An Acad Bras Cienc (2024) 96(1): e20230053 DOI 10.1590/0001-3765202420230053
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

Bone-marrow mononuclear cells and acellular human amniotic membrane improve global cardiac function without inhibition of the NLRP3 Inflammasome in a rat model of heart failure

ALINE L. TAKEJIMA, PAULO ANDRÉ B. MACHADO-JÚNIOR, GUSTAVO G. BLUME, ROSSANA BAGGIO SIMEONI, JULIO CESAR FRANCISCO, MURILO S. TONIAL, LUIS FELIPE B. MARQUEZE, LUCIA NORONHA, MARCIA OLANDOSKI, ELTYEB ABDELWAHID, KATHERINE A.T. DE CARVALHO, RICARDO A. PINHO & LUIZ CÉSAR GUARITA-SOUZA

Abstract: Recent studies have suggested that therapies with stem cells and amniotic membrane can modulate the inflammation following an ischemic injury in the heart. This study evaluated the effects of bone-marrow mononuclear cells (BMMC) and acellular human amniotic membrane (AHAM) on cardiac function and NLRP3 complex in a rat model of heart failure.On the 30th day,the echocardiographic showed improvements on ejection fraction and decreased pathological ventricular remodeling on BMMC and AHAM groups.Oxidative stress analysis was similar between the three groups,and the NLRP3 inflammasome activity were not decreased with the therapeutic use of both BMMC and AHAM,in comparison to the control group.

Key words: heart failure, Bone-marrow mononuclear cells, Amniotic membrane, NLRP3 inflammasome, oxidative stress.

INTRODUCTION

Acute myocardial infarction (AMI) remains the leading cause of cardiovascular death worldwide. Despite the benefits of current therapeutic strategies in ameliorating symptoms and improving patient survival rates, restoring the contractility of the necrotic cardiomyocytes remained an obstacle (Abdelwahid et al. 2016). Therefore, pathological ventricular remodeling and progression to heart failure are inevitable in most of the cases (Tanai et al. 2016).

Inflammation is known to play a major role in the pathologic processes leading to cardiac dysfunction after AMI, although certain levels of inflammatory response are necessary for myocardial repair (Fonseca et al. 2022, Shirazi et al. 2017). Thus, elevated levels of pro-inflammatory cells and cytokines may contribute to ventricular remodeling and functional deterioration of the heart via various mechanisms (Anzai 2013, Frangogiannis 2014, Briaud et al. 2001). Recently, the nucleotide-binding oligomerization domain (NOD)-Like-Receptor protein 3 (NLRP3 inflammasome), a multiprotein and cytosolic complex, has been shown to be a major regulator of the inflammatory response after AMI (Zheng et al. 2022, Butts et al. 2015, Chen & Frangogiannis 2013).

The inflammasomes are multimeric protein structures, and at least five types of inflammasomes have been identified, the most

well-characterized the nucleotide-binding domain, leucine-rich–containing family, pyrin domain–containing-3 (NLRP3) (Schroder & Tschopp 2010). The activation of the NLRP3 complex usually involves two signals: the first signal (priming) is mediated through the activation of the Nuclear Factor Kappa B (NF-kβ) signaling pathway, providing the transcriptional expression of pro interleukin-18 (pro-IL-18) , pro interleukin 1 β (pro-IL-1β), and NALP3. The second signal is dependent on DAMPs stimulus, PAMPs (pathogen- associated molecular patterns), and other substances such as reactive oxygen species (ROS), extracellular ATP, and potassium efflux after an ischemic injury in the heart (Frangogiannis 2014, Kelley et al. 2019, Walev et al. 2000).

ASC (Apoptosis-associated speck-like protein containing a caspase recruitment domain) and Caspase-1 are essential components of the NLRP3 inflammasome complex, acting as key proteins involved in the processing and release of the pro-inflammatory cytokines upon NLRP3 activation (Bracey et al. 2013, Taniguchi et al. 2007). Caspase-1 is a cysteine protease that is activated upon binding to the NLRP3, and ASC is a regulator protein that acts like a bridge between NLRP3 and pro-caspase-1, facilitating its autocleavage and activation to form active caspase-1 (Rühl & Broz 2015). These processes trigger the cleavage and release of Interleukin-1ß and Interleukin-18, which plays a critical role in the regulation of inflammatory responses and host defense mechanisms (He et al. 2016, Martinon et al. 2002).

This process has been shown to be associated with the development of atherosclerosis and heart failure (Butts et al. 2015, Takahashi 2014); therefore, ameliorating the inflammatory response through the blockade of the NLRP3 enzymes and cytokines had become a potential therapeutic target to preserve the global cardiac function after AMI, with studies suggesting that targeting the NLRP3 inflammasome may be a promising therapeutic strategy for preventing or treating AMI (Van Der Heijden et al. 2017, Marchetti et al. 2015, Mastrocola et al. 2016).

Current therapeutic approaches for AMI include: reperfusion therapies, medications, cardiac rehabilitation, lifestyle changes and surgery in selected cases. Stem cells and human amniotic membrane have emerged as a alternative therapeutic options for AMI due to their ability to differentiate into various cell types and regenerate damaged tissue (Machado-Júnior et al. 2020). Cell-based approaches with stem cells and human amniotic membrane was capable of reducing the inflammatory response and promoting significant improvements in global cardiac function after an ischemic injury in the heart (Guo et al. 2007, Khorramirouz et al. 2019). Despite this, the exact mechanisms underlying this association is not completely elucidated and, to date, there are lack of studies evaluating the possible evolvement of the NLRP3 complex with stem cells and human amniotic membrane.

Therefore, we assessed the hypothesis that both bone-marrow mononuclear cells (BMMC) and acellular human amniotic membrane (AHAM) would preserve the cardiac function and reduce the pathological ventricular remodeling through the inhibition of the NLRP3 Inflammasome in a model of heart failure in rats.

MATERIALS AND METHODS

All experiments were conducted according to the standards and ethical principles of the Brazilian College of Animal experimentation (COBEA) and approved by the CEUA (Committee of Ethics in Research Animals at PUCPR) number 01273.

Isolation of the bone marrow mononuclear cells

Animals were placed in lateral recumbency position with the posterior limb in flexion under general anesthesia with Ketamine (50 mg/kg) and Xylazine (10 mg/kg). The cell harvesting was achieved by the puncture and aspiration of the posterior iliac crest from the same animals, using 5-mL syringes containing 0.2 mL of heparin (5.000 UI/mL). After puncturing the iliac crest, the collected bone marrow blood 0,5 to 1,0mL was transferred to a 15 mL flask and mixed with sterile phosphate-buffered saline (PBS) (Boyum 1968, Takejima et al. 2021).

The tubes were centrifuged for 5 min at 350 G. After then, the supernatant was removed and mixed with 7 mL Dulbecco's Modified Eagle's Medium (DMEM). Seven milliliters (7 mL) of solution were then transferred to a 15 mL tube containing 3 mL density gradient Ficoll-Hypaque (density = 1.077 g/mL). The tube was centrifuged for 40 min at 400 G. After centrifugation and the appearance of a distinctive ring at the interface, the solution was transferred into another 15 mL tube, followed by adding up to 10 mL sterile PBS and centrifugation for 5 min at 400 G. The supernatant was then discarded, and the pellet was suspended in 1 mL of PBS/albumin 5%. Cell counting was done in a Neubauer chamber, and cell viability was tested using the Trypan Blue vital.

