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The effects of polyunsaturated fatty acids and antioxidant vitamins on atrial oxidative stress, nitrotyrosine residues, and connexins following extracorporeal circulation in patients undergoing cardiac surgery

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Abstract Cardiac surgery with extracorporeal circulation is characterized by different degrees of myocardial ischemia/reperfusion, which is often associated with postoperative atrial fibrillation (POAF). We have previously shown that a novel preventive therapy based on the reinforcement of the antioxidant system using omega-3 fatty acids plus antioxidant vitamin supplementation applied to patients undergoing cardiac surgery reduces POAF occurrence. We hypothesized that oxidative stress and nitrosative stress are involved in the development of an arrhythmogenic substrate by their effect on connexins (Cx40, Cx43 and Cx45) abundance and distribution pattern. Therefore, we have assessed the effect of redox status on atrial tissue in patients undergoing cardiac surgery. Placebo/POAF and supplemented/POAF patients showed 276 and 170% higher reactive oxygen species (ROS) levels and 223 and 96% higher nitrotyrosine residues levels, respectively, compared to sinus rhythm (SR). In POAF tissue, antioxidant supplementation prevented Cx40 and Cx43 lateralization on cardiomyocyte sarcolemma, keeping them at the intercalated disks. POAF samples showed Cx40 heterogeneous distribution pattern, presenting tissue areas lacking this protein (49 and 55% lower levels in placebo/POAF and supplemented/POAF groups, respectively, compared to SR). Of note, Cx45 overexpression occurred in POAF, being 211 and 167% higher in placebo/POAF and supplemented/POAF groups, respectively, compared to SR. It is concluded that treatment with omega-3 fatty acids and antioxidant vitamins reduces oxidative and nitrosative stress and prevents Cx40/Cx43 lateralization in atrial tissue likely contributing to POAF prevention. However, it failed to fully prevent POAF occurrence because these compounds have no effects on the normalization of Cx40 down-regulation and Cx45 up-regulation, which may promote POAF.

Keywords Postoperative atrial fibrillation · Omega-3 fatty acids · Vitamin C and E · Oxidative stress · Nitrotyrosine · Connexins

Introduction

Cardiac surgery with extracorporeal circulation is characterized by different degrees of myocardial ischemia and reperfusion, which are associated in up to 40% of patients with postoperative atrial fibrillation (POAF) [1, 2]. POAF is a common arrhythmia that in clinical practice is associated with increased hospital stay, risk of thrombosis, high care costs and potential mortality [3]. The pathophysiology of POAF is multifactorial, comprising processes related with both, the patient and the surgical procedure [3]. The patient-related factors include atrial dilation, age-related fibrosis, hypertension and myocardial structural damage [4]. Furthermore, surgery can increase the risk of POAF through handling, pericardial inflammation, atrial dilation, use of catecholamines, parasympathetic activation, electrolyte imbalance, cardioplegia, and ischemia-induced cooling [5].

Increasing evidence indicates that oxidative stress and inflammation can play an important role in the development

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of atrial fibrillation and POAF whereby changes in connexin (Cx) expression and distribution might be involved as recently reviewed [6]. Reactive oxygen species (ROS) may favor focal electrical activity, reentry phenomenon, alteration of ionic currents, and structural remodeling of the heart. In addition, ROS-induced remodeling in the electrical coupling of the heart is related to changes in potassium/calcium ion channels [7] and intercellular gap junctions formed by Cx proteins, which are transmembrane proteins that form hemichannels between cardiac cells allowing diffusion of small factors such as calcium, ATP and cAMP [8]. The connexons of one cardiac cell bind to their counterparts in the adjacent cardiac cell to form a gap junction channel. The most common Cx protein isoforms present in cardiac tissue are Cx40, Cx43 and Cx45 (reviewed in: [8–10]). Among them, Cx43 is the most abundant isoform in human hearts [9–12]. The most recognized mechanism of cardiac Cxs remodeling is the lateralization of these proteins at the cardiac cell membranes, reducing the proportion of Cxs at the intercalated disks [13–18].

The main therapeutic end-points for POAF control are the absence of symptoms, hemodynamic stability, thromboembolism prevention and recurrence. Pharmacological strategies include use of beta-blockers, antiarrhythmic drugs and anticoagulants [19]. On the other side, non-pharmacologic prophylactic strategies are atrial pacing and electrical cardioversion [19, 20]. Despite of the above available treatments, the antiarrhythmic efficiency remains low and new preventive and safe therapies are necessary in order to reduce the incidence of this atrial arrhythmia. In this context, the use of omega-3 polyunsaturated fatty acids (n-3 PUFA) is increasingly becoming a promising therapy to reduce the risk of POAF likely through the contribution of n-3 PUFA to the beneficial modulation of Cx43 expression and/or phosphorylation and their antiarrhythmic effects (reviewed in [21]). Previously, our study belonging to a clinical trial aimed at reducing the occurrence of POAF by strengthening the antioxidant defense systems postulated a possible antioxidant mechanism using n-3 PUFA and vitamins C and E [22]. Although, the effects of n-3 PUFA on cardiac Cxs have been investigated in diverse cardiac diseases [23–26] including POAF [27], more studies are needed to substantiate the consistency of these findings.

The present study, therefore, aimed at assessing changes in the expression and distribution of atrial Cxs by reducing oxidative and nitrosative stress in atrial tissue. The rationale of this study stems in the growing evidence linking oxidative stress, nitrotyrosine, and Cxs expression [18, 28–30]. The later has been shown to be responsible for atrial fibrillation [13, 14] including POAF [23, 27, 31, 32]. Therefore, we embark for the first time on studying all of these components in patients who remained in sinus rhythm with those who developed POAF after cardiac surgery.

Materials and methods

Patients

From July 2007 to March 2013, a randomized, a double-blinded, placebo-controlled clinical trial was performed with 203 patients undergoing cardiac surgery at the Cardiovascular Department of the clinical hospital of the University of Chile, Cardiac Surgery Department of San Juan hospital and San Borja Arriarán Hospital [22]. The protocol was approved by the respective hospital Ethics Committees and the management of the patients was in line with the principles of the Declaration of Helsinki, 2001. From 203 patients, right atrial appendage biopsies were obtained in 28 of cases for morphological analysis. All patients enrolled in the study signed informed consent. Inclusion criteria comprised the following: non-congenital heart diseases and sinus rhythm at the time of randomization. Exclusion criteria were as follows: chronic atrial fibrillation, previous heart operation, intake of vitamin C or E 1 month prior to the study, chronic kidney disease (creatininemia >2.0 mg/dL), hepatic disease (serum bilirubin >3.0 mg/dL or serum albumin <3.5 g/dL or prothrombinemia <60% in the absence of oral anticoagulant therapy or sonographic signs of chronic liver injury or presence of esophageal varices), pregnancy, congenital cardiac disease, emergency surgery. Patients included in the present study (mean age 54.5 ± 10.2 years) of both sexes were randomly assigned to receive n-3 PUFA, (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA, in 1:2 ratio, respectively) at a dose of 2 g per day or placebo, 7 days before heart surgery. Subsequently, 2 days before surgery, antioxidant vitamins C (1 g per day) and vitamin E (400 IU per day) were added. Both treatments were applied until hospital discharge. The placebo group received capsules, containing caprylic acid (825 mg per capsule), inert granules (500 mg), and vegetable oil (400 mg) replacing n-3 PUFA and vitamins C and E, respectively, provided by Procaps (Colombia) and Gynopharam CRF (Chile) laboratories.

