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Pro-oxidant and pro-inflammatory Micro-RNAS profile as a risk factor for postoperative atrial fibrillation

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Abstract

Atrial fibrillation (AF) is the most commonly encountered arrhythmia after cardiac surgery with extracorporeal circulation. Although usually self-limiting and represents an important predictor of increased patient morbidity, mortality and health care costs. Numerous studies have attempted to determine the underlying mechanisms of postoperative atrial fibrillation (POAF) with varied success. POAF comprises a multifactorial pathophysiology, in which inflammation and oxidative stress (OS) play a critical role. Studies have shown that microRNAs (miRNA), may be involved in the pathophysiology of AF determine a link between inflammation and OS occurrence. Also, in chronic patients, miRNAs have been implicated in AF-induced ion channel remodeling and fibrosis. However, the role of miRNA in POAF is not well defined. We will be tested the hypotheses that the development of POAF is associated with pro-inflammatory and pro-oxidant miRNA profile in blood, atrial and PF samples. These changes are attenuated by the administration of antagomiRs or microRNA mimics in *ex vivo* model of atrial fibrillation. In animal *ex vivo* model of AF, the effect of candidate miRNAs on the duration of arrhythmia and markers of oxidative stress, inflammation and mitochondrial dysfunction in cardiac tissue are presented. The contribution of miRNAs will be determined by blocking these candidate miRNAs with specific antagonists to establish effects on *ex vivo* AF duration and left ventricular function. The *ex vivo* model of AF could allow to define some molecular targets that would modulate these miRNAs, and contributes to cardiac arrhythmogenesis.

Introduction

The mechanisms underlying the development of complications associated with cardiac surgery are multifactorial and have been related to inflammation and oxidative stress, classically measured in the blood or plasma of patients. This is important since cardiac surgery alters the integrity of the pericardial membrane and causes significant alterations in the pericardial fluid (PF) composition. This can potentially have adverse effects on the thin-walled atria leading to postoperative atrial fibrillation (POAF). After cardiac surgery, the pericardium remains open, and chest drains are routinely placed to prevent fluid accumulation around the heart. It have been described that the concentration and trajectory of blood proinflammatory factors increased in the PF after cardiac surgery over time [1]. Kramer et al. demonstrated an increase in the neutrophil infiltration in PF after 4 and 48 h postcardiac surgery over PF levels at time 0. Lipid peroxidation products of arachidonic acid–derived isoprostane 8-iso-prostaglandin F2-α and its stereoisomer 8-iso-15-prostaglandin F2α (F2 isoprostanes) were elevated in PF after 4 and 12 h following surgery and returned to PCF levels at time 0 after 24 to 48 h. Such increase of the levels of these pathological stimulants coupled with underlying atrial myocardial pathology can amplify the direct myocardial insult of a cardiac operation and may potentially contribute to the risk for postoperative AF [2]. As an opposite mechanism, it is also suggested that the elimination of the FP by pericardial drainage would reduce the pro-inflammatory injury. However, there is clinical evidence that increases complications and POAF occurrence [3]. Therefore, current evidence of how the composition of PF influences POAF and its change during surgery is inconsistent and requires further study.

Post-operative atrial fibrillation pathophysiology

Postoperative AF (POAF) frequently occurs as a complication of cardiac surgery with extracorporeal circulation, associated with an increase hospital stay, medical costs and overall mortality [4]. This arrhythmia has a high incidence, between 27 and 40%, despite the optimization in anesthetic protocols, surgical techniques, medical treatment and the wide use of antiarrhythmics such as beta-blockers and amiodarone [5]. Therefore, due to the suboptimal efficacy of perioperative pharmacological treatment, the search for new markers and pharmacological targets becomes necessary. Although the exact pathophysiology of POAF remains unclear, it is multifactorial in its origin. Patient related factors known to contribute include atrial dilatation: age-related fibrosis, cardiac structural damage, hypertension, and other comorbid conditions [6,7] The concept of

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(structural) predisposition for AF seems to be true for vulnerability of certain patients to AF after cardiac surgery. The electrophysiological substrate may be pre-existing or may develop because of heterogeneity of refractoriness after surgery [8]. Furthermore, the role of ectopic beats from the pulmonary veins in the development of POAF, as in nonsurgical patients, is yet to be delineated [9]. Several factors related to the surgical procedure also potentially contribute to the development of AF. These include operative trauma from surgical dissection and manipulation, pericardial lesions (pericarditis), atrial dilatation (caused by left ventricular dysfunction and intraoperative volume overload), perioperative use of catecholamines, parasympathetic activation, and electrolyte imbalances [7,10,11]. Current cardioplegia techniques and inadequate atrial cooling may be responsible for atrial ischemia. This has led some to postulate that ischemic injury and subsequent oxidative stress on reperfusion are potential triggers for POAF [12].