Preparation of acellular human amniotic membrane

The amniotic membranes were obtained from patients undergoing cesarean delivery with signed consent, and serologically negative for HIV, Hepatitis B, Hepatitis C and Syphilis. The amniotic membrane was collected immediately after placental expulsion in the surgical environment, then it was separated from the chorion and handled within a laminar flow Class II. The membrane was washed with Phosphate buffer saline (PBS 2% streptomycin/penicillin), trimmed into approximately 12x12cm^2 parts, kept in a decellularization solution (0.1% sodium dodecyl sulfate, SDS) on a horizontal mechanical shaker (120 rpm), followed by gentle scraping. Membranes were washed five times with PBS, cut into 8mm in diameter circles with a surgical punch, stretched on culture dishes, kept with PBS, and exposed UV light for 1 hour inside the laminar flow. AHAM discs were incubated in cell culture medium (Dulbecco's modified Eagle's medium-F12, supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin) in standard conditions of 5% CO2 in air at 37°C for 72h (Francisco et al. 2016, Dziedzic et al. 2021).

Animals and surgery

Fifty male Wistar rats, 4 months old weighing between 250-300 grams were used in this study. The animals were housed in groups of four rats in polypropylene cages at ambient with constant temperature (23ºC ± 2), 12-hour light-dark cycle and free access to water andstandard commercial diet during the conduction of the study.

The coronary artery ligation was performed as previously described by Khorramirouz. Briefly, the rats were anesthetized by intraperitoneal injection of Ketamine (50 mg/kg) and Xylazine (10 mg/kg), then subjected to endotracheal intubation as well as to mechanical ventilation at a frequency of 60 cycles/min and a volume of 2.5 mL (683, Harvard® Apparatus, Inc, USA). The animals were then placed in the supine position and a left lateral thoracotomy was performed in the fourth intercostal space. The anterior interventricular coronary artery, identified between the left atrium and the pulmonary artery, was permanently ligated using 7-0 polypropylene thread. The ischemia was confirmed with paling and hypokinetic area in the

ventricular wall. All rats received perioperative antibiotics and analgesia (Cefazolin 40mg/kg and Carprofen Sterile injectable Solution 5-9 mg/mL, Pfizer Animal - 4,4mg/Kg).

Echocardiographic assessment

All animals were subjected to echocardiographic evaluations under the same anesthetic conditions on the 7th and 30th days after myocardial infarction, to analyze the ejection fraction (EF), left ventricular end-systolic (LVESV) and end-diastolic volumes (LVEDV). Bidimensional transthoracic evaluations were taken using Sonos 5500 (Hewlett Packard, USA) with S12 sectorial transducer (5-12 MHz) and 15L6 linear (7-15 MHz), allowing an analysis of up to 160 Hz. LVESV, LVEDV and EF were obtained with the transducer placed in the left anterolateral portion of the thorax using the following formula: ventricular volume (V) was 8×(S)2/(3×3.1415926×C), where V=volume, S=area and C=weight. EF was calculated as follows: EF=LEDV–LESV/LEDV 18. Measurements were blindly obtained three times by the same cardiologist.

Transplantation of the BMMC and AHAM

Seven days after myocardial infarction, animals with EF lower than 50% were randomly assigned into three groups: control group, BMMC group and AHAM group. The animals were again anesthetized (50 mg/kg Ketamine and 10 mg/kg Xylazine), intubated and subjected to median transsternal sternotomy. The BMMC group received $5x10^6$ mononuclear cells injected directly on the infarcted area, as previously described (Carvalho et al. 2008), and the AHAM group received the amniotic patch on the anterior surface of the heart, sutured with 7-0 polypropylene. The control group received only saline solution by transsternal sternotomy. After thirty days, a new echocardiographic evaluation was blindly performed on the three groups, followed by euthanasia with an overdose of the anesthetic drugs.

Sample preparation for immunohistochemical analyses

The apex of the heart was surgically removed and immediately frozen in liquid nitrogen and stored at -80ºC for biochemical analysis. The remaining tissue was fixed in 10% phosphate-buffered formaldehyde for 24 hours, embedded in paraffin blocks and 5 µm sections were made.

Tissue microarray (TMA) and immunohistochemistry (IHC)

Representative areas of the muscle were transferred from the histology block to a recipient TMA (Tissue Microarray) block. Next, two 4 µm thick paraffin-embedded sections of the TMA blocks were transferred to electrically charged Star Frost™ (Braunschweig, Germany) slides and incubated with a primary anti-NFKβ p105/50 (ab797; 1:200; Abcam, Cambridge, UK), TNFα (ab6671; 1:100; Abcam), ASC (ab70627; 1:200; Abcam) and Caspase1 (ab189796; 1:200, Abcam) overnight in a humidified chamber at a temperature between 2–8°C. We used Polyvalent HRP-DAB Detection System-Spring Bioscience™ (Pleasanton, CA, USA) to detect the antibody signal. The slides were incubated with the secondary antibody for 30 minutes at room temperature.

The immunoreactivity was developed by adding DAB chromogen-substrate solution (Spring) to the slides. Harris hematoxylin was used for counterstaining. Positive and negative controls were run in parallel with all reactions. The slides were scanned using the Axio Scan.Z1 scanner (Carl Zeiss, Germany), and approximately 25 images were selected for analysis. The areas of immunopositive markings for the antiprotein antibodies were quantified using Image-Pro Plus version 4.5 software (Media Cybernetics,

Rockville, MD, USA). The immunopositive objects were selected using a "mask" to standardize and automate the process. The analysis was blind, once the software randomly generated the images, with no investigator's interference. The numerical data of the immunopositivity were generated and subsequently exported to an Excel spreadsheet.

Oxidation of dichlorodihydrofluorescein (DCFH) and NALP3

The tissue was placed in eppendorf, and 100 µl of phosphate-buffered saline (PBS) was added and homogenized vigorously at 4°C with the use of a homogenizer. Afterwards, 200µl of trypsin-EDTA (0.25% - Sigma-Aldrich) was added for 30 minutes for digestion. These extracts were centrifuged at 11.000 rpm at 4°C for 40 minutes to remove insoluble material. Part of the supernatants from these tissues were used for protein quantification according to the Bradford method. About 500µl of the supernatant was carefully collected and incubated, in the dark, with a 100µl NALP3 solution, for 30 minutes at 37ºC. After this time, the samples were centrifuged at 1.200 rpm for 10 minutes and the pellet was resuspended in 400µl of PBS. The samples were kept at 4ºC and protected from light until the acquisition of the data on the cytometer (BD Accuri C6 Plus – Biosciences). The signals were obtained using the FITC channel. Data analysis was performed with the aid of the BD Accuri C6 Plus software, by determining the average fluorescence intensity of 30.000 events.

Total and oxidized glutathione

The total glutathione (GSH) levels were based on the reaction of GSH with DTNB (Ellman's reagent), which forms an oxidized glutathione-TNB product that is later reduced by glutathione reductase in the presence of Nicotinamide dinucleotide phosphate and adenine (NADPH) with the consequent synthesis of GSH. The total GSH concentration was determined using a regression curve that was plotted using various GSH standards. The oxidized (GSSG) levels were measured from the recycling of GSSG by the spectrophotometric monitoring of NADPH in the presence of 2-vinylpyridine. The total GSH and GSSG concentrations were determined using a regression curve plotted using various GSH standards (Rahman et al. 2007).

Interleukin-1β

IL1-β tissue concentrations were determined using commercial ELISA kits as recommended by the manufacturer (Bioalbra biotechnology, Brazil)

Statistical analysis

quantitative variables were reported as mean, standard deviation, median, minimum and maximum values. Analysis of variance (ANOVA) was applied in the echocardiographic evaluation for the comparison of the three groups at the baseline analysis, and the covariance analysis model (ANCOVA) was used for the 30th day and for the difference between baseline and 30^{th} day. Normality condition was examined by Shapiro-Wilk test. The non-parametric Kruskal-Wallis test was used for the immunohistochemical evaluations. P values <0,05 indicated statistical significance, and the analyses were conducted with statistical software STATA version 16.0 (StataCorpLp, Texas, United States).