The study groups reported here were placebo that remains in sinus rhythm (PI-SR, $n=7$) after surgery, placebo with POAF (Pl-POAF, $n=8$), n-3 PUFA and vitamins C and E supplemented that remains in sinus rhythm (supplemented-SR, $n=9$) or developed POAF (supplemented-POAF, $n=4$).

ROS and nitrotyrosine labeling and quantification

All tissue samples were snap-frozen in liquid nitrogen within a few minutes of collection. Cryosections, 10 µm thick, were air dried and fixed with 4% formaldehyde (freshly prepared from paraformaldehyde) in phosphate-buffered saline (PBS) and then incubated with 1% bovine

serum albumin for 30 min to block non-specific binding sites. After rinsing in PBS, the samples were incubated overnight with primary antibodies against nitrotyrosine (Merck Millipore, AB 06-264). Secondary antibody was a donkey anti-rabbit IgG-conjugated with Alexa Fluor® 488 (Molecular Probes). In situ ROS were determined using labeling with dihydroethidium as described [33, 34]. Tissue sections were examined by a laser scanning confocal microscope (Leica TCS SP2). Series of confocal optical sections were taken using a Leica Planapo x63/1.32 objective lens. Each recorded image was taken using multichannel scanning and consisted of 1024 × 1024 pixels. To improve image quality and to obtain a high signal to noise ratio, each image from the series was signal-averaged and was deconvoluted using AutoQuant X2 (Bitplane, Zürich, Switzerland) software. For three-dimensional image reconstructions, an Imaris 6.3.1 multichannel image processing software (Bitplane, Zürich, Switzerland) was used.

For quantification of ROS and nitrotyrosine residues, all tissue samples were labeled simultaneously with identical conditions of fixation and dilutions of primary and secondary antibodies. Ten randomly fields of vision were quantified using the three-dimensional quantification option of the Imaris program. For each quantification procedure, a specific setting was established and kept constant in all measurements. Quantification of ROS and nitrotyrosine was performed by measuring the fluorescence intensity using a range of 0–255 grey values. The quantity of ROS and nitrotyrosine was calculated as fluorescent arbitrary units per square millimeter myocardial area.

Connexins labeling

Atrial sample cryosections, 10 µm thick, were fixed in cold methanol or 4% formaldehyde and then incubated for double lablelings with antibodies: anti-Cx40 (Santa Cruz, SC20466 and Chemicon, MAB3068), anti-Cx43 (Sigma, C6219), anti-Cx45 (cQ14E (a generous gift from Dr N.J. Severs, Imperial College, London, UK) and Chemicon, AB1745), and anti-dystrophin (Sigma, MANDYS8 and Santa Cruz Biotechnology, SC15376). Anti-mouse and anti-rabbit IgG-conjugated with Alexa Fluor® 488 or Alexa Fluor® 555 (ThermoFisher Scientific) served as detection systems. DAPI (4′,6-diamidino-2-phenylindole) dye was used for nuclear staining (Molecular Probes), and Alexa Fluor® 555 Phalloidin or Alexa Fluor® 647 Phalloidin (ThermoFisher Scientific) was used for F-actin detection. All atrial tissue sections were subjected to identical antibody dilutions and reagent concentrations. To determine the cardiac connexin proteins (Cx40, Cx43, and Cx45) distribution in atrial tissue, co-localization labeling with dystrophin was performed. Five to ten selected fields per sample were obtained using either a Leica Planapo x40/1.00-0.50 or Leica Planapo x63/1.32 objective lens and confocal microscopy. Images were further processed using Imaris 6.3.1 multichannel image processing software (Bitplane, Zürich, Switzerland).

Western blot

Samples were processed for Western blot analysis as previously described [35]. In brief, frozen tissue was homogenized in RIPA buffer (containing 20 mmol/L Tris–HCl at pH 7.4, 100 mmol/L NaCl, 5 mmol/L ethylene-diamine tetraacetic acid, 1% Triton X-100, 10% glycerol, 0.1% sodium dodecylsulfate, 1% deoxycholate, 50 mmol/L NaF, 10 mmol/L $Na_4P_2O_7$, 1 mmol/L Na_3VO_4, 1 mmol/L phenylmethylsulfonylflouride, and mammalian protease inhibitor cocktail (Sigma) at pH 7.4 and centrifuged at 2000×*g* at 4 °C for 10 min. Atrial myocardial extracts were loaded onto 12% polyacrylamide gel and separated under the reducing conditions. Proteins were electrotransferred onto nitrocellulose membrane (Invitrogen) and blocked with 5% non-fat dry milk in Tris-buffered saline Tween-20 (TBST) at 4 °C. After washing with TBST, proteins were exposed overnight at 4 °C to antibodies against Cx43, CX40, and Cx45 diluted in TBS with 5% powdered milk. Bound antibodies were detected by IgG-conjugated with horseradish peroxidase. The SuperSignal WestFemto (Pierce) detection system was used and the membranes were exposed to X-ray film. Quantification of immunoblots was done by scanning on a STORM 860 (Amersham Pharmacia Biotech) using ImageQuant software. The immunoblotting values for the investigated connexins were normalized per actin (clone HHF-35, Sigma). The control values in the PL-SR group were set at 100%.

Statistical analysis

All data are presented as means ± SD. For multiple comparisons, we used ANOVA followed by analysis with the Bonferroni *t* test. Differences between groups were considered significant at $p < 0.05$.

Results

Oxidative stress

Figure 1 shows representative confocal microscopy images of ROS staining in right atrial tissue sections from placebo (PL-POAF) and supplemented-POAF (supplemented-POAF) patients and the average immunofluorescence intensity of ROS per tissue area in the studied groups. Atrial tissue sections group exhibited a 2.76-fold increase of ROS levels in patients from the

