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Ageing Related Down-Regulation of Myocardial Connexin-43 and Up-Regulation of MMP-2 May Predict Propensity to Atrial Fibrillation in Experimental Animals

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Summary

Mechanisms underlying atrial fibrillation (AF), the most common cardiac arrhythmia, particularly in aged population, are not fully elucidated. We have previously shown an increased propensity of old guinea pigs (GPs) heart to inducible AF when comparing to young animals. This study aimed to verify our hypothesis that susceptibility of aged heart to AF may be attributed to abnormalities in myocardial connexin-43 (Cx43) and extracellular matrix that affect cardiac electrical properties. Experiments were conducted on male and female 4-week-old and 24-week-old GPs. Atrial tissue was processed for analysis of Cx43 topology using immunohistochemistry, expression of Cx43 protein using immunobloting, and expression of mRNA of Cx43 and extracellular matrix metalloproteinase-2 (MMP-2) using real time PCR. Immunohistochemistry revealed uniform Cx43 distribution predominantly on lateral sides of the cardiomyocytes of young male and female GP atria. In contrast, non-uniform distribution, mislocalization and reduced immunolabeling of Cx43 were detected in atria of old GPs. In parallel, the atrial tissue levels of Cx43 mRNA were significantly decreased, while mRNA expression of MMP-2 was significantly increased in old versus young GPs. The changes were more pronounced in old GPs males comparing to females. Findings indicate that age-related down-regulation of atrial Cx43 and up-regulation of MMP-2 as well as disordered Cx43 distribution can facilitate development of AF in old guinea pig hearts.

Key words

Aged heart • Connexin-43 • Extracellular matrix • Atrial fibrillation

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Introduction

Occurrence of cardiac arrhythmias both ventricular and atrial is mostly associated with diseased heart due to its metabolic, structural and electrophysiological remodeling. Besides, arrhythmias can occur in structurally „intact“ heart in presence of genetically-related malformations of ion channels and, although not often, in healthy top-level athletes in which heart enlargement and autonomic regulation imbalance may be implicated. Severe ventricular rhythm disorders results in ventricular fibrillation (VF) that is prevalent cause of sudden cardiac death (Lopshire and Zipes 2006), while atrial rhythm disorders in sustained AF promote thrombus creation and stroke burden due to tromboembolism (January *et al.* 2014). Important risk factor for the development of AF is ageing (Tribulova *et al.* 2015a).

AF is an extremely common arrhythmia in human population, particularly in elderly people (Cracknell 2010), and presently with suboptimal therapeutic options (Kato *et al.* 2012). Efficacy of both invasive approaches to ablate arrhythmogenic foci or direct-current cardioversion and drug treatments is

limited due to relatively high rate of AF recurrence (January *et al.* 2014). Despite a great deal of research to understand the mechanisms of AF to reveal fundamental determinants of arrhythmogenesis there is still a task for further investigations that may challenge development of novel more successful treatments approaches.

As noted above, ageing is considered to be the highest risk factor for supraventricular cardiac arrhythmias. However, several animal models that have been developed to induce and explore AF have not taken into consideration this fact. In a goat model (Wijffels *et al.* 1997) using healthy adult females, electrically-induced AF always terminated spontaneously within a few seconds. Chronic AF (duration >24 h) could be achieved only when brief AF was repetitively re-induced by several days of pacing. Rapid atrial pacing decreases effective refractory period (ERP) and concomitant ion currents alterations likely promoted sustaining of AF. In healthy adult mongrel dog model AF could only be induced by prolonged rapid pacing and administration of acetylcholine that decrease ERP, conduction velocity, and wavelength (product of conduction velocity and ERP) (Gaspo *et al.* 1997). Young sheep of either sex were used in sophisticated Langendorff-perfused electrically paced sheep heart model in presence of acetylcholine (Chen *et al.* 2000). AF inducibility could be attributed to heterogeneities of refractoriness and in conduction away from the pacing site. Recently, *in vivo* adult porcine model of prolonged (>1 min) AF was introduced *via* burst pacing of right atrium and infusion of neostigmine (Lee *et al.* 2016) but there was inability to achieve 100 % success rate.