RESULTS

Morbidity and mortality of animals

From the initial 50 animals, 11 died shortly after the myocardial ischemia (mortality rate = 22%). Of the 39 remaining animals, 07 were excluded for presenting EF> 50%, thus 32 animals were included and randomized into three groups:

control (n=10), BMMC (n=11) and AHAM (n=11). After the BMMC and AHAM transplantation, three animals from the AHAM group and two animals from the control group died, related to postoperative complications (arrhythmia), resulting in a total of 27 animals included in the final analysis: control (n=8), BMMC (n=11), and AHAM (n=8).

Echocardiographic analysis

The left ventricular EF, LVESV and LVEDV, assessed by the baseline echocardiogram 7 days after AMI were not significantly different between the groups (p=0.122), thus the groups were considered homogenous. On the 30th day after myocardial ischemia, statical differences were observed in EF when comparing the control group versus BMMC (p=0.03) and the control versus AHAM groups (p=0.006). No difference between BMMC and AHAM was observed. No statistical difference was identified in respect to LVESV and LVEDV in the intergroup analysis.

The intragroup comparison showed that after thirty days the EF was significantly increased in both BMMC group (from 30.6±8.8% in baseline to 46.4±6.6% on the 30th day, p<0.001) and AHAM groups (from 33.2±7.6% in baseline to 49.7±9.9% on the 30th day, p=0.001), without significant changes in the control group. The LVESV was significantly decreased in the BMMC group (from 0.22±0.07mL to 0.12±0.05, p =0.004) and in the AHAM group (from 0.22±0.10mL to 0.09±0.04mL, p=0.001) over the same time interval. The LVED also decreased in the BMMC (from 0.31±0.08mL to 0.22±0.06, p=0.01) and AHAM groups (from 0.33±0.11mL to 0.18±0.06mL, p=0.002), over the time same interval. No significant benefits on LVESV and LVEDV were observed in the control group during the 30 days follow-up (figure 1).

Inflammation parameters

Immunohistochemical evaluations for TNF-α and NF-kβ were conducted to assess the activity of pro-inflammatory markers after 30 days in the infarcted heart. The results showed no difference in the levels of Tumor Necrosis Factor Alpha (TNF-α) between the three groups (figure 2a), and the levels of NF-kβ were significantly higher in both BMMC and AHAM groups in comparison to the control group (figure 2B), without statistical significance when compared to each other.

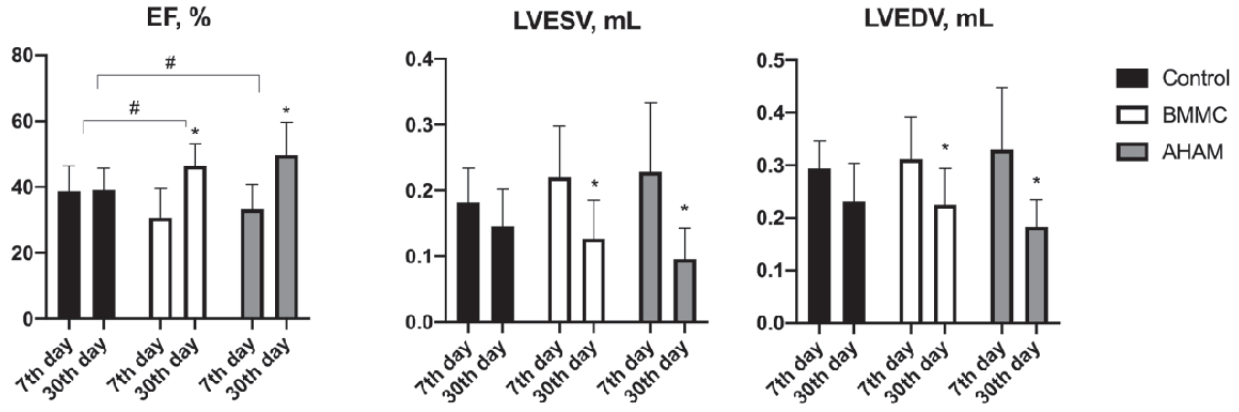

Figure 1. Comparison of the echocardiography results on the 7th and 30th days after myocardial infarction. The results are presented as mean +/- standard deviation. p<0,05 denoted statistical significance versus control group (#) or intragroup baseline analysis (*). Sample size: control = 8; BMMC = 11; AHAM = 8. EF (ejection fraction, in %); LVESV (left ventricle end-systolic volume, in mL); LVEDV (left ventricle end-diastolic volume, in mL); BMMC (bone marrow mononuclear cells); AHAM (acellular human amniotic membrane).

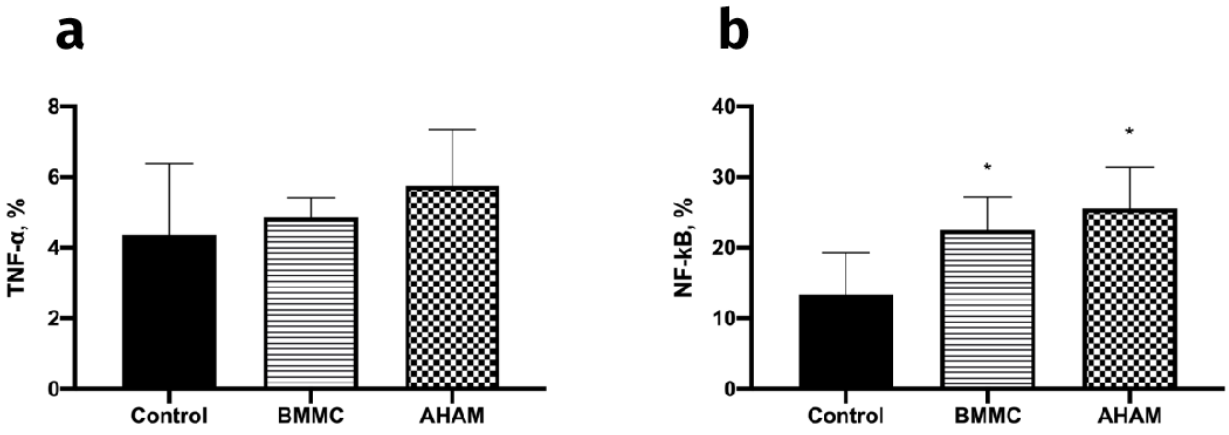

Figure 2. Effects of BMMC and AHAM on inflammatory parameters 30 days after myocardial infarction. The results of TNF-α (a) and NF-kβ (b) are presented as mean +/- standard deviation. p<0,05 denoted statistical significance versus the control group (*). Sample size: control = 8; BMMC = 11; AHAM = 8. BMMC (bone marrow mononuclear cells); AHAM (acellular human amniotic membrane).

Oxidative stress parameters

Reactive oxygen species levels were measured by detecting the oxidation of the 2',7 dichlorodihydrofluorescein diacetate (DCFH-DA) in a fluorescent 2',7'- dichlorodihydrofluorescein (DCF), and as shown in figure 3A, no significant difference was observed among the groups after the 30 days analysis. The GSH (figure 3B) and GSSG (figure 3C) levels were also similar between the groups, and the ratio GSH/GSSG remained unchanged in all groups (figure 3D).

Inflammasome NLRP3 activity

Heart tissues from the BMMC and AHAM groups significantly elevated the expression of NALP3 enzyme 30 days after AMI (figure 4A) in comparison to the control group (p=0.032 and 0.017, respectively). The immunohistochemistry demonstrated similar levels of ASC (figure 4B, p=0.07) and Caspase-1 (figure 4C, p=0.116) between the groups, and the levels of interleukin-1B (figure 4D) were significantly reduced in the AHAM group in comparison to both the control and BMMC groups (p=0.03 and p<0.01, respectively). Representative images of cross sections of the studied hearts are shown in figure 5.