ROS generation is appreciated to occur during ischemia despite the low oxygen tension, from a likely mitochondria source, and ROS-induced ROS release may amplify its signal. The burst of ROS seen during reperfusion may originate from a different cellular source than during ischemia and is not yet fully identified [13,14]. This oxidative burst could cause and determine, in part, electrical remodeling processes that would trigger greater re-entry activity and, therefore, greater susceptibility to developing AF [15]. Regarding the inflammation, in atrial tissue from AF patients with valvular heart disease, there were significant positive correlations among NF- κB activity, serum TNF-α and IL-6 levels, and collagen volume fraction [16]. Serum levels of the fibro-inflammatory biomarkers MMP-9, type III procollagen, and hs-CRP, were greater in persistent-AF patients than in SR controls, and positively correlated with echocardiographic left atrial volume, an index of atrial remodeling [17,18]. It is important to distinguish between the contribution of inflammatory cells that migrate due to the phenomenon of surgical injury *vs.* The generation of local inflammation in the atrial tissue [19].

Participation of oxidative stress in the mechanisms inducing POAF

The technical procedure applied in cardiac surgeries implies an injury against the myocardial tissue, fundamentally derived from the changes of perfusion, and therefore, oxygenation, giving rise in this way to the formation of reactive oxygen species (ROS). Several mechanisms such as mitochondrial respiration and neutrophils activation generate ROS [14,20]. The production of free radicals in the early phase of reperfusion, combined with the decrease in antioxidant defences induced by ischemia reperfusion (IR), makes the myocardial tissue extremely vulnerable to oxidative damage. Among these, the superoxide anion (O2-), the hydroxyl radical (OH-) and the peroxynitrite (NOO-) are key species underlying the mechanism of damage in different experimental models, as well as in individuals subjected to post-infarction thrombolysis and stroke [21,22], to percutaneous angioplasty [23,24] or to cardiothoracic surgery [25,26]. As the cell membranes are composed mainly of phospholipids and proteins, alterations in membrane proteins by these ROS are important factors in the evolution of atrial tissue damage by IR. In the case of lipids, lipoperoxidation and the loss of membrane integrity trigger drop ATP levels and cytosolic calcium overload, which lead to cell death [27]. In addition, ROS act in the form of mediators or messengers, triggering intracellular signals. Thus, transcriptional factors, such as NF-κB, can be activated by ROS, which in turn activates the expression of pro-inflammatory genes [28,29]. Once the inflammatory process is initiated, transmigration and activation of the leukocytes takes place, which contributes to enhance local oxidative stress. The release of mediators such as cytokines, chemokines and adhesion molecules, all of which exacerbate the tissue damage, even areas of necrosis of the myocardial fiber can be generated [30,31]. The next step, the repair involves the risk of collagen deposition in the extracellular matrix, a process of interstitial fibrosis that would affect the functional properties, both electrical and mechanical, of the myocardium. Thus, *in vitro* studies, increase ROS concentration have shown affect the contractile function of cardiomyocytes associated with calcium overload and major sensitivity of myofilaments, as a arrhythmogenic mechanism [32,33].

Mitochondrial function

Recent experimental evidence suggests that changes of levels of phosphocreatine, electron transfer chain proteins and differences in mitochondrial distribution further imply that mitochondria play a role in AF [34]. Mitochondrial dysfunction leading to mitochondrial ROS production is implicated in ryanodine receptor oxidation facilitating Ca^{2+} leak and AF development [35,36]. Also, an interesting *ex vivo* study using atrial tissue from patients with and without AF showed that inward calcium L-type channels remodeling contributes to mitochondrial oxidative stress and increased expression of oxidative markers and adhesion molecules while antioxidants and inhibition of NF-｜B attenuate these changes [37,38]. Myeloperoxidase (MPO), an enzyme released from activated polymorphonuclear neutrophils has been linked to atrial fibrosis and remodeling [39]. MPO catalyzes the generation of reactive species like hypochlorous acid which affect intracellular signaling cascades in various cells and advance activation of pro-metaloproteinases and deposition of atrial collagen resulting in atrial arrhythmias. In an experimental setting MPO-deficient mice or rabbits were protected from AF [40,41]. In the same study, humans with AF had higher plasma concentrations of MPO and a larger MPO burden in right atrial tissue compared to control subjects. Furthermore, a recent study examining right atrial tissues from patients undergoing cardiac surgery indicated that monoamine oxidase represents an important source of ROS in human myocardium associated with POAF along with glutathione peroxidase [42]. However, it is currently unknown whether the mechanisms of mitochondrial dysfunction and eventual calcium overload would have a relevant pathogenic role in the development of POAF.