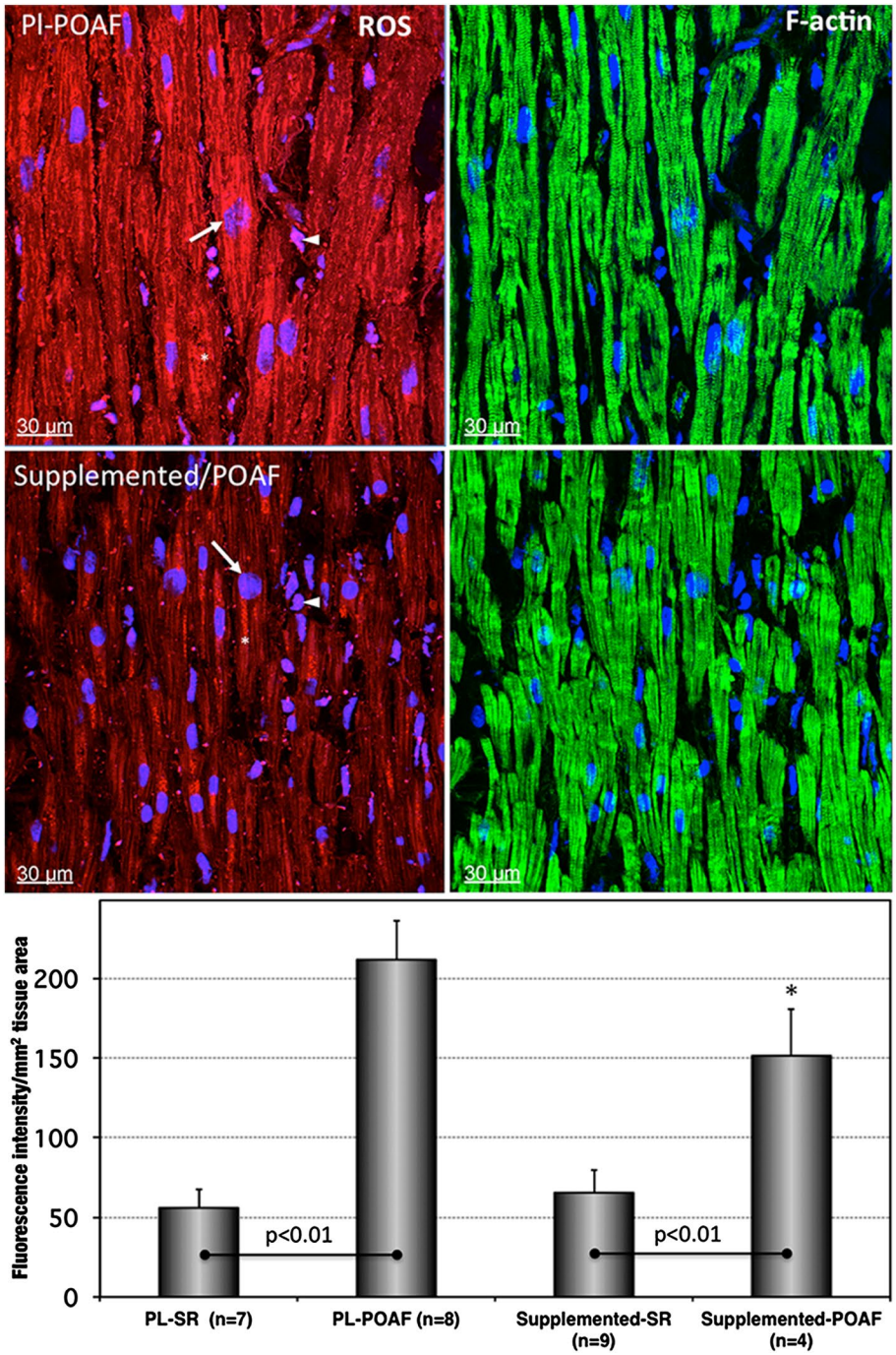

Fig. 1 Representative confocal images from POAF atrial tissue sections from placebo (Pl-POAF, *upper panels*) and supplemented (supplemented/POAF, *middle panels*) groups. Dihydroethidium staining for ROS is shown in *red color*, *left panels*. Note the ROS signal confined to the nuclei of cardiomyocytes (*arrows*) and non-cardiomyocytes (*arrowheads*), which co-localizes with DAPI (*blue*) resulting in *purple color*. *Asterisks* indicate the mitochondrial pattern of ROS staining. Cardiomyocytes are stained *green* with F-actin (*right panels*). Nuclei are labeled blue with DAPI. The *lower diagram* shows the quantitative results of ROS staining expressed as fluorescence intensity units per mm^2 atrial tissue area. ROS levels are indicated as means ± SD in sinus rhythm group receiving placebo (Pl-SR), POAF group receiving placebo (PL-PAF), sinus rhythm group receiving supplement (Supplemented-SR), and POAF group receiving supplement (Supplemented-POAF). *$p < 0$ 0.05 PL-POAF versus Supplemented-POAF

PL-POAF group as compared with patients who received placebo and remained in sinus rhythm (PL-SR). Atrial ROS levels in the supplemented-POAF group were 2.3-fold higher than those observed in patients who received n-3 PUFA supplemented with vitamins and remained in sinus rhythm (supplemented-SR). POAF atrial sections from the PL-POAF group showed 39% higher ROS levels compared to the supplemented-POAF group.

Nitrotyrosine

Figure 2 displays representative images of nitrotyrosine in right atrial tissue sections from patients in sinus rhythm (PL-SR), placebo, and supplemented-POAF patients and the average immunofluorescence intensity of nitrotyrosine per tissue area in the studied groups. Quantitative analysis of tissue sections in the PL-POAF group showed 223%

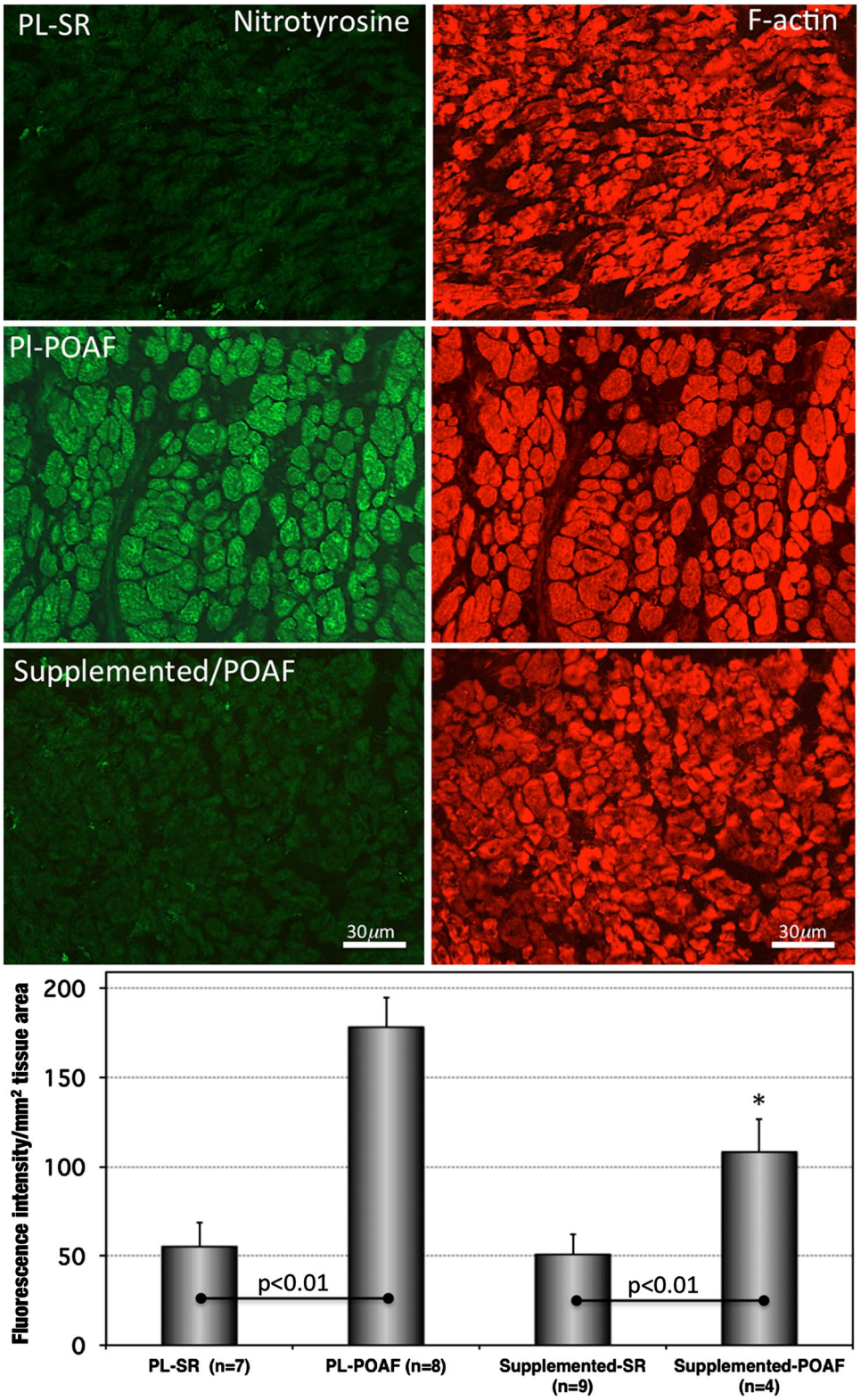

Fig. 2 Representative immunofluorescent images of nitrotyrosine (*green color*, *left panels*) obtained from right atrial tissue sections from patients in sinus rhythm group receiving placebo (Pl-SR), POAF group receiving placebo (PL-PAF), and supplemented (supplemented/POAF) groups. *Right panels* are corresponding images for F-actin actin (*red color*) and DAPI (*blue color*) for nuclei staining. *Scale bars* 50 μm. The *lower diagram* shows the quantitative results of nitrotyrosine staining expressed as fluorescence intensity units per mm^2 atrial tissue area. Nitrotyrosine levels are indicated as means ± SD. *$p < 0$ 0.05 PL-POAF versus Supplemented-POAF

more nitrotyrosine levels compared to PL-SR. Atrial nitrotyrosine levels in the supplemented-POAF group were 2.2-fold higher than in the supplemented-SR group. The atrial tissue from the PL-POAF group showed 64% higher