DISCUSSION

In the current study, we investigated whether cell-therapy with BMMC and AHAM could inhibit the NLRP3 inflammasome activity in a rat model of chronic ischemia. The BMMC and AHAM patch were injected and engrafted, respectively, seven days after coronary ligation in animals with EF < 50% to simulate the clinical scenario of heart failure.

Bone-marrow cells and human amniotic membrane has been regarded as potential therapeutic-agents for cardiac regeneration after myocardial ischemia (Roy et al. 2016, Ntege et al. 2020, Fisher et al. 2016). The proposed mechanism of action is based on paracrine and immunomodulatory effects that are able to reduce the cardiac dysfunction after coronary occlusion by promoting various ventricular benefits in the heart (Machado-Júnior et al 2020, Blume et al. 2021). Previous studies have suggested that the benefits observed in global cardiac function would be caused in consequence

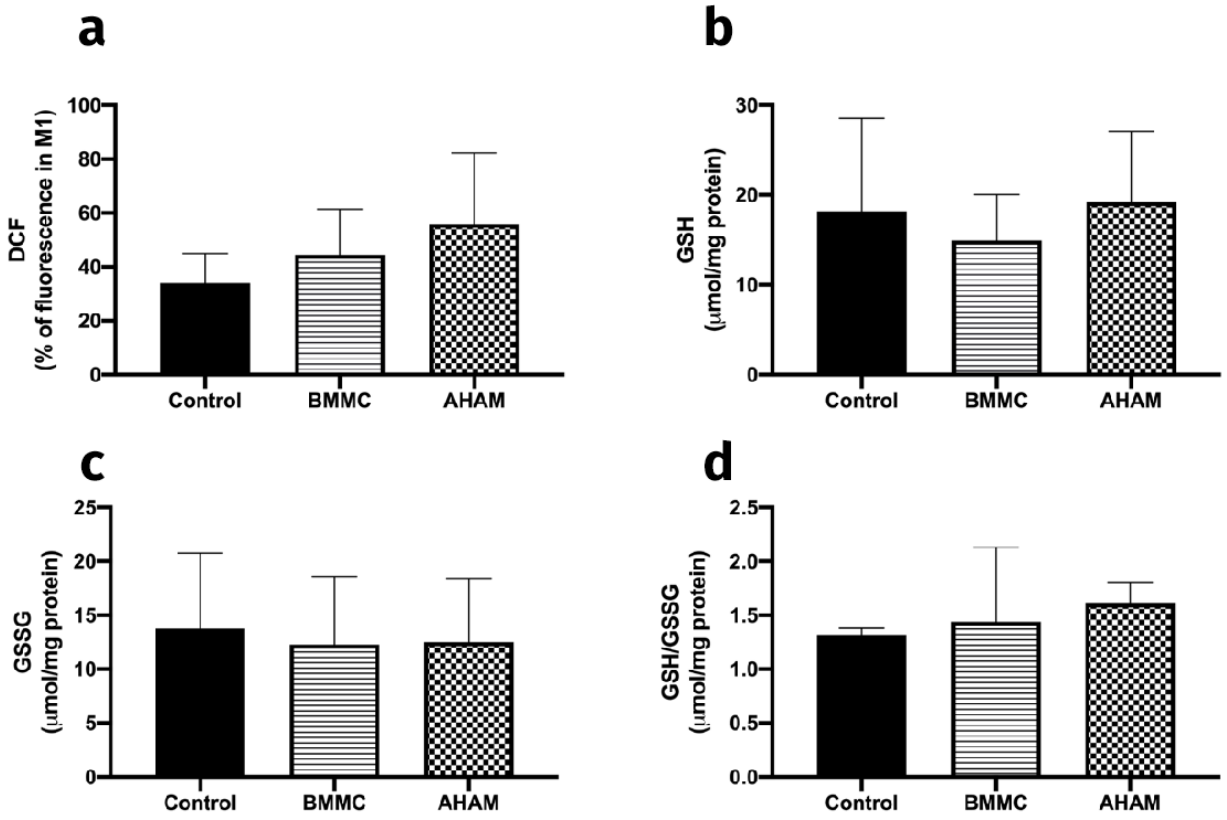

Figure 3. Effects of BMMC and AHAM on oxidative stress parameters after 30 days of myocardial infarction. The values of DCF (a), total GSH (b), GSSG (c) and GSH/GSSG ratio (d) are presented as mean +/- standard deviation. p<0,05 denoted statistical significance. Sample size: control = 8; BMMC = 11; AHAM = 8. BMMC (bone marrow mononuclear cells); AHAM (acellular human amniotic membrane).

of the reduction of the inflammatory parameters in the myocardium (Guo et al. 2007); despite this, the exact mechanism underlying this effect is still not completely elucidated (Khorramirouz et al. 2019, Danieli et al. 2015).

The pathogenesis of AMI involves several complex mechanisms that ultimately lead to a sudden interruption of blood flow to a part of the heart, leading to damage or death of heart muscle cells. The subsequent death of cardiomyocytes after AMI release to the extracellular space a group of substances which include HMGB1, Heat shock proteins, and DNA fragments, able to trigger immunological reactions caused by activation of the pattern recognition receptors (PRRs) (Jin et al. 2014, Bianchi 2007). These receptors are found in the cell membranes of macrophages and dendritic cells or in the cytoplasm, causing a process of sterile inflammation in the heart (Saparov et al. 2017).

The NLRP3 complex is a cytosolic PRR, and its activation involves two signals (Schroder & Tschopp 2010). The first signal (priming) is mediated through the stimulation of toll-like receptors and activation of the NF-kβ signaling pathway, which provides the transcriptional expression of pro-IL-18, pro-IL-1β, and NALP3. The second signal, dependent on DAMPs stimulus, PAMPs and substances such as ROS, extracellular ATP, and potassium efflux (Kelley et al. 2019, Mariathasan et al. 2006), trigger the oligomerization of the complex (modulated by ASC), activate the enzyme Caspase-1 and