Different from these young or adult large animal heart models, AF could be induced also in small but aged animals. Indeed, we have previously reported (Tribulova *et al.* 1999, 2008, 2015a) that prolonged AF is elicited in >1-year-old guinea pigs already after short-lasting burst pacing of isolated Langendorff-perfused heart while AF was not induced in young <3-month-old animals. Young guinea pig heart atria is characterized by high number of gap junctions that are responsible for synchronized electrical impulse propagation. In contrast, the overall density of gap junctions is reduced and intercellular space is widened due to collagen deposition in the old guinea pig atria. Moreover, old heart is much prone to Ca^{2+} overload that is highly arrhythmogenic and deteriorates gap junctions function (Tribulova *et al.* 2009). We postulated that one aspect of susceptibility to AF is the level of cardiac cell-to-cell coupling at the gap junction connexin channels. This view is supported by the observations that AF is difficult to induce even in the old rat heart without the impairment of connexin channel mediated intercellular coupling by heptanol (Haysahi *et al.* 2002) and AF could be induced in anesthetized adult guinea pig only by topical application of aconitine that promotes Ca^{2+} overload (Suzuki *et al.* 2014).

Taking into consideration mentioned data we aimed to explore the expression and localization of principal cardiac connexin, connexin-43 (Cx43) in atria of old and young male and female guinea pigs. Because myocardial fibrosis due to disease or ageing deteriorates cell-to-cell coupling the expression of MMP-2 as an indicator of extracellular matrix alterations was examined as well.

Material and Methods

The study was conducted on male and female young 4-week-old (n=16) and elderly more than 24-week-old (n=16) Dunkin-Hartley guinea pigs. All the experiments were performed in accordance with the rules issued by the State Veterinary Administration of the Slovak Republic, legislation No. 289/2003 and they conform to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (1985, Council of Europe No. 123, Strasbourg). Animals were housed under conventional laboratory conditions, fed *ad libitum* with standard laboratory rodent chow and had a free water supply 24 h/day. Animals were euthanized by stunning and the chest was quickly opened for the rapid heart excision into ice-cold saline and separation of the atria followed by quenching into liquid nitrogen. Frozen atria were stored at –80 °C until used for specific analysis. They were used for real-time PCR and immunoblotting to determine Cx43 mRNA and protein expression respectively. Cryostat sections were used to detect *in situ* myocardial gap junction-related Cx43 distribution and subsequent qualitative and quantitative image examination. Randomly taken small 1 mm^3 blocks of atria were used for routine electron microscopy examination of subcellular localization of gap junctions. The body weight and whole-heart weight of guinea pigs were registered.

Analysis of Cx43 and MMP-2 mRNAs expression by real-time PCR

RNA isolation from 50 mg of each heart tissue was performed using TRISure reagent (Sigma-Aldrich,

Bioline, USA). Each tissue was incubated for 5 min with 1 ml of TRISure reagent at room temperature. Thereafter 1 ml of chloroform (Centralchem, Slovakia) was added to the sample, mixed properly, and left for 2-3 min at room temperature. After specified time, samples were centrifuged for 15 min at 4 °C and 12 000 g. The upper phase of the sample was used to determin the concentration of RNA. RNA concentration and purity was determined using a NanoDrop ND1000 spectrophotometer (Thermo scientific, USA). The absorbance was measured at 260-280 nm. We used 2 µg of RNA for the reverse transcription. The result of reverse transcription was 20 µl of cDNA. Obtained single-chain DNA was used for real-time PCR. 18.5 µl of SYBR Green PCR Master Mix (Applied Biosystem, USA) containing 30 pM of each primer was added to the 1.5 µl of cDNA. For amplification of GJP43, β-actin (housekeeping gene) and MMP-2 gene fragments, the following primers were used: GJP43, 5'-TCCTTGGTGTCTCTCGCTTT-3' (sense) and 5'-GAGCAGCCATTGAAGTAGGC-3' (antisense); β-actin, 5'-TCATCACTATCGGCAATGAGC-3' (sense) and 5'-GGCCAGGATAGAGCCACCA-3' (antisense); MMP-2, 5´-AGGGCACCTCCTACAACAAGC-3' (sense) and 5´-CAGTGGACATAGCGGTCTCG-3' (antisense). Amplification was performed on a 7500 fast Real-Time PCR system (Applied Biosystems, USA). The amplification program consisted of an initial AmpliTaq GoldR DNA polymerase activation step at 95 °C for 10 min, followed by 50 cycles of denaturation (95 °C for 15 s), annealing, and elongation (56 °C for 60 s). For control of specificity, a dissociation stage was conducted by the sequential increase of temperature from 56 to 99 °C, recording the drop in the double-stranded DNA-SYBR Green complexes' fluorescence strength. We performed calculations using the 7500 fast system SDS software provided (7500 Fast System SDS 1.4 software, Applied Biosystems, USA). The cycle threshold is defined as the number of cycles required for the fluorescence signal to exceed the detection threshold. We calculated the expression of the target gene relative to the housekeeping gene (β-actin) as the difference between the threshold values of the 2 genes.