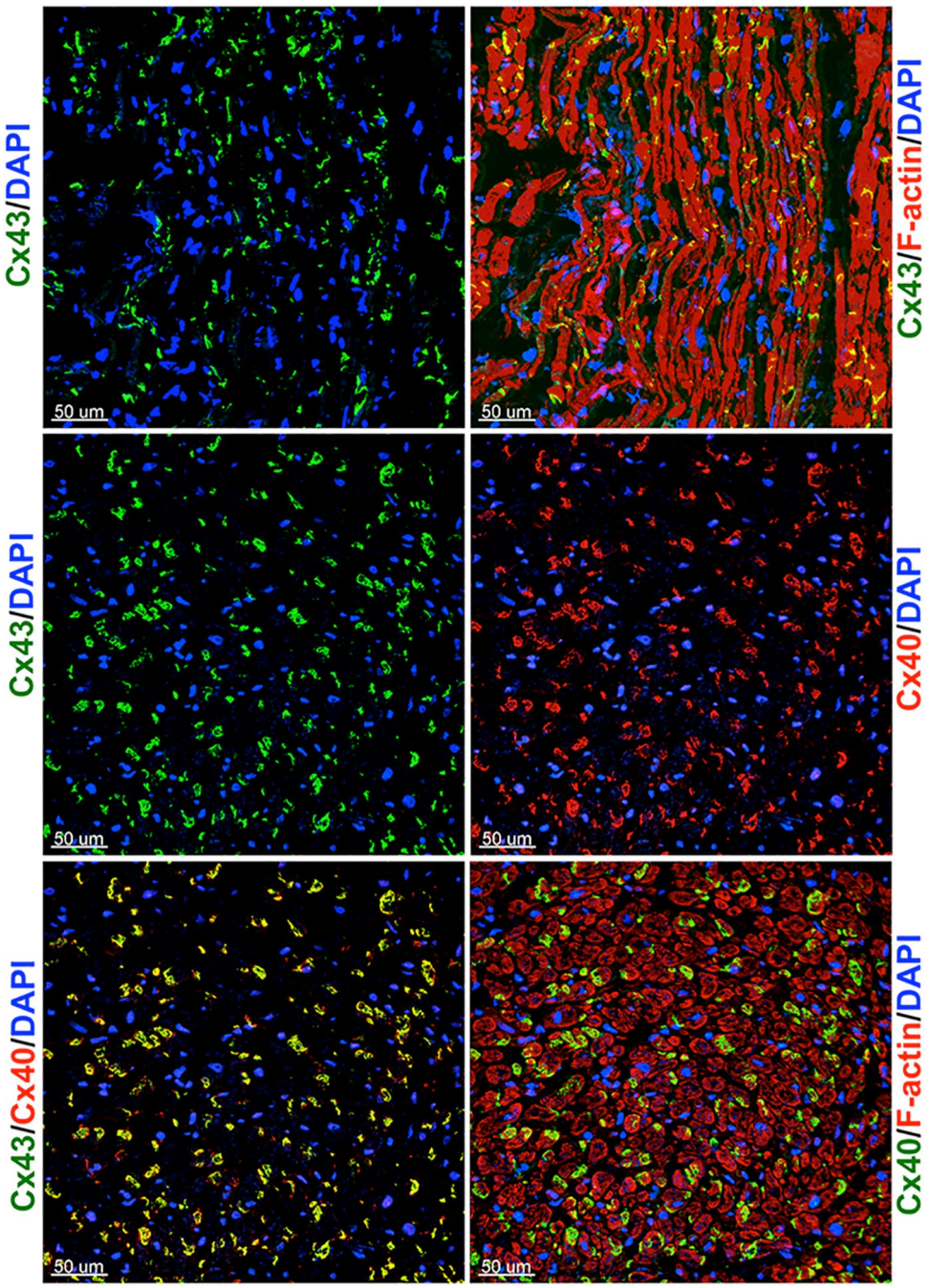

Fig. 3 Confocal images of Cx43 distribution (*green, left upper panel*) in relation with cardiomyocytes labeled in *red* with F-actin (*right upper panel*) in right atrial appendages sectioned in longitudinal planes in patients in SR. Note that Cx43 gap junctions are mainly located at the cell termini as *short transverse lines* representing intercalated disks with little side-to-side interconnections. *Middle* and *lower panels* are confocal images of Cx43 (*green*) and Cx40 (*red*) distribution in atrial myocardium sectioned in a transverse plane in patients in SR. Notice in the merged image (*lower right panel*), a uniform distribution of both, Cx43 and Cx40. The *lower left panel* shows the localization of Cx40 in relation with myofibrils which are stained *red* with F-actin. In all images, nuclei are stained *blue* with DAPI

nitrotyrosine levels compared to the supplemented-POAF group.

Tissue distribution of atrial connexins

As shown in Fig. 3, Cx43 and Cx40 proteins showed a homogeneous expression seen in longitudinal and cross atrial tissue sections of both placebo and supplemented group that remains in sinus rhythm after surgery. In longitudinal sections, Cx43 was mainly observed as transverse lines at cell poles corresponding to the pattern of the intercalated disks.

Figure 4 shows representative confocal images of Cx43 protein and dystrophin protein in POAF atrial tissue sections. In supplemented-POAF atrial sections, Cx43 protein is mainly located at the intercalated disks of myocardial fibers. In marked contrast, in the placebo group, Cx43 protein shows a higher degree of disorganization being confined to the lateral sarcolemma of cardiomyocytes. Similar observations have been documented for Cx40 distribution (Fig. 5).