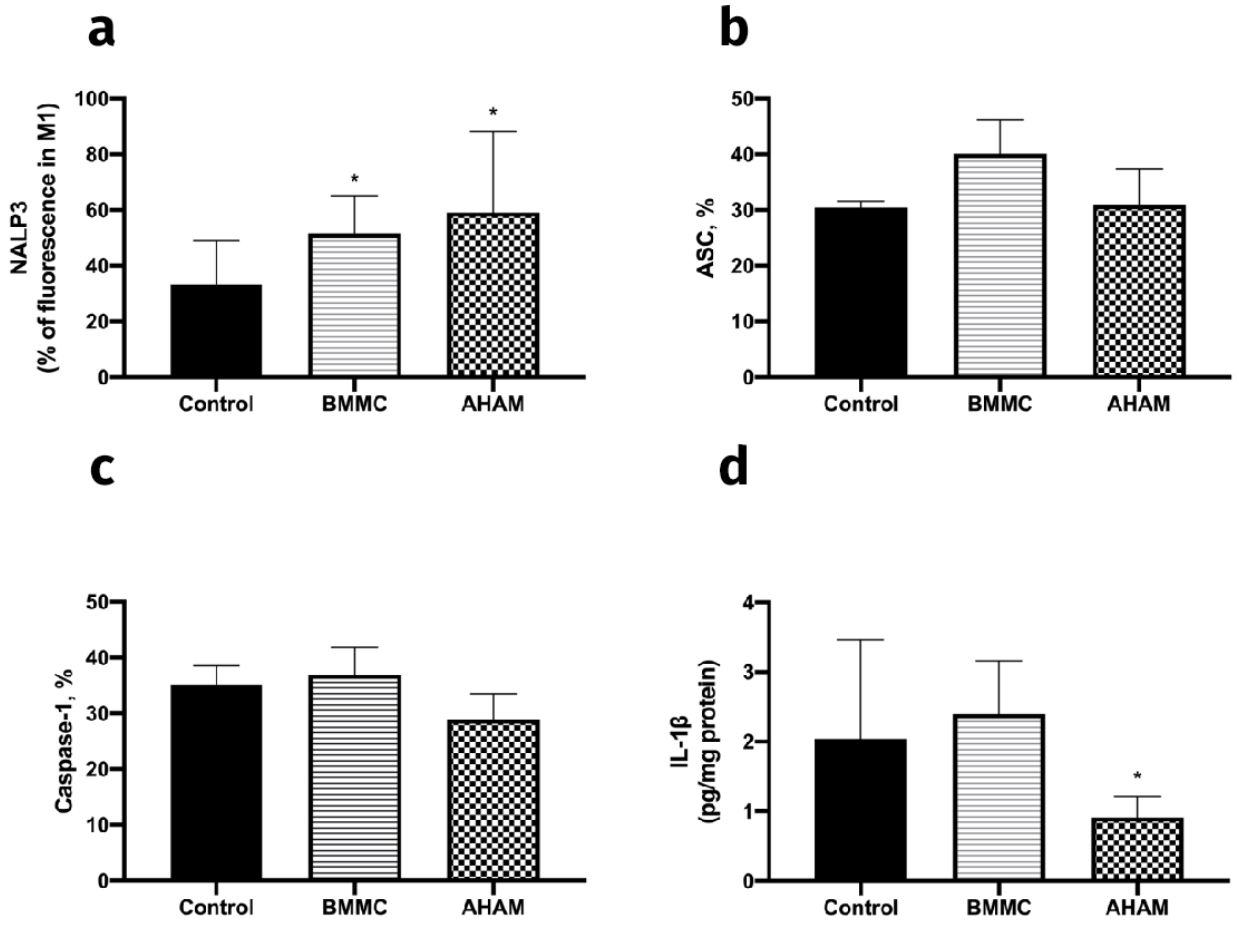

Figure 4. Effects of BMMC and AHAM on the NLRP3 inflammasome activity 30 days after myocardial infarction. The values of NALP3 (a), ASC (b), Caspase-1 (c) and interleukin-1β (d) are presented as mean +/- standard deviation. p<0,05 denoted statistical significance. Sample size for ASC, Caspase-1, NALP3 and interleukin- 1β: control = 8; BMMC = 11; AHAM = 8.

causes cleavage and release of Interleukin-1ß and Interleukin-18, leading to gasdermin-D-mediated cell death, in a process known as pyroptosis (He et al. 2016, Thornberry et al. 1992).

The activation of the NLRP3 inflammasome has been shown to contribute to the pathogenesis of AMI by promoting inflammation, oxidative stress, and cell death in the heart tissue (Liao et al. 2022). There is growing interest in the development of therapeutics that target the NLRP3 inflammasome as a potential treatment for MI. Several small molecule inhibitors of the NLRP3 inflammasome are currently being tested in preclinical and clinical trials for the treatment of MI and other inflammatory diseases (Takahashi 2014, Van Der Heijden et al. 2017, Silvis et al. 2020). The agents most commonly used as NLRP3 inhibitors are Canakinumab (a monoclonal antibody targeting interleukin B), Anakinra (a recombinant interleukin-1 receptor antagonist), colchicine, OLT1177, IZD344, MCC-950, and muscone (Toldo et al. 2019, Van Hout et al. 2017, Zahid et al. 2019, Coll et al. 2019, Silvis et al. 2022, Ismailani et al. 2022, Buckley & Libby 2019).

Du et al. (2018) showed that sequential doses of muscone after permanent coronary occlusion in rats were capable of inhibiting the

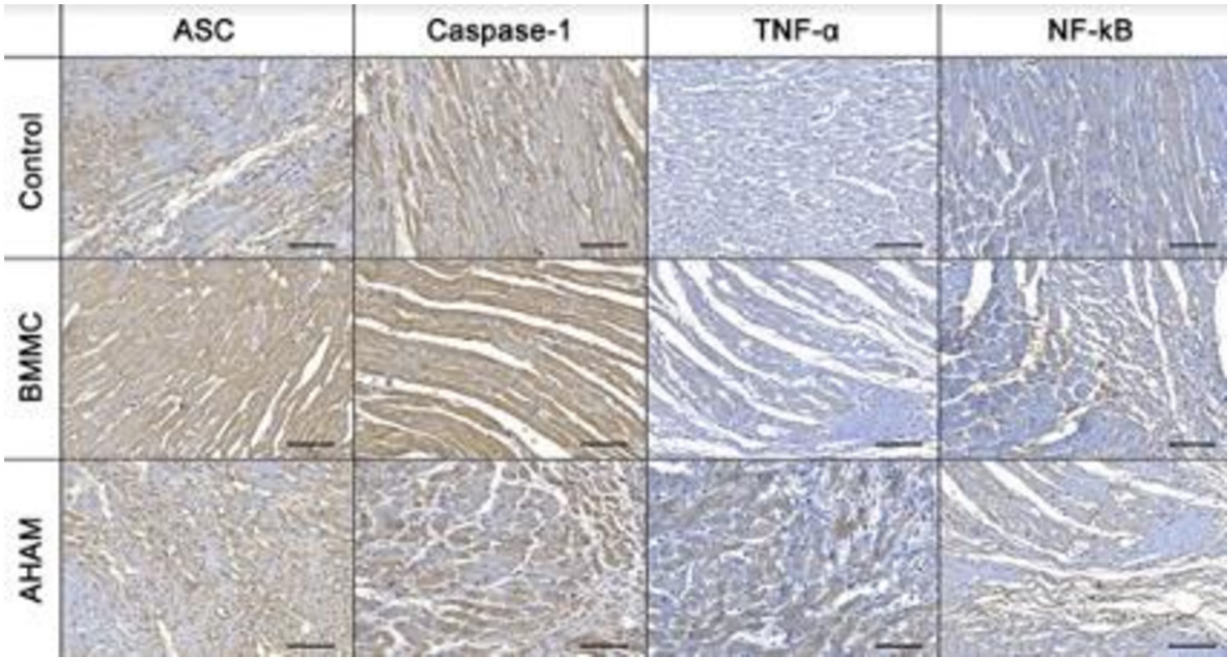

Figure 5. Representative images of histological analysis (cross sections) of the hearts of animals subjected to myocardial infarction. BMMC (bone marrow mononuclear cells); AHAM (acellular human amniotic membrane). Acquisition of images in 10x objective.

NLRP3 inflammasome activity and decreasing the expression of NF-kβ, with improvements in cardiac function after four weeks. In a similar analysis, Fujisue et al. (2017) demonstrated that oral administration of colchicine for five days resulted in attenuation of the left ventricular remodeling and reduced inflammatory response and NLRP3 activity after AMI.

In the present study, we showed that both BMMC and AHAM have upregulated the levels of NALP3 enzyme 30 days after myocardial infarction (figure 4A), and no modulation of ASC or Caspase was observed in comparison to the control group. These elevated levels of NALP3 are consistent with the high levels of NF-kß observed in both BMMC and AHAM groups (figure 2B), once the activation of the NF-kß pathway is required for NALP3 transcription (He et al. 2016). Despite this, the levels of IL-1β were similar in both control and BMMC groups, indicating no modulation of these therapies over the NLRP3 enzymes after thirty days. Only the AHAM group showed significantly lower levels of IL-1β (figure 4D), suggesting that the inhibition IL-1β might be a possible mechanism by which the human amniotic membrane modulates ventricular remodeling after myocardial ischemia.