Analysis of Cx43 protein expression by Western blot

Frozen atria tissue was powdered and solubilized in SB20 solution (20 % SDS, 10 mmol/l EDTA, 100 mmol/l Tris, pH 6.8) using a UP100H sonicator (Hielscher, Teltow, Germany), for analyzing Cx43 protein expression. An equal amount of total protein in each sample was separated in 10 % SDS-PAGE and transferred electrophoretically to the nitrocellulose membrane. For Cx43 determination, the membrane was incubated with primary rabbit polyclonal antibodies (anti-connexin 43 C 6219, diluted 1:1000; Sigma-Aldrich, St. Louis, Missouri, USA) to recognize the phosphorylated (functional) (P-Cx43) as well as unphosphorylated forms (N-P-Cx43) of Cx43. Overnight incubation at 4 °C was followed by incubation for 1 h at room temperature with the secondary donkey antibody (peroxidase-labeled anti-rabbit immunoglobulin, diluted 1:2000, for Cx43; Amersham Biosciences, Ltd., UK). Bound antibodies were detected by the enhanced chemiluminescence method. After this, membranes were dried, put into a special device (KODAK In-Vivo Multispectral System FX) and the expression of studied proteins was quantitatively evaluated using Carestream Molecular Imaging Software (USA) program. Finally, the individual measured values were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) protein (rabbit polyclonal antibody anti-GAPDH, FL-335, sc-25778, Santa Cruz, Biotechnology, Inc., Dallas, Texas, USA, 1:750) as an internal control.

Myocardial immunostaining of gap junction-related Cx43

Cryostat sections from guinea pig heart atria were used for *in situ* immunodetection of cardiomyocyte distribution of Cx43. Following short fixation in freshly prepared paraphormaldehyde and washing in PBS, the sections were incubated overnight at 4 °C in primary, mouse monoclonal anti-Cx43 antibody (Chemicon International, Inc., USA, 1:200) and subsequently exposed for 1.5 h (in a dark place) to secondary FITC-conjugated goat anti-mouse antibody (Chemicon International, Inc., USA, 1:200). Immunostained sections were examined using a fluorescence microscope (Axiostar; Carl Zeiss, Jena, Germany), and digitalized images were stored for subsequent quantitative image analysis (Soft Imaging System, GmBh, Germany). Twenty randomly selected myocardial areas were investigated per heart. The area of positive Cx43 indication was defined as the number of pixels with the Cx43 signal intensity exceeding a threshold of 30 on the 0-255 gray scale. The total number of Cx43 positive pixels was expressed as integral optical density (IOD) per area. The latter parameter, expressed in arbitrary units, was compared between young and old guinea pigs.

Transmission electron microscopy for subcellular localization of gap junctions

Small, 1-2 mm^3, atrial tissue blocks (five per each heart) were fixed in buffered 2.5 % glutaraldehyde, postfixed in 1 % OsO_4, dehydrated *via* ethanol series, infiltrated by propylene oxide, embedded in Epon 812, and ultrathin sections were examined using transmission electron microscopy (Tesla 500; Tesla, Brno, Czech Republic). Details in tissue processing are described previously (Tribulova *et al.* 1999).

Statistical analysis

Data are expressed as the mean ± standard deviation (SD). A comparison between 2 groups was performed using 2-tailed Student's t-test. $P<0.05$ values were considered to be statistically significant. The normality of data distribution was verified using the Shapiro-Wilk test. One-way ANOVA was also used to analyze statistical significance among the selected parameters of rats.