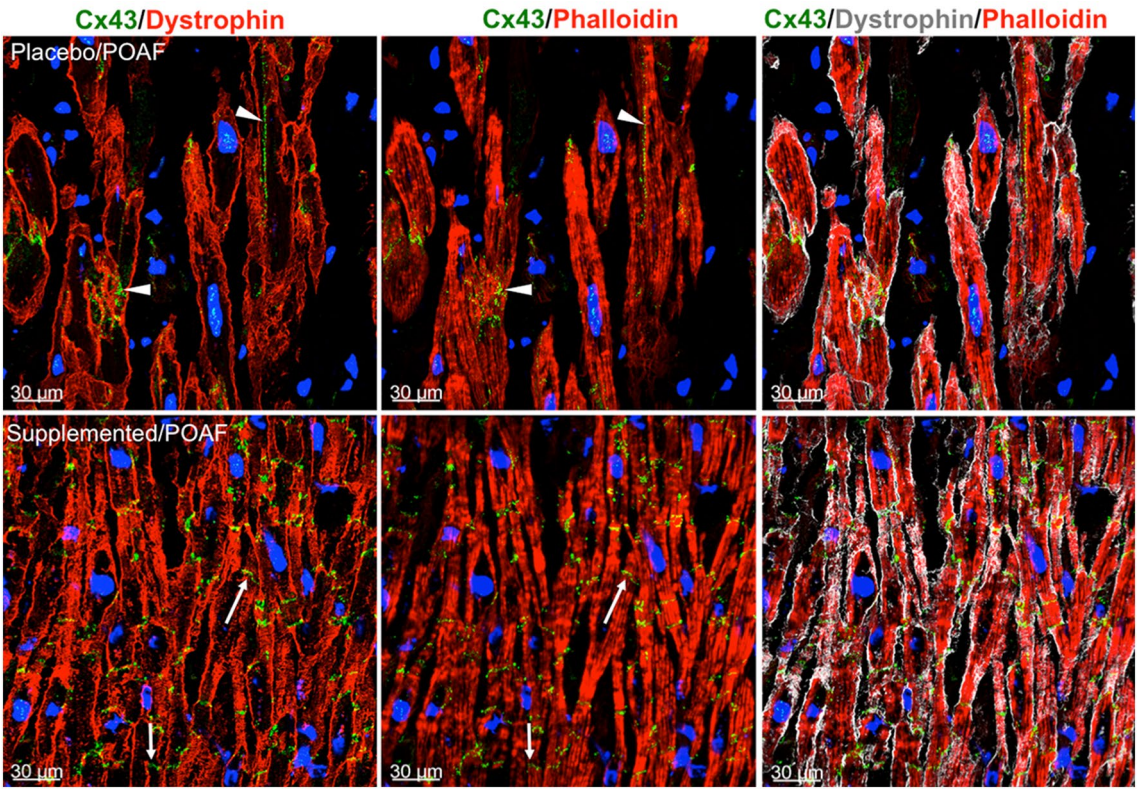

Fig. 4 Typical confocal images of Cx43 distribution (*green*) in placebo (*upper panels*) and in supplemented group who developed PAOF (*lower panels*). Dystrophin and F-actin are shown in *red color*, while in the merged images (*right panels*), dystrophin is pseudocolored in *gray color*. *Arrows* indicate the Cx43 signal confined to the intercalated disks, *arrowheads* point to the lateral Cx43 immunostaining

Heterogeneous expression pattern of Cx40 protein in POAF

Figure 6 shows expression pattern of Cx40 and Cx43 proteins in atrial sections from patients with POAF. Cx43 protein expression maintains a homogeneous pattern similar to that seen in sinus rhythm atrial samples. However, in these patients, Cx40 protein expression shows a heterogeneous pattern with tissue areas completely lacking this protein.

It is important to mention that in atrial sections from patients developing POAF, tissue areas lacking Cx40 protein, expresses abundantly Cx45 protein (Fig. 7).

It should be noted that heterogeneous pattern of Cx40 distribution, although to a lesser degree, was also observed in POAF patients who received PUFA and antioxidant vitamins (Fig. 8). This pattern was also associated with increased in Cx45 expression (data not shown).

Expression of atrial connexins

Figure 9 shows a representative western blots of Cx40, Cx43, and Cx45 protein in atrial samples in the studied groups. Quantitative analysis revealed that POAF atrial tissue samples show a 49.2 and 54.7% lower Cx40 protein abundance in placebo and supplemented group, respectively, compared to those samples in sinus rhythm with the same protocol. There were no significant differences between placebo and supplement effect on Cx40 expression. There were no significant differences in Cx43 protein abundance between experimental groups. However, a 39.1% increase of Cx43 relative abundance was observed in the supplemented-POAF group as compared to Placebo-SR ($p < 0.05$). Figure 9 shows also the relative abundance of Cx45 in atrial tissue samples for each experimental group. POAF samples displayed a 211 and 167.1% Cx45 higher protein levels in placebo and supplemented group, respectively, compared to those samples in sinus rhythm with the same protocol. There were no significant differences between placebo and supplement effect on Cx40 relative abundance.

Discussion

The present study is a part of a double-blinded, placebo-controlled clinical trial, which supported the hypothesis

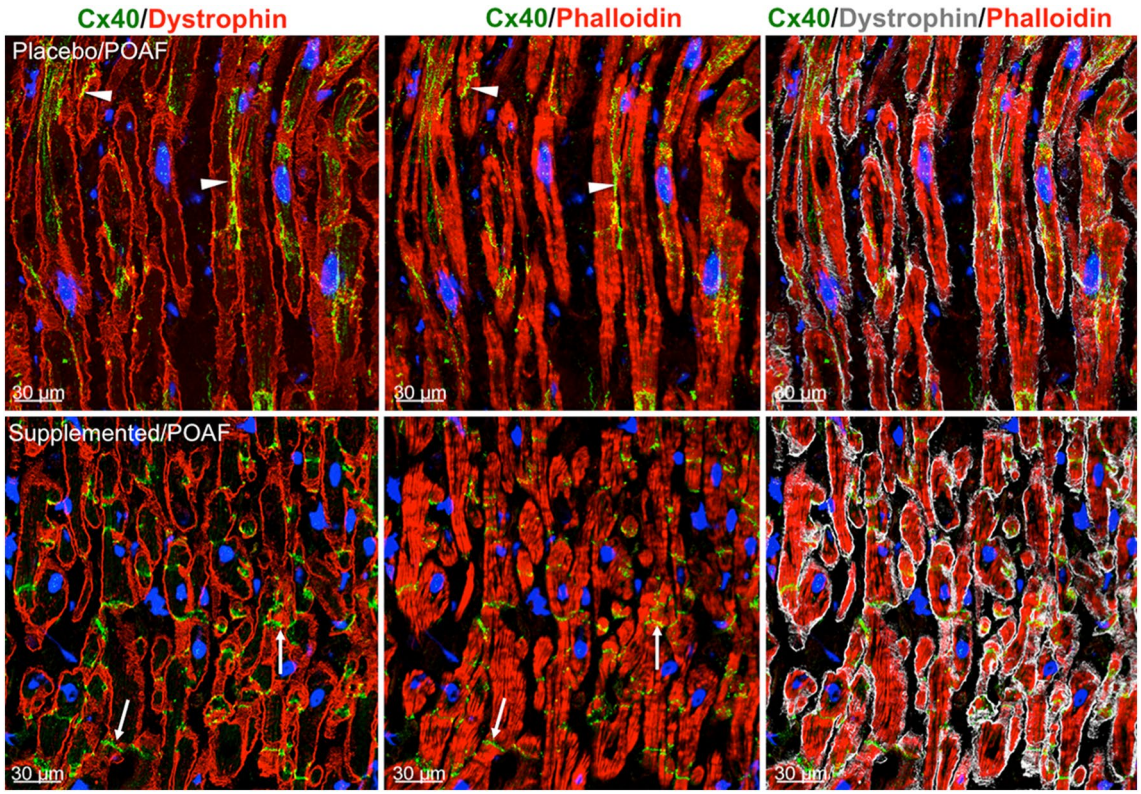

Fig. 5 Representative confocal images of Cx40 distribution (*green*) in placebo (*upper panels*) and in supplemented group who developed PAOF (*lower panels*). Dystrophin and F-actin are shown in *red color*, while in the merged images (*right panels*), dystrophin is pseudocolored in *gray color*. In all images, nuclei are stained blue with DAPI. Note a striking rearrangement of Cx40 at the lateral sarcolemma (*arrowheads*) in the placebo/POAF group. In marked contrast Cx40 signal in the supplemented/POAF group is localized mainly at the cardiomyocytes poles (*arrows*)

that strengthening local and systemic antioxidant potential is associated with a reduction on POAF incidence [22]. This treatment strategy was based on two consecutive steps: (1) production of mild oxidative stress as a result of n-3 PUFA administration to induce and enhance the endogenous antioxidant systems and (2) the strengthening of the non-enzymatic antioxidant defense system through vitamins C and E supplementation [36]. Cardiac surgery with extracorporeal circulation results in ischemia/reperfusion of the heart tissue favoring thus the production of ROS [37, 38]. We have also recently shown that our new antioxidant therapeutic protocol abolishes the deleterious effect of ROS on heart rhythm and function in hypoxic rat hearts [39] or after experimental myocardial ischemia/reperfusion [40].

