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Positive effects of acellular aortic scaffold on antioxidant activities and cellular homeostasis: an experimental study on cardiomyocytes of infarcted mice

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Abstract

Myocardial infarction (MI) has increased in the recent years due to aging as well as lifestyle changes in the population. In the last decades, regenerative medicine has been considered to provide modern and efficient methods for MI patients' therapies. The current study was aimed at investigating the effect of acellular aortic scaffold (AAS) on MI cardiomyocytes to identify the therapeutic potential of this natural biomaterial for MI patients. The study was conducted in five main steps: Preparing MI animal model, preparing MI cells, culture of animal MI cell on AAS, assessment of cell viability (MTT assay) as well as antioxidant and catalytic activities (ROS, TAC, SOD, GPX, and CAT assays) and molecular study on apoptotic and anti-apoptotic factors (qRT-PCR). AAS positively affected the viability rate of MI cells and the GPX and SOD levels were significantly increased in MI cells due to the culture on AAS. The RT-PCR quantification showed a decrease in the levels of of *Cox8* and *Caspase3* expression genes levels in MI cells, cultured on AAS, while an increase in miR-24 expression level was observed in MI cells as a result of culture on AAS. We concluded that acellular aortic scaffold can positively control the catalytic and antioxidant activities, cellular hemostasis, and cell viability of cardiomyocytes after myocardial infarction, demonstrating the potential of such natural biomaterials for cardiac tissue reconstruction. However, to achieve favorable results and ideal therapeutic applications, further studies are required.

Key words: Myocardial infarction, Regenerative medicine, Acellular aortic scaffold, Antioxidant Activity

Introduction

As one of the main health problems, heart attack still leads to morbidity and mortality worldwide (Jawad et al, 2007; Khan and McCann, 2017; Plakht et al, 2015; Roth et al, 2015). A heart attack, generally known as a myocardial infarction (MI), leads to the formation of scar tissue that cannot function as normal myocardium (Buja and Vela, 2008; Moghaddam et al, 2019). Due to the weaknesses in the present therapies for MI and despite the abundant pharmaceutical and medical device advances, the MI mortality rate is still high. Hence, recently, regenerative medicine has been considered to provide modern and efficient methods for the prevention and treatment of cardiac diseases (Moghaddam et al, 2019).

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In the recent decades, cardiac tissue engineering has been proposed to repair or regenerate damaged cardiac tissue, using a combination of living cells and biomaterials. Cardiac tissue engineering involves performing 3D scaffolds, in the form of patch, foam, or mesh being cultured with either cardiac or non-cardiac cells (Arnal-Pastor et al. 2013). The biocompatibility and biodegradability as well as microenvironment adaptation make the biomaterials (natural and synthetic) as an optimal option to create scaffolds. Some natural (such as chitosan, collagen, alginate, fibrin, and hyaluronic acid) (Geng et al, 2018) and synthetic (such as polyester, polylactic acid, poly caprolactone, and polypropylene) (Fujimoto et al, 2009; Mukherjee et al, 2011; Wang et al, 2009) polymers have been investigated as scaffolds for cardiac regeneration. The excellent strength and durability, better uniformity, and also lower risk of infection, are advantages of synthetic materials compared with natural biomaterials. However, toxicity and biocompatibility, as the main concerns, limit the application of synthetic materials to create scaffolds (Geng et al, 2018); And to address these limitations, cardiac regenerative medicine has certainly moved towards finding the ideal natural biomaterials (Reis et al. 2016).

The natural acellular extracellular matrix for cardiac regeneration has been considered in the material sciences (Badylak, 2007; Ravi et al, 2012). In general, decellularization is a process in which the cellular and nuclear components of tissue are completely removed while the extracellular matrix (ECM) is preserved because of its native components and structure (Pellegata et al, 2013). Acellular scaffolds represent well-known biochemical and biomechanical properties (Badylak, 2007) and are able to promote the adhesion, differentiation, and proliferation of cells. Therefore, they have been used for the replacement of various tissues with favorable clinical results (Barnes et al, 2011).

Decellularized extracellular matrix from various sources has been used for tissue engineering of heart valves and blood vessels (Beattie et al, 2009; Eitan et al, 2010). But, extracellular matrices isolated from different sources have different properties in terms of content and density, collagen type, and glycosaminoglycans composition. Therefore, the cardiac-derived extracellular matrix could be more effective for tissue engineering of infarcted hearts due to the similarity in molecular composition (Badylak et al, 2009). Herein, the effect of acellular aortic scaffold (AAS) on infarcted cardiomyocytes in comparison with healthy cardiomyocytes under the same conditions was studied. First, cardiomyocytes and aortic tissue were isolated from healthy mice. Then infarction and decellularization processes were performed for cardiomyocytes and aortic tissue, respectively. The infarcted cardiomyocytes were cultured on AAS as a three- dimensional system and efficiency of this scaffold on the cell viability, catalytic and antioxidant activities as well as gene expression of infarcted cardiomyocytes was investigated. The flowchart of study is present in Figure 1.