Table 1. Biometrical parameters of experimental guinea pigs.

	Young male	**Old male**	**Young female**	**Old female**
Body weight (g)	202.8 ± 13.4	986.3 ± 62.9*	194.7 ± 9.2	897.5 ± 100.9*
Heart weight (g)	1.10 ± 0.13	2.97 ± 0.23*	0.86 ± 0.04	2.66 ± 0.14*
Heart weight/ Body weight (mg)	0.005 ± 0.0009	0.003 ± 0.0001*	0.004 ± 0.0003	0.002 ± 0.0002*

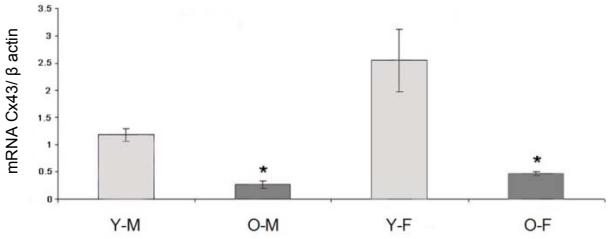

Fig. 1. Connexin-43 (Cx43) mRNA expression normalized to β-actin in the atria of young and old male and female guinea pig hearts. Y-M, young male guinea pigs; O-M, old male guinea pigs; Y-F, young female guinea pigs; O-F, old female guinea pigs. Results are the mean ± SD of 6 hearts. *, $p<0.05$ O-M compared with Y-M and O-F compared with Y-F.

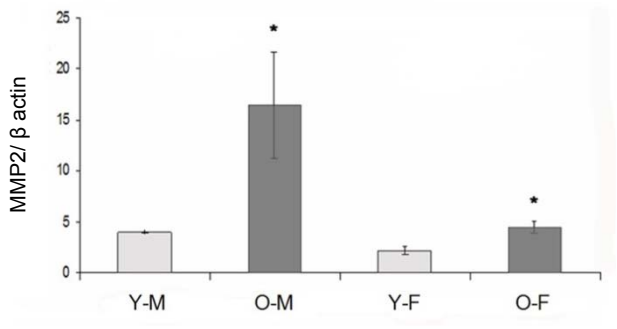

Fig. 2. MMP-2 mRNA expression normalized to β-actin in the atria of young and old male and female guinea pig hearts. Y-M, young male guinea pigs; O-M, old male guinea pigs; Y-F, young female guinea pigs; O-F, old female guinea pigs. Results are the mean ± SD of 6 hearts. *, $p<0.05$ O-M compared with Y-M and O-F compared with Y-F.

Results

There were significant differences in body and heart weights between young and old as well as male and females guinea pigs (Table 1).

Real-time PCR analysis revealed that atrial tissue Cx43 gene transcript was significantly increased in young comparing to old guinea pigs heart regardless the sex of animals (Fig. 1). There were no differences in Cx43 mRNA expression between old male and female guinea pigs, but the increase in young female was more pronounced than in males (Fig. 1). In contrast to Cx43, the atrial MMP-2 gene transcript was significantly increased in old guinea pigs of both sexes compared to young counterpart (Fig. 2). Of note, the increase of MMP-2 mRNA expression was more pronounced in male compared to female guinea pigs heart atria (Fig. 2).

Immunobloting analysis showed that Cx43 protein expression is significantly reduced in old comparing to young guinea pigs atria regardless the sex of animals (Fig. 3). Moreover, the level of functional phosphorylated forms of Cx43 was also decreased in old comparing to young animals (Fig. 3). There were no sex-related differences in Cx43 protein expression either in atria of young or old guinea pig hearts.