To further test this hypothesis, we have analyzed ROS production in situ and detection of nitrotyrosine residues as a marker of proteins nitration in atrial tissues in human patients. Our results have shown that POAF atrial samples have higher ROS levels as compared to those samples from patients remaining in sinus rhythm. In addition, pre-treatment with omega-3 fatty acids and antioxidant vitamins significantly reduced ROS levels in POAF atrial tissue sections compared to those of placebo. A similar phenomenon was demonstrated by the detection of nitrotyrosine residues in proteins, as POAF atrial samples had significantly higher levels of nitration compared to those from patients remaining sinus rhythm or to placebo group. Taken together, the effects of our preventive regimen on myocardial status redox suggest that the reduction on POAF occurrence, as previously documented in our clinical trial [22], is related to the antioxidant potentiation of atrial tissue prior to surgery and thus preventing oxidative damage [37].

The pathophysiology of POAF certainly comprises the presence of a preoperative myocardial structural remodeling that supports the development of an arrhythmogenic substrate [14, 35]. Gap junction remodeling is an important component of atrial structural remodeling and therefore has been investigated in the present study. The knowledge pertaining to the relationships between POAF and CXs expression and distribution is very limited and controversial. Dupont et al. [31] examined the expression of Cx40, Cx43, and Cx45 in patients who underwent cardiac surgery and reported elevated Cx40 mRNA and protein levels in patients susceptible to POAF. Similar to our

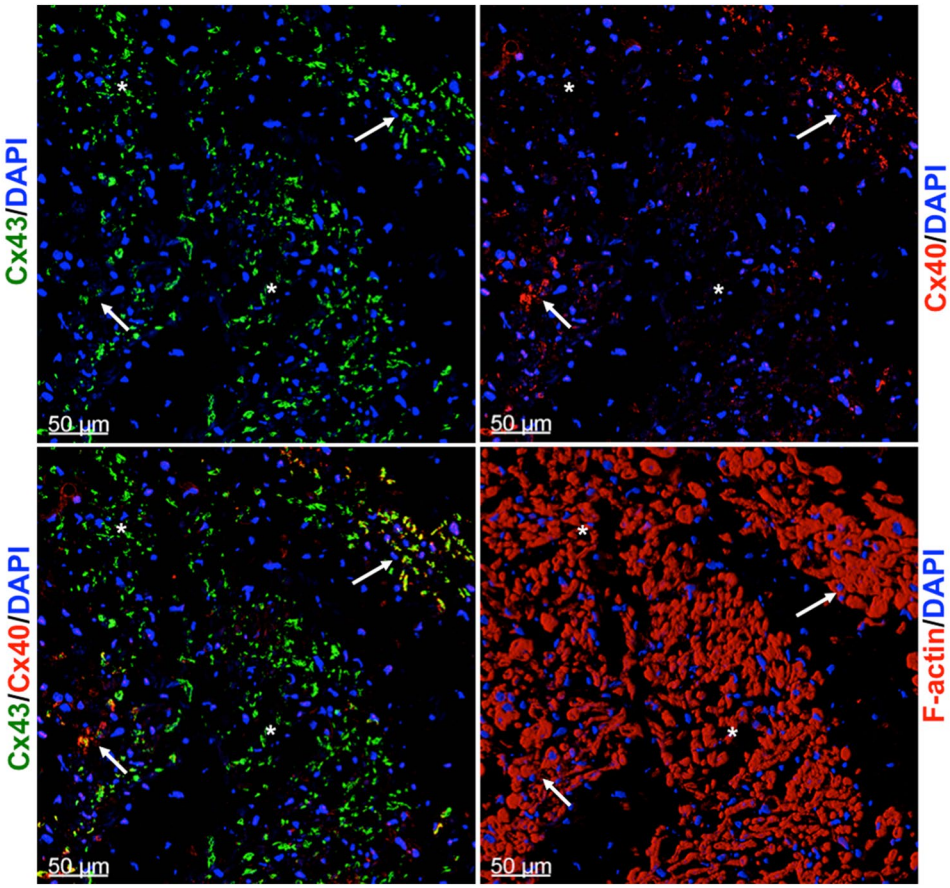

Fig. 6 Confocal images of Cx43 (*green*) and Cx40 (*red*) distribution in right atrial tissue sections obtained from placebo/POAF patients. Note a homogeneous distribution of Cx43 which contrasts with a heterogeneous distribution of Cx40 in terms that large tissue areas are lacking Cx40 signal (*asterisks*). This feature is even more evident in the superimposed image (*lower left panel*). *Arrows* indicate small groups of atrial cardiomyocytes with preserved Cx40 expression. The *right lower panel* is a corresponding image showing the entire population of cardiomyocytes stained in *red* with F-actin. Nuclei are stained *blue* with DAPI

study, Saravanan et al. [27] have compared placebo and n-3 PUFA-treated patients who developed POAF and found no differences in Cx40 and Cx43 expression levels between groups. However, in this study, the supplementation protocol differed from our since it was not used the same EPA:DHA ratio, doses of n-3 PUFA, and did not include C and E vitamins as non-enzymatic antioxidant reinforcement [22, 41]. In another study, Wilhelm et al. [32] examined the expression of Cx40 and Cx43 and reported a trend of diminished Cx40 and increased of Cx43 density in POAF patients. Moreover, this study demonstrated a reduction of Cx40/Cx43 protein ratio (~50%) in POAF samples. These data concur well with our observations documenting that in POAF patients, decreases in Cx40 were accompanied with increased Cx43 by 39% (Fig. 9). Collectively, these results suggest that the occurrence of POAF is associated with an increase in Cx43 abundance with a preferential distribution at the lateral membrane of the cardiomyocyte.

A peculiar and common feature documented in all the above-mentioned studies was the heterogeneous pattern of Cx40 distribution. The functional consequence of Cx40 heterogeneity would be predicted to provide a non-uniform pattern of wave-front propagation. Direct evidences linking the heterogeneous pattern of gap junction distribution and loss of Cx40 with impulse conduction defects are coming from murine models of heterogeneous gap junction distribution [42] and Cx40 knock-out mice [43]. It is important to emphasize that Cx40 expression levels and Cx40 heterogeneity persisted also in supplemented group who developed PAOF suggesting that pre-treatment with omega-3 fatty acids and antioxidant vitamins is not capable of suppressing this pathological mechanism.

Apart from heterogeneous distribution of Cx40, we found significant reductions in Cx40 expression in POAF patients irrespective whether the patients received supplementation or placebo. In this context, Gemel et al. [44] showed that decreased expression levels of Cx40 protein and reductions of the Cx40/Cx43 protein ratio are associated with paroxysmal and chronic atrial fibrillation. In addition, Wakili et al. [45] showed that altered Cx40 and Cx43 protein distribution in cardiac tissue can affect the electrical refractoriness, thus favoring reentry phenomenon and arrhythmias. However, one should consider that POAF pathophysiology differs from other types of atrial fibrillation, involving different degrees of structural and electrical tissue remodeling [45].