Despite these observations, the cell redox-cell state was not different among the three groups, reinforcing the hypothesis that cell-therapy with BMMC or AHAM does not provide modulation of the NLRP3 enzymes, considering that increased ROS behaves like the second signal for the NLRP3 oligomerization (Toldo & Abbate 2018, Liu et al. 2014). The lack of association between the analyzed therapies and the NLRP3 inflammasome in the context of chronic myocardial ischemia suggests that either the immunomodulatory action of the BMMC and AHAM occurs by other mechanisms not including NLRP3 inflammasome inhibition or the activity of the NLRP3 complex was not suppressed in a long-term analysis such as 30 days after AMI.

This potential time-related association may also justify the similarity of the redox-cell state between the three groups. This is supported by an important randomized clinical trial, the BOOST-trial, on which 60 patients with a recent history of myocardial infarction were randomized 1:1 to receive bone-marrow cells or placebo, and the results after 18 months suggested that the beneficial effects of the cells in cardiac function would be only transient (Meyer et al. 2006). This finding may be related to a low percentage of viable cells after delivery into the myocardium due to cardiac contraction, with a consequent decrease in cardiac function after months (Mäkelä et al. 2009, Sheng et al. 2013); this scarcity of viable cells on the delivery site may justify the similarity observed on between the groups, suggesting that the possible immunomodulatory action of both BMMC and AHAM may not be occurring after 30 days of AMI.

In the present study, we opted for the direct myocardial injection as an alternative to decrease this loss of viable cells on the delivery site. However, other studies had shown that this approach was associated with a transient inflammatory response on the myocardium (Suzuki et al. 2004), which can explain the elevated levels NF-kβ observed in both BMMC and AHAM groups. On the other hand, these levels may also be related to the physiological process of myocardial repair after AMI, considering that previous data indicated that certain levels of the inflammatory response are necessary for myocardial healing (Yan et al. 2019), as well as for the process of bone marrow cell activation after delivery into the myocardium, with consequent benefits in cardiac function and ventricular remodeling (Yan et al. 2019, Vagnozzi et al. 2020).

Despite the absence of inhibition of the NLRP3 inflammasome or the inflammatory response, the echocardiographic assessment in our study showed that cell therapy with either BMMC or AHAM could improve cardiac function during the 30 days follow-up, compared to baseline results. The LV ejection fraction improved about 16% in the BMMC group ($p<0.001$) and almost 20% in the AHAM group ($p=0.001$), without difference when these two groups were compared to each other (figure 1). Also, the LV remodeling, measured by LVESV and LVEDV, was improved in both BMMC and AHAM groups, without significant changes in cardiac function and LV remodeling in the control group. Similar benefits in global cardiac function were also observed in other experimental studies (Henry et al. 2020, Roy et al. 2015, Gorjipour et al. 2019).

To our knowledge, this is the first study to evaluate the possible relationship between the bone marrow cells, the human amniotic membrane, and the NLRP3 inflammasome complex in the context of chronic myocardial ischemia. The recovery of the global heart function in a model of severe ventricular dysfunction observed in our study suggests that both bone marrow cells and human amniotic membrane could be valuable therapeutic approaches for the treatment of heart failure. However, absence of modulation of the inflammatory response and the NLRP3 inflammasome enzymes after 30 days suggest that the immunomodulatory action over the NLRP3 inflammasome may not be sustained in a long period of time, or that the mechanism of action of both BMMC and AHAM may be independent of the NLRP3 inflammasome inhibition.

Despite this, some considerable limitations should be expressed. Beyond the limitations of the rat model, the analysis after myocardial ischemia without reperfusion differs from previous studies that proposed to evaluate the inhibition of the NLPR3 Inflammasome as a therapeutic target after AMI. Most of the studies aimed to assess the short-terms effects of the

NLRP3 inhibition, usually during hours or few days after myocardial infarction (Marchetti et al. 2015, Van Hout et al. 2017, Toldo & Abbate 2018); therefore, we could not predict the short-term consequences of BMMC and AHAM over the NLRP3 complex. Also, treatment with either BMMC or AHAM occurred only once during the period of evaluation in animals with severe ventricular disfunction; a single dose of these agents may be not sufficient to inhibit the NLRP3 Inflammasome after 30 days, although could improve cardiac function and ameliorating LV-remodeling. Future studies should explore the benefits of BMMC and AHAM on NLRP3 complex inhibition in a shorter period.

CONCLUSIONS

Despite the improvements observed in cardiac function and the reduction of pathological ventricular remodeling, treatment with bone-marrow mononuclear cells or human amniotic membrane was not able to suppress the inflammatory response nor the NLRP3 inflammasome activity 30 days after myocardial infarction.

REFERENCES

ABDELWAHID E, KALVELYTE A, STULPINAS A, DE CARVALHO KAT, GUARITA-SOUZA LC & FOLDES G. 2016. Stem cell death and survival in heart regeneration and repair. Apoptosis: doi 10.1007/s10495-015-1203-4.

ANZAI T. 2013. Post-infarction inflammation and left ventricular remodeling - A double-edged sword. Circ J: doi 10.1253/circj.CJ-13-0013.

BIANCHI ME. 2007. DAMPs, PAMPs and alarmins: all we need to know about danger. J Leukoc Biol: doi 10.1189/jlb.0306164.

BLUME GG, MACHADO-JÚNIOR PAB, PALUDO BERTINATO G, SIMEONI RB, FRANCISCO JC & GUARITA-SOUZA LC. 2021. Tissue-engineered amniotic membrane in the treatment of myocardial infarction: a systematic review of experimental studies. Am J Cardiovasc Dis 11(1): 1-11.

BOYUM A. 1968. Isolation of mononuclear cells and granulocytes from human blood. Isolation of monuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest Suppl.: 77-89.

BRACEY NA ET AL. 2013. The Nlrp3 inflammasome promotes myocardial dysfunction in structural cardiomyopathy through interleukin-1β. Exp Physiol 98(2): 462-472. doi: 10.1113/expphysiol.2012.068338.

BRIAUD SA, DING ZM, MICHAEL LH, ENTMAN ML, DANIEL S & BALLANTYNE CM. 2001. Leukocyte trafficking and myocardial reperfusion injury in ICAM-1/P-selectin-knockout mice. Am J Physiol Heart Circ Physiol 280(1): H60-7. doi: 10.1152/ajpheart.2001.280.1.H60.

BUCKLEY LF & LIBBY P. 2019. Inhibiting NLRP3 Inflammasome Activity in Acute Myocardial Infarction: A Review of Pharmacologic Agents and Clinical Outcomes. J Cardiovasc Pharmacol 74(4): 297-305. doi:10.1097/FJC.0000000000000701.

BUTTS B, GARY RA, DUNBAR SB & BUTLER J. 2015. The Importance of NLRP3 Inflammasome in Heart Failure. J Card Fail 21(7): 586-593. doi:10.1016/j.cardfail.2015.04.014.

CARVALHO KATC ET AL. 2008. Immunophenotypic Expression by Flow Cytometric Analysis of Cocultured Skeletal Muscle and Bone Marrow Mesenchymal Stem Cells for Therapy Into Myocardium. Transplant Proc 40(3): 842-844. doi:10.1016/j.transproceed.2008.02.053.

CHEN W & FRANGOGIANNIS NG. 2013. Fibroblasts in post-infarction inflammation and cardiac repair. Biochim Biophys Acta 1833(4): 945-953. doi:10.1016/j.bbamcr.2012.08.023.

COLL RC ET AL. 2019. MCC950 directly targets the NLRP3 ATP-hydrolysis motif for inflammasome inhibition. Nat Chem Biol 15: 556-559. doi: 10.1038/s41589-019-0277-7.

DANIELI P ET AL. 2015. Conditioned Medium From Human Amniotic Mesenchymal Stromal Cells Limits Infarct Size and Enhances Angiogenesis. Stem Cells Transl Med 4(5): 448-458. doi:10.5966/sctm.2014-0253.

DU Y ET AL. 2018. Muscone improves cardiac function in mice after myocardial infarction by alleviating cardiac macrophage-mediated chronic inflammation through inhibition of NF-κB and NLRP3 inflammasome. Am J Transl Res 10(12): 4235-4246.