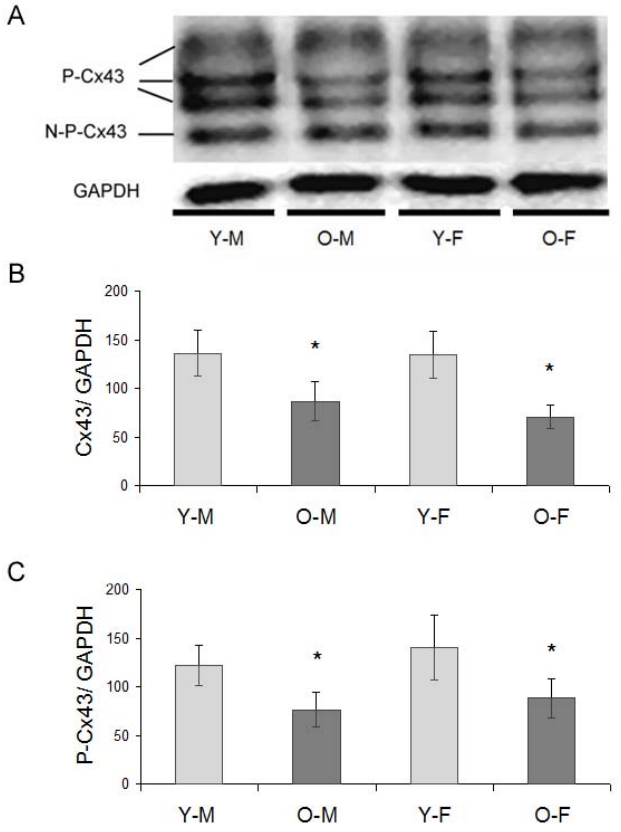

Fig. 3. Representative immunoblot showing 3 forms (**A**) of connexin-43 (Cx43) and densitometric quantification (**B**) of total Cx43 expression and its phosphorylated forms (**C**) normalized to GAPDH in the atria of young and old male and female guinea pig hearts. P-Cx43, phosphorylated forms of Cx43; N-P-Cx43, unphosphorylated form of Cx43; Y-M, young male guinea pigs; O-M, old male guinea pigs; Y-F, young female guinea pigs; O-F, old female guinea pigs. Results are the mean ± SD of 6 hearts. *, $p<0.05$ O-M compared with Y-M and O-F compared with Y-F.

Myocardial immunostaining of gap junction-related Cx43 revealed its distinct cardiomyocyte localization in the atria of young comparing to old guinea pigs regardless the sex (Fig. 4). Cx43 immunopositive fluorescence signal was seen predominantly at the intercalated discs and less frequently on lateral sides of the cardiomyocytes in the atrial tissue of old male and female guinea pigs. This topology differs when comparing to young male and females guinea pigs in which Cx43 immunopositivity was seen predominantly on lateral sides of the cardiomyocytes and less often at the intercalated discs. Quantitative image analysis determined that Cx43 related immunofluorescence signal was reduced in old comparing to young male or female guinea pigs atria (Fig. 5).

Subcellular atrial tissue examination revealed focal interstitial fibrosis with widened extracellular spaces and degradation of lateral junctions, i.e. adhesive junctions fascia adherens and desmosome as well as gap junctions in atria of old male and female guinea pigs (Fig. 6). In contrast, well developed lateral junctions, including gap junctions were frequently seen in young guinea pigs of both sexes (Fig. 6). In addition, widened extracellular space at the intercalated discs related adhesive junctions and lower amount of gap junctions were observed in old animals comparing to enhanced adhesive and gap junctions coupling seen in young guinea pigs atria (not shown).