A noteworthy immunohistochemical observation was that atrial fibers with large diameters (up to 1 mm)

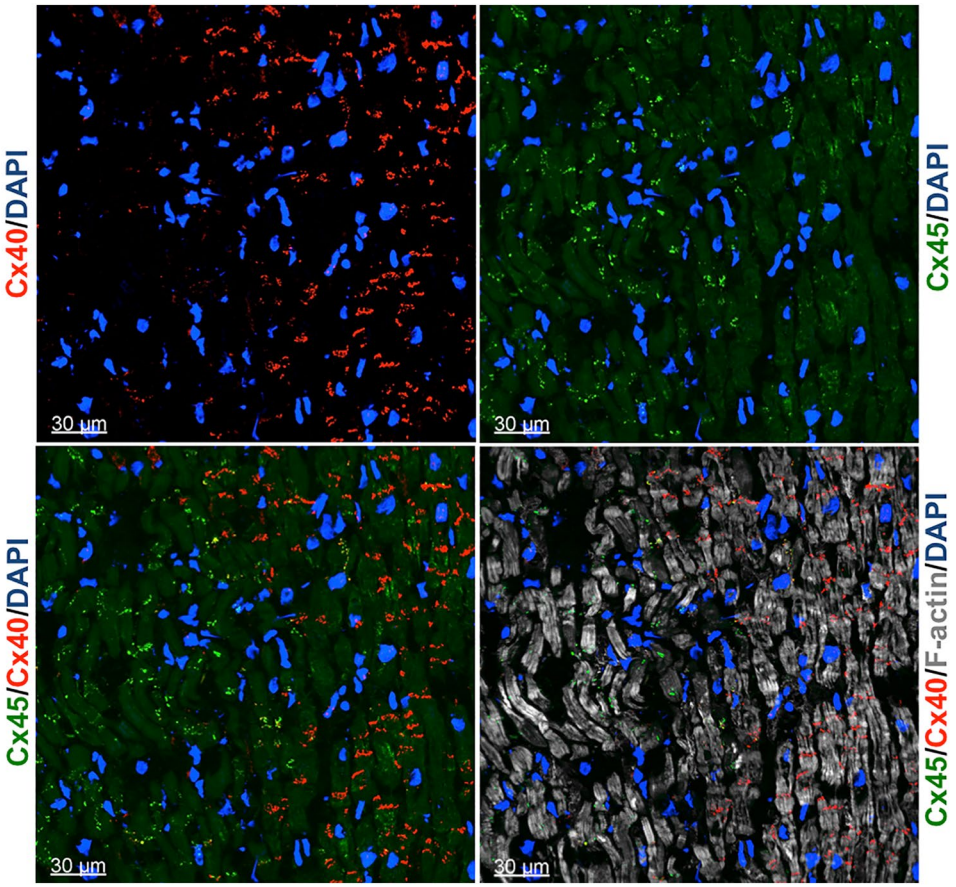

Fig. 7 Representative images of Cx40 (*green*) and Cx45 (*red*) distribution in right atrial tissue sections obtained from placebo/POAF patients. *Asterisks* denote patches of atrial myocytes with markedly reduced Cx40 signal (*asterisks*). Note that in such patches of myocytes lacking Cx40, Cx45 is abundantly expressed. The difference between these proteins in the distribution patterns of labeling intensities is even more evident in the superimposed image (*left lower image*). The *right left panel* is a merged image of all immunofluorescent signals. Nuclei are stained *blue* with DAPI

lacking Cx40 and expressed high amount of Cx45 protein. Such areas might represent a potential arrhythmogenic substrate due to the slow conductance properties of Cx45. Therefore, changes in conduction properties associated with low Cx40 and high Cx45 expression levels, in addition to lateralization of Cx40 and Cx43 at cardiomyocyte sarcolemma, would be expected to develop an electrical and structural arrhythmogenic substrate predisposing to reentry circuits and initiation of POAF. In addition, higher Cx45 expression levels have been related to the development of an arrhythmogenic substrate by forming heterotypic and heteromeric gap junctional channels in association with Cx43 [46]. Importantly, Cx45 is the dominant factor in conduction and propagation velocity properties of heteromeric gap junctions formed by Cx43/Cx45 dimerization [46, 47]. It has also been reported that an increase of Cx45 abundance relative to normal Cx43, as observed in our patients with POAF, is associated with decreased in gap junction size at intercalated disks [12]. These factors and a Cx43/Cx45 lower ratio have been demonstrated to be responsible for the occurrence of cardiac arrhythmias [48]. In line with this evidence, we observed 211% higher levels of Cx45 protein in POAF tissue group compared to those in sinus rhythm (Fig. 9) implying that up-regulation of Cx45 might in theory lead to a slow atrial conduction thereby promoting POAF. On the other hand, it has been shown that in the absence of Cx40, up-regulation of Cx45 in the heart results in reduced conduction velocity in the left atrium [49]. Moreover, genetic studies have shown that one aminoacid mutation (Cx46A96S) in Cx40 protein decreases atrial conduction velocity and connexin replacement by Cx45 protein occurs in atrial tissue lacking Cx40 protein [50]. The latter is similar to what was observed in our study, where heterogeneous expression pattern of Cx40 is related to overexpression of Cx45 protein in human atrial tissue from patients developing POAF (Figs. 7, 9).

In the present study, pre-treatment with omega 3 and antioxidant vitamins before cardiac surgery was associated with a distribution of Cx40 and Cx43 mainly at the intercalated disks as previously reported in non-fibrillating atria [13, 14]. This distribution pattern was also present in the supplemented-POAF group indicating the potential of our treatment to reverse gap junctional remodeling. Therefore, our preventive treatment which reduces oxidative damage of the atrial tissue thereby resulting in reduced POAF incidence [22] could be related at least "in part" to the preservation of a normal Cx distribution pattern.