Inflammation and POAF

There is consistent evidence to support the influence of a surgery-related acute inflammation on the pathogenesis of POAF. This is largely based on association between levels and activity of white blood cells and incidence of POAF. Patients who have higher postoperative leukocytes count are significantly more likely to develop POAF [43-46] and patients developing POAF tend to have greater degree of monocyte activation as seen by higher expression of CD11b [47,48]. Moreover, the elevated pre and postoperative neutrophils/lymphocytes ratio in patients undergoing coronary bypass graft surgery can be associated with an increased incidence of POAF [49,50]. Exactly how these blood components can trigger POAF is not known. Previous work using animal models has shown that when activated neutrophils bind to cardiac myocytes they can cause changes in myocyte electrical activity that could be arrhythmogenic [51,52]. Cardiac surgery can induce a systemic inflammatory state (systemic inflammatory response syndrome, SIRS), whose cellular mechanisms of generation include the participation of ROS [45,53]. This systemic response is associated with the activation of cytoplasmic transcription factors such as NF-κB, which is key in the regulation of the inflammatory, immune, proliferative and apoptotic

response [45,54]. In the case of NF-κB, the ROS, especially H_2O_2, would act at least at two levels: 1) oxidation of key kinases in the activation of IκB Kinase which activates the NF-κB [55,56] and, 2) Modulation of the transport of this factor from the cytoplasm to the nucleus [45,57,58]. Systemic inflammatory response syndrome has a mild modality, but on the occasion of prolonged surgeries or exaggerated elevation of serum cytokines, especially IL-6, it can progress to a severe systemic inflammatory state, with lethal consequences [3,59] and it is likely that oxidative stress determines this difference. Markers of oxidative stress and inflammation are usually very ubiquitous in cardiac IR injury, therefore other markers that increase the specificity of the diagnosis in this type of arrythmias are clinically needed. The contribution of oxidative stress and inflammation are shown in Figure 1.

mi-RNA and cardiovascular pathology

MicroRNAs (miRNA) are a class of small non-coding RNA (20-25 nucleotides) that participate in gene regulation. In recent years, miRNAs have emerged as a key epigenetic mechanism in the development and functionality of the cardiovascular system. These molecular species regulate basic functions in virtually all cell types and therefore are directly associated with the pathophysiology of a large number of cardiovascular diseases [60,61]. Since its relatively recent discovery in extracellular fluids, miRNAs have been studied as potential biomarkers of several diseases. There are numerous studies that propose miRNAs as circulating biomarkers of different cardiovascular pathologies (myocardial infarction, coronary heart disease or heart failure, among others), even with physicochemical and biochemical properties superior to the conventional protein indicators currently used in clinical practice. They can be isolated from a variety of samples such as cell-conditioned media, plasma, serum, and other bodily fluids using a range of different methods such as sequential ultracentrifugation, density gradient separation, ultrafiltration, and commercial kits [62]. Currently, cardiovascular risk assessment is based exclusively on established classical risk factors such as hypertension, dyslipidemia, diabetes or smoking. Unfortunately, these traditional risk factors do not fully explain the risk of a cardiovascular event. Most of the events occur in patients with a low or intermediate risk that present a reduced number of classic cardiovascular risk factors. On the contrary, a large part of the individuals classified according to these factors as high risk

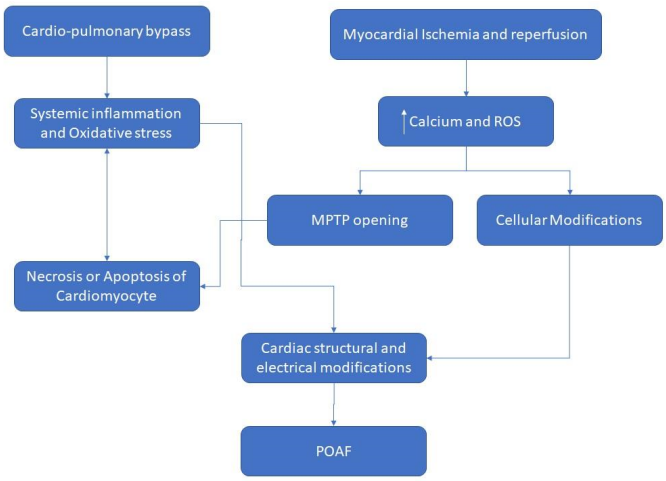

Figure 1. Proposed hypothesis for the role of oxidative stress and inflammation in the pathophysiology of postoperative atrial fibrillation in patients scheduled for cardiac surgery with cardiopulmonary bypass. ROS, reactive oxygen species; MPTP, mitochondrial permeability transition pore; POAF, postoperative atrial fibrillation

do not experience any cardiovascular episode; not even in the long term. Thus, there is a clear clinical interest in the development of new non-invasive and easily accessible biomarkers that significantly improve the predictive capacity of the algorithms developed to date, beyond the traditional risk factors [63].