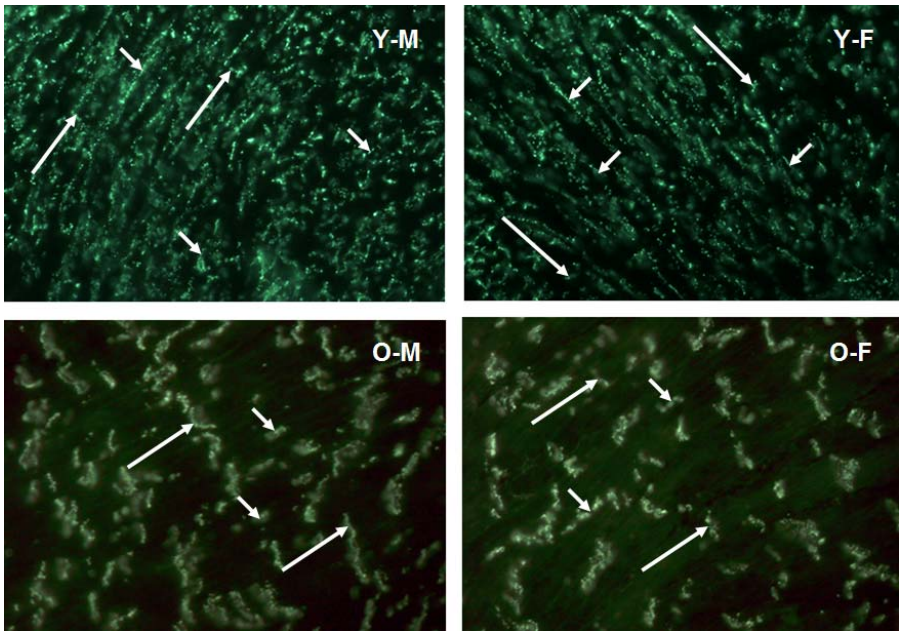

Fig. 4. Representative images of connexin-43 (Cx43) immunolabeling in the atria of young/male and female (Y-M and Y-F) and old/male and female (O-M and O-F) guinea pigs. Note the regular distribution of Cx43-positive gap junctions predominantly at the intercalated discs (long arrows) and rarely on lateral surfaces (short arrows) of the cardiomyocytes in all groups of guinea pigs. The lateralization is enhanced in young guinea pigs´ hearts. Magnification ×40.

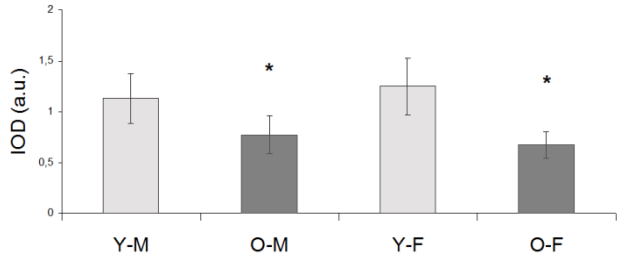

Fig. 5. Quantitative image analysis of the connexin-43 immunofluorescence signal of young and old male and female guinea pigs. IOD, integral optical density; Y-M, young male guinea pigs; O-M, old male guinea pigs; Y-F, young female guinea pigs; O-F, old female guinea pigs. Results are the mean ± SD of 20 images per heart. *, p<0.05 O-M compared with Y-M and O-F compared with Y-F.

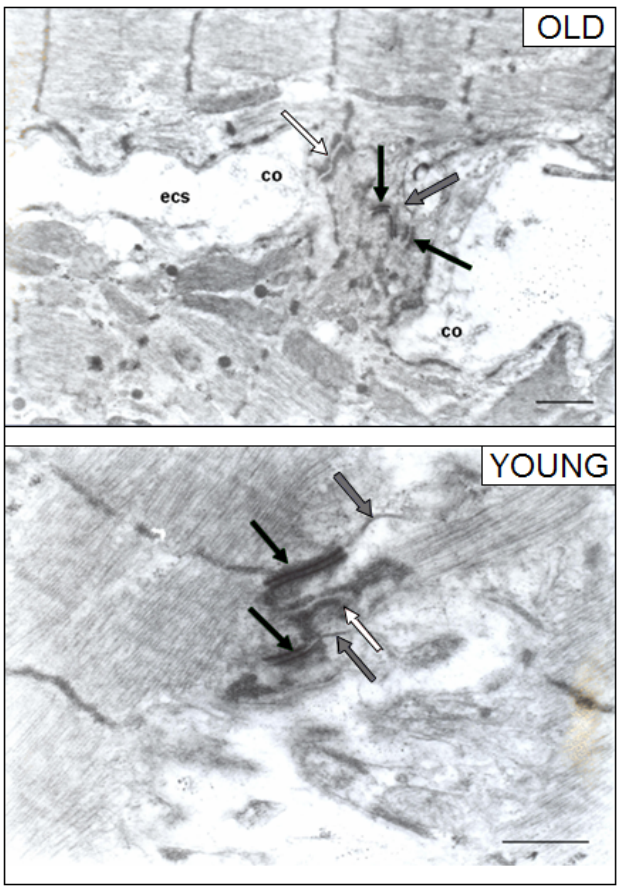

Fig. 6. Electron microscopic images demonstrate lateral, side-to-side type of intercellular connections in atria of old and young guinea pig hearts. Note lost of gap junctions (grey arrows) as well as degradation of adherens junctions (white arrows) and desmosome (black arrows) in old animals, comparing to well organized junctions in young animals. Scale bar – 1 mm.