DZIEDZIC DSM ET AL. 2021. Combined Biomaterials: Amniotic Membrane and Adipose Tissue to Restore Injured Bone as Promoter of Calcification in Bone Regeneration: Preclinical Model. Calcif Tissue Int 108(5): 667-679. doi:10.1007/s00223-020-00793-1.

FISHER SA, DOREE C, MATHUR A, TAGGART DP & MARTIN-RENDON E. 2016. Stem cell therapy for chronic ischaemic heart disease and congestive heart failure. Cochrane Database Syst Rev: CD007888-CD007888. doi:10.1002/14651858.CD007888.pub3.

FRANCISCO JC ET AL. 2016. Decellularized Amniotic Membrane Scaffold as a Pericardial Substitute: An In Vivo Study. Transplant Proc 48(8): 2845-2849. doi:10.1016/j.transproceed.2016.07.026.

FRANGOGIANNIS NG. 2014. The inflammatory response in myocardial injury, repair, and remodelling. Nat Rev Cardiol 11(5): 255-265. doi:10.1038/nrcardio.2014.28.

FONSECA FA & IZAR MC. 2022. Role of Inflammation in Cardiac Remodeling After Acute Myocardial Infarction. Front Physiol 13: 927163. doi: 10.3389/fphys.2022.927163.

FUJISUE K ET AL. 2017. Colchicine improves survival, left ventricular remodeling, and chronic cardiac function after acute myocardial infarction. Circ J 81(8): 1174-1182. doi:10.1253/circj.CJ-16-0949.

GORJIPOUR F ET AL. 2019. Mesenchymal stem cells from human amniotic membrane differentiate into cardiomyocytes and endothelial-like cells without improving cardiac function after surgical administration in rat model of chronic heart failure. J Cardiovasc Thorac Res 11(1): 35-42. doi:10.15171/jcvtr.2019.06.

GUO J, LIN GS, BAO CY, HU ZM & HU MY. 2007. Anti-inflammation role for mesenchymal stem cells transplantation in myocardial infarction. Inflammation 30(3-4): 97-104. doi:10.1007/s10753-007-9025-9033.

HE Y, HARA H & NÚÑEZ G. 2016. Mechanism and Regulation of NLRP3 Inflammasome Activation. Trends Biochem Sci 41(12): 1012-1021. doi: 10.1016/j.tibs.2016.09.002.

HENRY JJD ET AL. 2020. Development of Injectable Amniotic Membrane Matrix for Postmyocardial Infarction Tissue Repair. Adv Healthc Mater 9(2): e1900544. doi:10.1002/adhm.201900544.

ISMAILANI US, BUCHLER A, MACMULLIN N, ABDIRAHMAN F, ADI M & ROTSTEIN BH. 2023. Synthesis and Evaluation of [^{11}C] MCC950 for Imaging NLRP3-Mediated Inflammation in Atherosclerosis. Mol Pharm. 20(3):1709-1716. doi: 10.1021/acs.molpharmaceut.2c00915.

JIN C ET AL. 2014. Human myocardium releases heat shock protein 27 (HSP27) after global ischemia: The proinflammatory effect of extracellular HSP27 through toll-like receptor (TLR)-2 and TLR4. Mol Med 20(1): 280-289. doi:10.2119/molmed.2014.00058.

KELLEY N, JELTEMA D, DUAN Y & HE Y. 2019. The NLRP3 inflammasome: An overview of mechanisms of activation and regulation. Int J Mol Sci 20(13): doi10.3390/ijms20133328.

KHORRAMIROUZ R, KAMELI SM, FENDERESKI K, DARYABARI SS & KAJBAFZADEH AM. 2019. Evaluating the efficacy of tissue-engineered human amniotic membrane in the treatment of myocardial infarction. Regen Med 14(2): 113-126. doi:10.2217/rme-2018-0024.

LIAO Y, LIU K & ZHU L. 2022. Emerging Roles of Inflammasomes in Cardiovascular Diseases. Front Immunol 13: 834289. doi: 10.3389/fimmu.2022.834289.

LIU Y ET AL. 2014. TXNIP mediates NLRP3 inflammasome activation in cardiac microvascular endothelial cells as a novel mechanism in myocardial ischemia/reperfusion injury. Basic Res Cardiol: doi 10.1007/s00395-014-0415-z.

MACHADO-JÚNIOR PAB, BLUME GG, FRANCISCO JC & GUARITA-SOUZA LC. 2020. Cell-Based Therapies for Myocardial Regeneration in Heart Failure: 20 Years of Debate. Brazilian J Cardiovasc Surg 35(4): VIII-XI. doi:10.21470/1678-9741-2020-0362.

MÄKELÄ J ET AL. 2009. Acute homing of bone marrow-derived mononuclear cells in intramyocardial vs. intracoronary transplantation. Scand Cardiovasc J 43(6): 366-373. doi:10.1080/14017430903045350.

MARCHETTI C ET AL. 2015. Pharmacologic Inhibition of the NLRP3 Inflammasome Preserves Cardiac Function after Ischemic and Nonischemic Injury in the Mouse. J Cardiovasc Pharmacol 66(1): 1-8. doi:10.1097/FJC.0000000000000247.

MARIATHASAN S, WEISS DS, NEWTON K, MCBRIDE J, O'ROURKE K, ROOSE-GIRMA M, LEE WP, WEINRAUCH Y, MONACK DM & DIXIT VM. 2006. Cryopyrin activates the inflammasome in response to toxins and ATP. Nature 440: 228-232. doi: 10.1038/nature04515.

MARTINON F, BURNS K & TSCHOPP J. 2002. The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta. Mol Cell 10(2): 417-426. doi: 10.1016/s1097-2765(02)00599-3.

MASTROCOLA R ET AL. 2016. Pharmacological inhibition of NLRP3 inflammasome attenuates myocardial ischemia/reperfusion injury by activation of RISK and mitochondrial pathways. Oxid Med Cell Longev: doi 10.1155/2016/5271251.

MEYER GP ET AL. 2006. Intracoronary Bone Marrow Cell Transfer After Myocardial Infarction. Circulation 113(10): 1287-1294. doi:10.1161/CIRCULATIONAHA.105.575118.

NTEGE EH, SUNAMI H & SHIMIZU Y. 2020. Advances in regenerative therapy: A review of the literature and

future directions. Regen Ther 14: 136-153. doi:10.1016/j.reth.2020.01.004.

RAHMAN I, KODE A & BISWAS SK. 2007. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nat Protoc 1(6): 3159-3165. doi:10.1038/nprot.2006.378.

ROY R ET AL. 2016. Decellularized amniotic membrane attenuates postinfarct left ventricular remodeling. J Surg Res 200(2): 409-419. doi:10.1016/j.jss.2015.08.022.

ROY R ET AL. 2015. Epithelial-to-mesenchymal transition enhances the cardioprotective capacity of human amniotic epithelial cells. Cell Transplant 24(6): 985-1002. doi:10.3727/096368913X675151.

RÜHL S & BROZ P. 2015. Caspase-11 activates a canonical NLRP3 inflammasome by promoting K(+) efflux. Eur J Immunol 45(10): 2927-2936. doi: 10.1002/eji.201545772.

SAPAROV A ET AL. 2017. Role of the immune system in cardiac tissue damage and repair following myocardial infarction. Inflamm Res 66(9): 739-751. doi:10.1007/s00011-017-1060-4.

SCHRODER K & TSCHOPP J. 2010. The Inflammasomes. Cell 140(6): 821-832. doi:10.1016/j.cell.2010.01.040.

SHENG CC, ZHOU L & HAO J. 2013. Current stem cell delivery methods for myocardial repair. Biomed Res Int: doi 10.1155/2013/547902.