mi-RNA and atrial fibrillation

The role of miRNA in cardiac arrhythmogenesis is in growing study in clinical and basic models [64]. miRNA targeting pathways associated with the regulation of cardiomyocyte metabolism (miR-208a and miR-223) may alter the provision of energy substrate required to maintain AF [65], whereas other miRNAs are thought to play a central role in changes associated with structural (miR-133, miR-590, miR-29b, miR-208, miR-638 and miR-150) and electrical remodeling of the cardiac tissue (miR-328, miR-1 and miR-26) [66]. Most of the studies to date examine miRNA expression in right or left atrial tissue, however, there is scarce evidence on the surrounding circulating miRNA in human AF [67-69]. Furthermore, current studies address the circulating miRNA signature in long-standing and paroxysmal AF and do not examine the role of miRNA in the new onset post-operative form of this arrhythmia. MiRNAs involved in cardiac electrical remodelling are miR-1, miR-26, miR-208a, miR-328 and miR-499. Their target genes are encoding ion channels, connexins or proteins involved in calcium signaling resulting in conduction slowing or shortening of the action potential duration, which are hallmarks of AF pathophysiology. In addition, mi-RNAs involved in cardiac structural remodelling are miR-21, miR- 26, miR-29b, miR-30, miR-133 and miR-590. These miRNAs regulate genes encoding proteins that are involved in extracellular matrix turnover and pro- or anti-fibrotic signaling cascades leading to atrial fibrosis as the anatomical substrate for re-entry. Several options to agonize or antagonize miRNA effects were developed and successfully evaluated *in vivo* in AF-related animal models [68,70,71]. Overexpression of a miRNA that is downregulated in disease can be achieved by miRNA mimics. Mimics are synthetic double- stranded RNAs that are incorporated and processed by the cell-like endogenous miRNAs and therefore 'mimic' their effects [72]. However, mimics are not tissue-or cell-type specific and can therefore create undesirable off target effects. This can be avoided by using cardiotropic adenovirus-mediated miRNA transfer that has been shown for the treatment of heart failure in mice [73,74] and cardiac hypertrophy in rats [75,76]. For antagonizing a pathological miRNA upregulation, several knockdown approaches are available including anti-miRNA oligonucleotides (antagomiRs) [77] or locked nucleic acid, [78] miRNA sponges, erasers or masks. AntagomiRs are synthetic oligonucleotides with miRNA complementary sequences that bind to endogenous miRNAs and thus, competitively inhibit them to bind to their target genes. MiRNA sponges [79] and erasers [77] are sequences of multiple miRNA sequences incorporated into a vector (e.g. a (cardiotropic) virus). While sponges contain only the seed sequence and might therefore inhibit various miRNAs, erasers are complementary to specific miRNAs. MiR masks, however, are single-stranded oligonucleotides that are complementary to a miRNA target sequence and can therefore specifically block single miRNA–mRNA interactions [80,81]. All potential therapeutic interventions are currently based on an intramuscular or systemic application of these agents *in vivo*. In summary, progress in miRNA research has opened a window for establishing a new potential therapeutic intervention in the context of translational medicine. The future will show whether mi-RNAs can help to close the translational gap between underlying causes and specific treatment, which is currently thought to be one major problem in AF disease management. Also, the stability and the detection in biological fluids, such as PF, can increase the specificity of

other markers such as the classical oxidative stress and inflammation, generating a new type of diagnostic cluster in POAF.

Conclusion

Despite the evidence of oxidative stress, inflammation and apoptosis occurrence in the atrial tissue and plasma of POAF patients post cardiac surgery with extracorporeal circulation, miRNA expression profiling results revealed a clear dissociation in expression levels between the atrial appendage and blood circulation. Whereas, an increase in some miRNA candidates such as higher levels of miR-1 and a decreased in miR- 133A that specifically negatively correlated with apoptosis was observed in the atrial tissue; however, the miR plasma equivalents were similar to their pre-CABG levels bringing into question the reliability of circulating miRs to serve as potential biomarkers for POAF in cardiac patients [82]. Nevertheless, an improved understanding of miR function would facilitate the design of novel strategies for cardio-protection against atrial tissue remodeling in POAF patients.

Finally, the use and validation of new markers such as miRNA in cardiac tissue and different fluids is important since their different expression profiles could bring us in a non-invasive way to what is induced by ischemia-reperfusion in myocardial tissue, its relationship with atrial remodeling and probable pharmacological targets that can be modelled.

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