Discussion

This study provides further data in support of our postulation that one important aspect of vulnerability of the heart to AF is the level of myocardial cell-to-cell coupling at the gap junction connexin channels. Accordingly, down-regulation of Cx43 as shown by its reduced mRNA as well as protein levels and abnormal topology of Cx43 in old guinea pigs atria most likely contribute to the occurrence of inducible AF previously demonstrated in those animals (Tribulova *et al.* 2008, 2014). However, young guinea pigs in which AF was difficult to induce, were characterized by high level of cardiomyocytes coupling due to up-regulation of Cx43 mRNA and protein as well as by uniform Cx43 distribution. In addition, not only total Cx43 protein expression but also its functional phosphorylated forms were reduced in old comparing to young guinea pigs atria. Furthermore, we demonstrated that in contrast to young male and female guinea pigs atria with well developed junctions among the cardiomyocytes, the aged hearts exhibit deterioration not only gap junctions but also adhesive junctions (Fig. 6). Thus, it appears that both impairment of gap junctions mediated coupling and loss of integrity of adhesive junctions might be implicated in arrhythmogenesis as recently reviewed (Tribulova *et al.* 2015a). Compared to marked deterioration of Cx43 and gap junctional coupling in atria of aged guinea pig hearts, the expression of atrial Cx43 in young animals reflects most likely intensive growth of cardiomyocytes. It is also suggested by enhanced expression of lateraly distributed Cx43-positive gap junctions that is typical in hypertrofied adult heart (Peters 1996, Tribulova *et al.* 2015b) and immature human heart (Peters *et al.* 1994). Besides, subcellular examination revealed focal areas of interstitial fibrosis as well as widened extracellular space at the adhesive junctions in atria of old guinea pigs. Such ageing related progressive structural remodeling of heart muscle resulted in conduction abnormalities and uncoordinated contraction facilitating occurence of AF in old guinea pigs (Jones *et al.* 2015). Even moderate changes in gap junctional conductance directly correlate with changes in cardiac conductance velocity highlighting their key role in ensuring normal conduction in the heart (Dhillon *et al.* 2014). Areas of reduced conduction or even conduction block due to fibrosis and deficiency of connexins have been established to promote myocardial electrical instability and development of re-entrant arrhythmias, including AF (Kato *et al.* 2012).

Regulation of connexin degradation and turnover may be an important mechanism for adjusting intercellular coupling in the heart under normal and pathophysiological conditions (Saffitz *et al.* 2000). Besides, regulation of phosphorylation state of Cx43 that determines gap junction

conductance, is another important process implicated in cardiac electrical stability or instability. The activation of stress-associated signaling within the heart progressively increases during ageing (Jones *et al.* 2015, Tomaselli *et al.* 2010, Tribulova *et al.* 2015a) with some of intracellular pathways implicated in the reduction of cardiac Cx43 and its phosphorylation (Yan *et al.* 2013) that may impair conduction. However, effects of Cx43 phosphorylation are complex and protein kinase-specific. Enhanced phosphorylation has the potential to alter channel gating by increasing or reducing conductance (Sovari *et al.* 2011). In addition, atrial Cx43 channels conductance can be reduced by intracellular phosphatases (e.g. calcineurin) suggesting their role in the modulation of Cx43 channel's function (Hatch *et al.* 2014).

Recently, it has been reported (Jansen *et al.* 2012) that reduced cardiomyocyte coupling resulted in more excessive collagen deposition during aging due to enhanced fibroblast activity, leading to increased conduction inhomogeneity and proarrhythmia. In addition to those observations during physiological aging, down-regulation of Cx43 along with increased collagen content (interstitial and reactive fibrosis), have been found under various pathophysiological conditions. These alterations have impaired conduction velocity of the cardiac impulse, by increasing the anisotropic ratio and heterogeneity of conduction (Kawara *et al.* 2001). Indeed, with increasing age of dogs the direction of conduction block in atria changes from longitudinal to transverse (Koura *et al.* 2002).