SHIRAZI LF, BISSETT J, ROMEO F & MEHTA JL. 2017. Role of Inflammation in Heart Failure. Curr Atheroscler Rep 19(6): doi 10.1007/s11883-017-0660-3.

SILVIS MJM ET AL. 2020. Immunomodulation of the NLRP3 Inflammasome in Atherosclerosis, Coronary Artery Disease, and Acute Myocardial Infarction. J Cardiovasc Transl Res 1-12: doi 10.1007/s12265-020-10049-w.

SILVIS MJM, DEMKES EJ, TIMMERS L, ARSLAN F, DE JAGER SCA, SLUIJTER JPG, MOSTERD A, DE KLEIJN DPV, BOSCH L & VAN HOUT GPJ. 2022. NLRP3-Inflammasome Inhibition with IZD334 Does Not Reduce Cardiac Damage in a Pig Model of Myocardial Infarction. Biomedicines 10(12): 3056. doi: 10.3390/biomedicines10123056.

SUZUKI K ET AL. 2004. Role of interleukin-1β in acute inflammation and graft death after cell transplantation to the heart. Circulation 110(11 Suppl.): doi10.1161/01.CIR.0000138388.55416.06.

TAKAHASHI M. 2014. NLRP3 inflammasome as a novel player in myocardial infarction. Int Heart J 55(2): 101-105. doi:10.1536/ihj.13-388.

TAKEJIMA AL ET AL. 2021. Role of mononuclear stem cells and decellularized amniotic membrane in the treatment of skin wounds in rats. Tissue Barriers 10(2): doi 10.1080/21688370.2021.1982364.

TANAI E & FRANTZ S. 2016. Pathophysiology of heart failure. Compr Physiol 6(1): 187-214. doi:10.1002/cphy.c140055.

TANIGUCHI S & SAGARA J. 2007. Regulatory molecules involved in inflammasome formation with special reference to a key mediator protein, ASC. Semin Immunopathol 29(3): 231-238. doi: 10.1007/s00281-007-0082-3.

THORNBERRY NA ET AL. 1992. A novel heterodimeric cysteine protease is required for interleukin-1 beta processing in monocytes. Nature 356(6372): 768-774. doi: 10.1038/356768a0.

TOLDO S & ABBATE A. 2018. The NLRP3 inflammasome in acute myocardial infarction. Nat Rev Cardiol 15(4): 203-214. doi:10.1038/nrcardio.2017.161.

TOLDO S ET AL. 2019. The NLRP3 Inflammasome Inhibitor, OLT1177 (Dapansutrile), Reduces Infarct Size and Preserves Contractile Function after Ischemia Reperfusion Injury in the Mouse. J Cardiovasc Pharmacol 73(4): 215-222. doi:10.1097/FJC.0000000000000658.

VAGNOZZI RJ ET AL. 2020. An acute immune response underlies the benefit of cardiac stem cell therapy. Nature 577(7790): 405-409. doi:10.1038/s41586-019-1802-2.

VAN DER HEIJDEN T ET AL. 2017. NLRP3 Inflammasome Inhibition by MCC950 Reduces Atherosclerotic Lesion Development in Apolipoprotein E-Deficient Mice - Brief Report. Arterioscler Thromb Vasc Biol 37(8): 1457-1461. doi:10.1161/ATVBAHA.117.309575.

VAN HOUT GPJ ET AL. 2017. The selective NLRP3-inflammasome inhibitor MCC950 reduces infarct size and preserves cardiac function in a pig model of myocardial infarction. Eur Heart J 38(11): 828-836. doi:10.1093/eurheartj/ehw247.

WALEV I, KLEIN J, HUSMANN M, VALEVA A, STRAUCH S, WIRTZ H, WEICHEL O & BHAKDI S. 2000. Potassium Regulates IL-1β Processing Via Calcium-Independent Phospholipase A2. J Immunol 164: 5120-5124. doi: 10.4049/jimmunol.164.10.5120.

YAN W, ABU-EL-RUB E, SARAVANAN S, KIRSHENBAUM LA, ARORA RC & DHINGRA S. 2019. Inflammation in myocardial injury: Mesenchymal stem cells as potential immunomodulators. Am J Physiol - Hear Circ Physiol 317(2): H213-H225. doi:10.1152/ajpheart.00065.2019.

ZAHID A, LI B, KOMBE AJK, JIN T & TAO J. 2019. Pharmacological inhibitors of the nlrp3 inflammasome. Front Immunol 10(OCT). doi:10.3389/fimmu.2019.02538.

ZHENG Y, XU L, DONG N & LI F. 2022. NLRP3 inflammasome: The rising star in cardiovascular diseases. Front Cardiovasc Med. 9:927061. doi: 10.3389/fcvm.2022.927061.

How to cite

TAKEJIMA AL ET AL. 2024. Bone-marrow mononuclear cells and acellular human amniotic membrane improve global cardiac function without inhibition of the NLRP3 Inflammasome in a rat model of heart failure. An Acad Bras Cienc 96: e20230053. DOI 10.1590/0001-3765202420230053.

Manuscript received on January 18, 2023; accepted for publication on April 19, 2023

ALINE L. TAKEJIMA[1]
https://orcid.org/0000-0002-2608-0171

PAULO ANDRÉ B. MACHADO-JÚNIOR[1]
https://orcid.org/0000-0003-1577-9195

GUSTAVO G. BLUME[1]
https://orcid.org/0000-0002-2936-8541

ROSSANA BAGGIO SIMEONI[1]
https://orcid.org/0000-0002-9976-5809

JULIO CESAR FRANCISCO[1]
https://orcid.org/0000-0003-1970-6399

MURILO S. TONIAL[1]
https://orcid.org/0000-0001-6302-7014

LUIS FELIPE B. MARQUEZE[2]
https://orcid.org/0000-0002-4738-1440

LUCIA NORONHA[1]
https://orcid.org/0000-0003-0310-7164

MARCIA OLANDOSKI[1]
https://orcid.org/0000-0002-0979-3604

ELTYEB ABDELWAHID[3]
https://orcid.org/0000-0002-2964-6927

KATHERINE A.T. DE CARVALHO[4]
https://orcid.org/0000-0003-4860-2855

RICARDO A. PINHO[2]
https://orcid.org/0000-0003-3116-4553

LUIZ CÉSAR GUARITA-SOUZA[1]
https://orcid.org/0000-0003-2781-9705

[1]Pontifícia Universidade Católica do Paraná (PUCPR), Experimental Laboratory of Institute of Biological and Health Sciences, 1555 Imaculada Conceição Street, 80215-901 Curitiba, PR, Brazil

[2]Pontifícia Universidade Católica do Paraná (PUCPR), Laboratory of Exercise Biochemistry in Health, School of Medicine, 1555 Imaculada Conceição Street, Prado Velho, 80215-901 Curitiba, PR, Brazil

[3]Northwestern University, Feinberg School of Medicine, Feinberg Cardiovascular Research Institute, 303 E. Chicago Ave., Tarry 14-725, 60611 Chicago, IL, USA

[4]The Pelé Pequeno Príncipe Institute, Cell Therapy and Biotechnology in Regenerative Medicine Department, Child and Adolescent Health Research & Pequeno Príncipe Faculties, 1632 Silva Jardim Ave., Água Verde, 80240-020 Curitiba, PR, Brazil

Correspondence to: **Aline Luri Takejima**
E-mail: alinetakejima@yahoo.com.br

Author Contributions

MACHADO-JÚNIOR, SIMEONI, BLUME, TONIAL, MARQUEZE, NORONHA, OLANDOSKI: acquisition, analysis and interpretation of data; TAKEJIMA, ABDELWAHID, CARVALHO, FRANCISCO, PINHO, GUARITA-SOUZA: drafting and revising the manuscript; final approval of the version to be published.