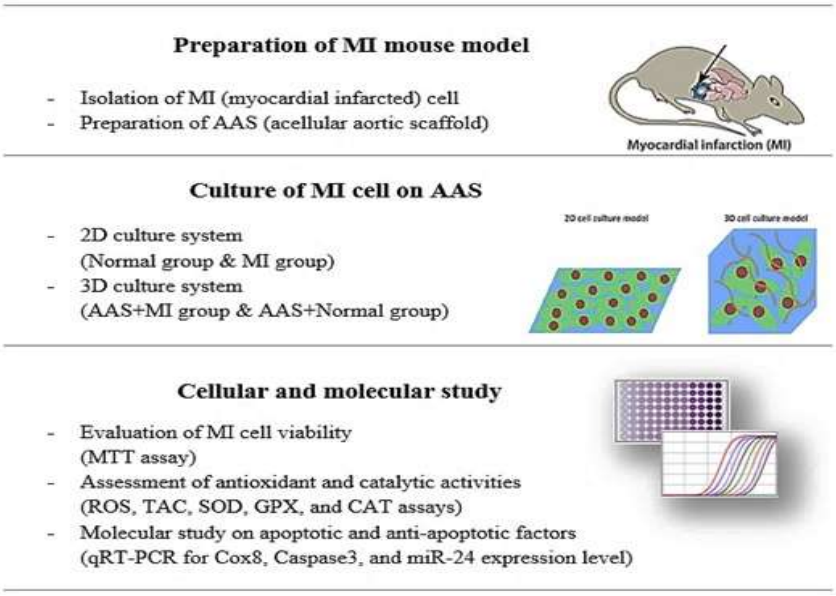

Figure 1: Flowchart of study

Materials and methods

Preparation of MI animal model

Animal model. Ten healthy adult male NMRI (Naval Medical Research Institute) mice, weighing 25-30 g, were purchased from Razi Institute (Karaj, Iran). The animals were housed at cages with a constant temperature (22±2°C) and humidity (55±5%), under a controlled 12 h light/dark cycle. The unlimited access to chow and water based on ad libitum standard laboratory diet (5% lipid, 25% protein, and 70% carbohydrate) was provided for animals. They were acclimated to laboratory conditions one week before the experiment. To create the MI model, animals were randomly divided into control and model groups. Five healthy mice were used as MI model by intraperitoneal injection of 85 mg/kg isoproterenol HCL (Sigma-Aldrich, USA) diluted with DPBS - Dulbecco's phosphate-buffered saline- (Sigma-Aldrich, USA). Five other mice were injected with DPBS at equivalent volume and considered as a control group. The injection procedure was repeated after 24 h for both MI and control groups. Following 24 h after the second injection (to ensure the model development), the mice hearts and thoracic aorta were removed under anesthesia (intraperitoneal injection of 80 mg/kg ketamine 10% and 5 mg/kg xylazine 2%) and used for further analyses (Forte et al, 2021).

Evaluation of cardiac indicators in serum. To confirm the induction of myocardial infarction in mice, 24 hours after the last injection of isoproterenol, the serum levels of Cardiac troponin-I (cTnI) and Creatine kinase-MB (CK-MB) were measured in both MI and control groups. Initially, peripheral whole blood (for each group) was collected into an EDTA tube and centrifuged (1000×g, 15 min) to obtain the serum. CK-MB was measured using CK-MB Commercial kits (Pars Azmoon,Tehran, Iran) and absorbance was measured at 450 nm by a colorimetric assay, using an automatic analyzer according to the manufacturer's instruction (the CK-MB results expressed in U/L). The cTnI levels in serum samples were determined by an Enzyme –linked immune-Sorbent assay (ELISA) kit by ELISA reader (BioTek, ELx 50, USA), according to the manufacturer's instruction (Shanghai Crystal Day Biotech CO., LTD, China) and its results reported as (ng/ml).

Ethics. All animal treatments and experimental procedures were carried out

based on the US NIH guidelines for the care and use of laboratory animals. The Ethical Committee of Science and Research Branch, Azad University (Karaj, Iran) approved this project with the ethical code: IR.IAU.K.REC.1399.052