Besides reduced Cx43 expression another factor implicated in extracellular matrix alterations and myocardial fibrosis is activation of angiotensin II (Ang II). Experimental studies have identified that cardiac RAS up-regulation appears to play an important role in ageing hearts (Lindpaintner and Ganten 1991). Expression of angiotensinogen and ACE is selectively elevated in the hearts of aged male rodents despite an age-induced reduction in systemic RAS activity (Heymes *et al.* 1994). Myocardial expression of Ang II receptor subtypes AT1R and AT2R is also up-regulated in aged males, potentially mediating age-related cardiac pathology (Heymes *et al.* 1998). Atrial fibrosis is one component of the atrial substrate that has garnered recent attention based on newer MRI techniques that have been applied to visualize atrial fibrosis in humans with prognostic implications regarding the success of treatment (Goldberger *et al.* 2015). Despite of this fact, there is no current evaluation to identify the preclinical atrial myopathy. Ageing related atrial fibrosis in our study was suggested by subcellular interstitial alterations, deposition of collagen and increased levels of MMP-2 gene transcripts. Increase in expression of MMP-2 that cleaves the extracellular matrix proteins and thereby play a central role in tissue remodeling can promote atria dilatation and its enlargement, hence to facilitate occurrence of AF. Noteworthy, the brain natriuretic peptide (BNP) that is important marker of failing heart and dilated cardiomyopathy, has been shown produced by cardiac fibroblasts and it induced matrix MMP-2 (Tsuruda *et al.* 2002). MMP-2 activities were reported to be increased during ageing (Bonnema *et al.* 2007). Markers of collagen turnover, including MMP-2, were also elevated in patients who experienced AF recurrence after ablation (Okumura *et al.* 2011). Perhaps, assessment of fibrosis likewise in dilated cardiomyopathy (Masci *et al.* 2013) the detection of late gadolinium enhancement in atria remodeling by cardiovascular magnetic resonance may be a useful marker for predicting propensity of aged human heart to AF. In addition to atrial wall fibrosis, increased wall stress might contribute to AF development in long-standing AF (Aldhoon *et al.* 2013).

Another interesting aspect to discuss is cardiomyocyte distribution of Cx43 related gap junctions in young versus old guinea pigs. Immunolabeling revealed uniform dominant Cx43 distribution on lateral surfaces of the cardiomyocytes. It is consistent with other studies showing that myocardial tissue of young animals (Koura *et al.* 2002) and even human up to 6 years (Peters 1996) is characterized by a punctate distribution of Cx43 over the entire surface of the cardiomyocytes. This (immature) pattern of distribution differs comparing to healthy adult myocardial pattern that is characterized by prevalence of polar intercalated discs related localization and less frequent in lateral position. Topology of Cx43 in young intact heart has not been found to be arrhythmogenic. In contrast, enhanced Cx43 lateralization along with reduced polar, intercalated disc related distribution in diseased heart ventricles promotes malignant arrhythmias (Tribulova *et al.* 2015b). However, rather than lateralization, we observed degradation of lateral gap junctions in aged guinea pig heart atria as well as deposition of collagen in extracellular space. These changes can explain, at least in part, how AF spontaneously emerges when the transverse cell-to-cell coupling decreases, as occurs with age (Christensen *et al.* 2015, Kato *et al.* 2012). Along with the age-dependent structural anisotropy, a zigzag

propagation based on lateral uncoupling would predispose the elderly to multiple reentry and a higher incidence of AF (Koura *et al.* 2002).

Althogether, available data and our findings suggest that a combination of reduced Cx43 expression and increased collagen content is required to increase atrial arrhythmogenicity promoting occurence of AF. Because ageing is accompanied by inflammation, Ang II up-regulation, oxidative and nitrosative stress (Tribulova *et al.* 2015a), it is most likely that these risk factors contributed to alterations in Cx43, MMP-2 and deterioration of gap junctional coupling demonstrated in this study.

Conclusions

Findings provide further evidence in support of our hypothesis that propensity of aged heart to AF might be attributed to down-regulation of atrial Cx43 and extracellular matrix alterations resulting in deterioration of cell-to-cell coupling at the gap junctions.

Conflict of Interest

There is no conflict of interest.

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