td>AF incidence (%)</td>
<td>0.82 ± 1.30</td>
<td>2.30 ± 2.94</td>
<td>NS</td>
</tr>
</tbody>
</table>

Conduction time

Table 4  Effect of PUFAs on conduction time in dogs paced for 2 days: global atrial conduction time in sinus rhythm at 400ms cycle length in ms (measured between SVC and LAA)

<table>
<thead>
<tr>
<th></th>
<th>Paced 2 days, Olive oil 9 days</th>
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<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Conduction time (ms)</td>
<td>64.58 ± 6.61</td>
<td>64.67 ± 8.46</td>
<td>NS</td>
</tr>
</tbody>
</table>

Atrial effective refractory period

<table>
<thead>
<tr>
<th></th>
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<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AERP (ms)</td>
<td>115.2 ± 3.11</td>
<td>115.2 ± 6.82</td>
<td>NS</td>
</tr>
</tbody>
</table>

Echocardiography

Table 5  Effect of PUFAs on AERP in dogs paced for 2 days: AERP at 200ms cycle length

<table>
<thead>
<tr>
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</tr>
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<tbody>
<tr>
<td>LASV (Δ%)</td>
<td>−6.52 ± 15.50</td>
<td>−15.65 ± 9.60</td>
<td>NS</td>
</tr>
<tr>
<td>LADV (Δ%)</td>
<td>−2.03 ± 11.98</td>
<td>−8.32 ± 11.53</td>
<td>NS</td>
</tr>
<tr>
<td>LVEG (Δ%)</td>
<td>43.4 ± 35.87</td>
<td>46.09 ± 27.26</td>
<td>NS</td>
</tr>
<tr>
<td>LVEDV (Δ%)</td>
<td>4.12 ± 7.88</td>
<td>7.56 ± 10.58</td>
<td>NS</td>
</tr>
<tr>
<td>LVEF (Δ%)</td>
<td>−39.51 ± 12.71</td>
<td>−32.05 ± 18.27</td>
<td>NS</td>
</tr>
</tbody>
</table>

Histology
Cellular hypertrophy

Table 6  Effect of PUFAs on echocardiographic parameters in dogs paced for 2 days: % change between baseline and sacrifice

<table>
<thead>
<tr>
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</tr>
</tbody>
</table>

Table 7  Effect of PUFAs on cellular hypertrophy in dogs paced for 2 days: myocyte cross-sectional area

<table>
<thead>
<tr>
<th></th>
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<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cross-sectional area (μM²)</td>
<td>198.2 ± 59.55</td>
<td>199.3 ± 24.64</td>
<td>NS</td>
</tr>
</tbody>
</table>

Table 8  Effect of PUFAs on cellular hypertrophy in dogs paced for 2 days: collagen area fraction

<table>
<thead>
<tr>
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<th>Paced 2 days, PUFAs 9 days</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen area fraction (%)</td>
<td>8.40 ± 1.08</td>
<td>8.97 ± 1.91</td>
<td>NS</td>
</tr>
</tbody>
</table>

Reference List