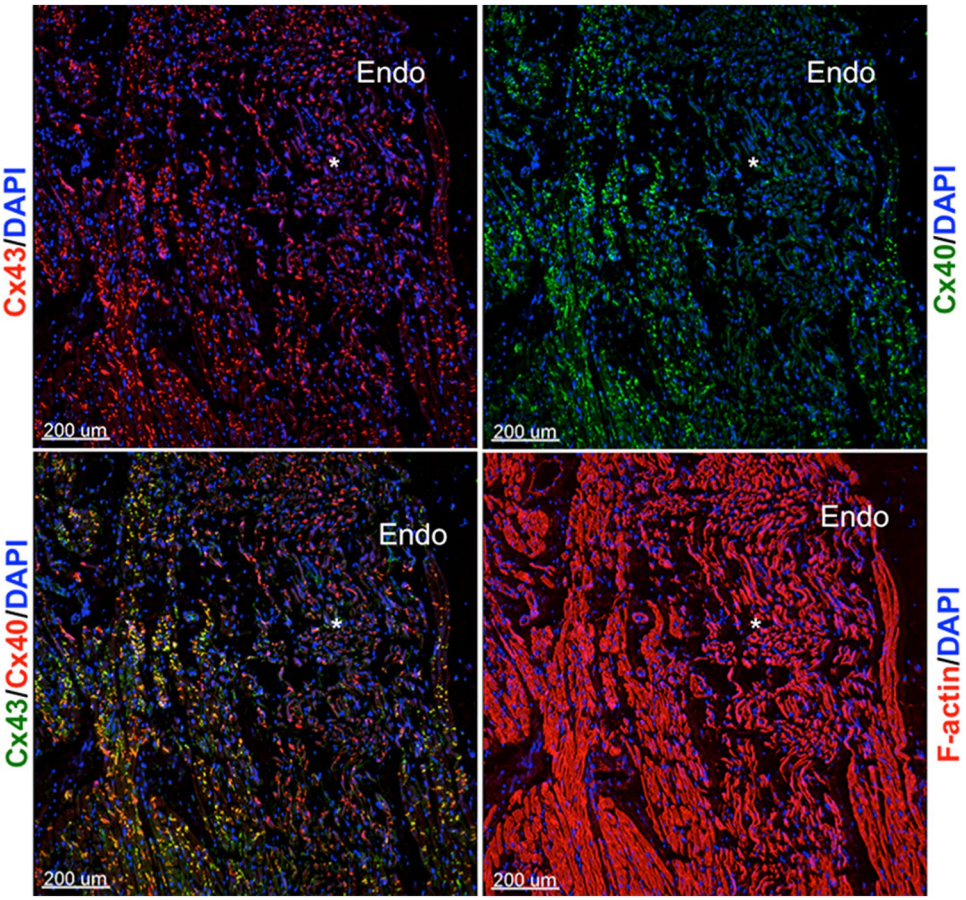

Fig. 8 Confocal images of Cx43 (*red*) and Cx40 (*green*) distribution in right atrial tissue sections obtained from supplemented-POAF patients. Note a homogeneous distribution of Cx43 which is in marked contrast with a reduced Cx40 signal (*asterisk*) in a subendocardial (*Endo*) patch of atrial myocytes. The *right lower panel* is a corresponding image showing cardiomyocytes stained in *red* with F-actin. Nuclei are stained *blue* with DAPI

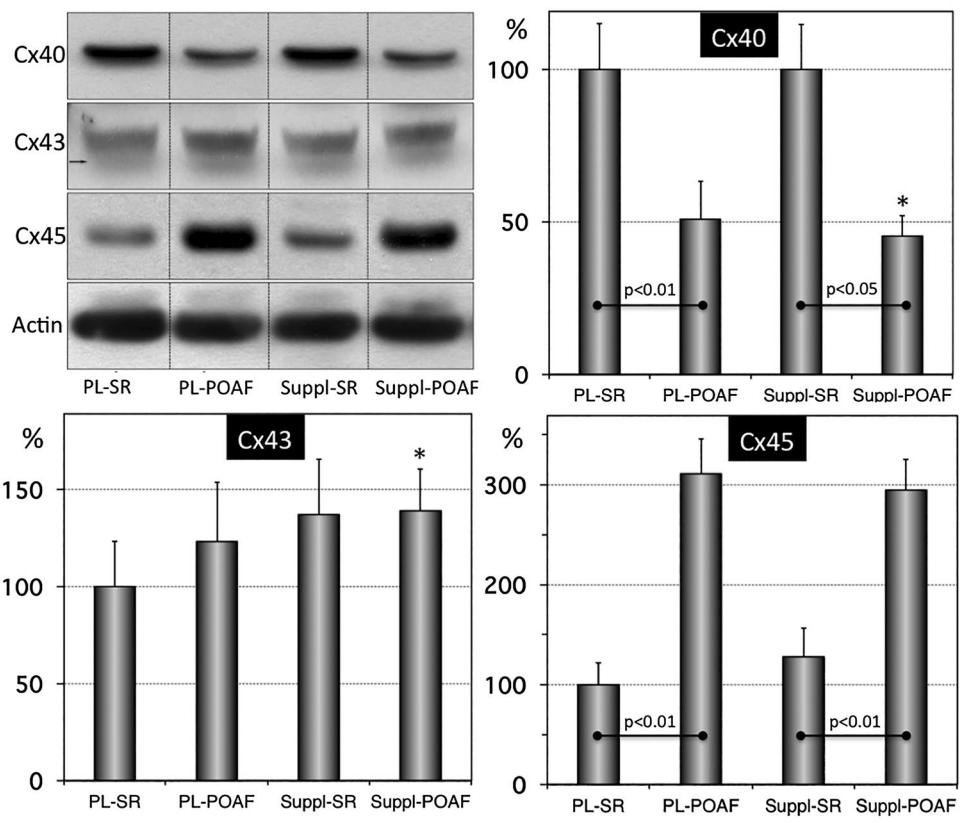

Fig. 9 Representative WB and quantitative data of Cx40, Cx43 and Cx45 expression in the studied groups: Placebo-sinus rhythm (PL-SR, $n=7$), placebo-POAF (PL-POAF, $n=8$), supplemented-sinus rhythm (Suppl-SR, $n=9$), and supplemented-POAF (Suppl-POAF, $n=4$). Relative abundance is expressed as percentage of loading control (Actin protein abundance corresponding to 100%). *$p<0.05$, Supplemented-POAF versus PL-SR

The redistribution of Cx40 and Cx43 at the intercalated disks upon application of n-3 PUFA is likely due to its ability to be incorporated into the lipid membranes [51] favoring thus the migration of Cx hemichannels along the plasma membrane to the sites of cell-to-cell junctions. In support to this hypothesis, recent animal studies in rats with diabetes type 1 demonstrated that n-3 PUFA incorporation at the cardiomyocyte cell membranes provides their protection and integrity attenuating Cx43 lateralization [26]. In addition, similar to our observations, n-3 PUFA incorporation increases the proportion of Cx43 at the intercalated disks and is related to increased Cx43 phosphorylation and Cx43 mRNA expression levels [26].

It should also be mentioned that in addition to normalization of Cx40 and Cx43 distribution, the mechanism of the beneficial effects of n-3 PUFA in preventing POAF would also lie in modifying the kinetics of ion channels, through binding to specific sites in proteins [52] and the ability to modify the ionic flow patterns of the plasma membrane [12].

Study limitations

The findings of the present study should be interpreted in light of several limitations. First of all, further studies are needed to directly examine the atrial tissue conduction and arhythmogenic substrates comprising atrial Cxs distribution and expression in the context of POAF and its treatment. Next, we have studied only biopsies from the right atrial appendages. The most important issue is whether this tissue represents the right atrial wall. In our previous studies, we have compared structural remodeling in right and left atria in human patients with atrial fibrillation and have concluded that structural and biochemical changes observed in the right atrial appendages closely reflect those observed in the right and left atrial free walls [14, 35]. Therefore, it is highly plausible that the same holds true also for POAF. Moreover, in comparison with atrial fibrillation, the triggers of POAF are most likely acute and perioperative alterations, including oxidative stress, inflammation, elevated catecholamines (reviewed in [6]). These factors would be predicted to equally affect both, the right and the left atrium.

Another limitation is that the supplemented-POAF group comprised only four patients, which is too small to be representative for a larger cohort of patients. The reason is that the present study was a part of a prospective clinical trial, which might have given unpredictable incidence of POAF. On the other hand, this study demonstrates again that the administration of omega-3 fatty acids and antioxidant vitamins applied to patients undergoing cardiac surgery decreases the occurrence of POAF as we have previously demonstrated on a larger cohort of patients in a double-blinded clinical trial [22].

Conclusions

This study demonstrates for the first time that a novel therapeutic protocol based on omega-3 fatty acids and antioxidant vitamins applied to patients undergoing cardiac surgery is able to reduce oxidative and nitrosative stress and to prevent Cx40 and Cx43 lateralization in atrial tissue likely contributing to POAF prevention. This protocol, however, failed to fully prevent POAF occurrence and normalization of Cx40 down-regulation and Cx45 up-regulation as observed in our placebo-POAF patients.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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