Preparation of MI cells and acellular aortic scaffold

Infarcted and healthy cardiomyocytes. The hearts, dissected from MI and control groups, were perfused using ice-cold DPBS supplemented with $CaCl_2$ (0.9 mM) to rinse out the blood from heart chambers and vessels. For cardiomyocytes' isolation, the ventricle of hearts was harvested, finely minced, and suspended into 10 mL digestion media (DMEM -Dulbecco's Modified Eagle's Medium- supplemented with collagenase/dispase, trypsin and, DNase). The suspension was incubated for 40 min at 37°C, then centrifuged (1000×g, 20 min) at 4°C. The supernatant was removed and the cell pellet was suspended in 2 mL of fresh digestion media and incubated for 20 min at 37°C. The cells were diluted with 5 mL culture media containing DMEM, FBS -Fetal Bovine Serum- 10%, and penicillin/streptomycin 1%. Then, the cell suspension was centrifuged (1000×g, 20 min) at 4°C. The supernatant was removed and the cell pellet was suspended in 5 mL culture media (DMEM, FBS 10%, and penicillin/streptomycin 1%). T25 culture flasks were used for cell culture and 5 mL culture media containing the cells were plated into the flasks (5 mL medium per culture flask). Cells were cultured and attached to the flask during 2 h incubation under standard conditions (temperature 37°C, humidity 95%, and CO_2 5%). Then non-adherent cells were removed and washed by PBS. The medium was replaced every two or three days per week, and cardiomyocytes' cultures were typically confluent in 4–7 days. Finally, the proliferation and adhesion of cells were monitored by inverted light microscopy.

Acellular aortic scaffold. A fresh thoracic aorta was obtained from healthy male NMRI mice and washed twice with DPBS supplemented with amphotericin B and antibiotics. Then, preparation of the acellular scaffold was performed as follows: 18 h washing with deionized water at 4°C. After, washing with sodium deoxycholate 4% for 4 h at room temperature following placed in DNase-I (2000 kU) in NaCl (1M) for 3h at room temperature (Meezan, Hjelle et al, 1975). The whole process was carried out under agitation and between each step the aorta was rinsed 3 times in DPBS. The treatment was repeated twice to ensure that all cells were completely removed. The acellular scaffold was stored in DPBS at 4 °C until use (All reagents were purchased from Sigma-Aldrich, USA).

Culture of animal MI cell on the acellular aortic scaffold

To investigate the effect of an acellular aortic scaffold on infarcted cardiomyocytes, the culture of MI cells on the acellular aortic scaffold was studied. For this purpose, four main cultural groups were defined:

Normal group: culture of healthy cardiomyocytes in a two-dimensional (2D) culture system

MI group: culture of infarcted cardiomyocytes in the 2D culture system

AAS_+MI group: culture of infarcted cardiomyocytes on acellular aortic scaffold as a three-dimensional (3D) culture system

AAS_+Normal group: culture of healthy cardiomyocytes and acellular aortic scaffold as the 3D culture system

Before the cell seeding process in the AAS_+MI and AAS_+ Normal groups, acellular aortic scaffold samples were divided into 1×1 cm^2 pieces, then put into 24-well cell culture plates. All wells containing AAS were washed with DPBS twice and then incubated in DMEM medium under standard conditions (temperature 37°C, humidity 100%, and CO_2 5%) for 10 min. Cardiomyocytes were

seeded onto the luminal surface of AAS and kept in the DMEM media under standard conditions for 14 days. Cardiomyocytes were also seeded in the 2D culture system for the Normal and MI groups.

MTT assay for cell viability evaluation

For cardiomyocytes viability evaluation, MTT assay was performed on normal and MI cardiomyocytes cultured in 2D and 3D culture systems. First, cardiomyocytes obtained from each group (defined in the previous step) were seeded in 96-well plates at a density of 1×10^4 cells/well and incubated for 14 days. In the following, 5 mg/ml MTT solution (3-(4,5-dimethylthiazol- 2-yl) -2, 5-diphenyltetrazolium bromide) was added and plates were incubated for 4 h at 37 °C. After incubation, the contents of all wells were discarded and 200 µl DMSO was added to each well. Finally, optical absorbance of each well at 570 nm was recorded by ELISA reader and viability percent relative to the control values was calculated for all groups.

Assessment of antioxidant and catalytic activities

The ROS, TAC, SOD, GPX, and CAT biochemical tests were performed based on fluorometric assays to record the changes in antioxidant and catalytic activities due to the culture of MI cells on AAS.

ROS (Reactive Oxygen Species). The evaluation of ROS levels in cardiomyocytes (obtained from four defined groups) was performed using DCFH-DA -2′, 7′-dichlorofluorecin diacetate- as an oxidative stress indicator. First, the medium from each well was removed and washed with DMEM. Then 500 µL of DCFH-DA (10 µM) was added to each well and incubated for 30 min at 37°C. The fluorescence intensity of DCFH to fluorescent product DCF -2′,7′-dichlorofluorescein- was assayed by the fluorescent spectrophotometer DTX 880 detector (Beckman, USA). The wavelengths of 480 and 520 nm were considered as excitation and emission wavelengths, respectively. The data from each sample was analyzed, and protein content was evaluated. Then results were expressed in concentration based on a standard sample using H_2O_2 serial dilution.

TAC (Total Antioxidant Capacity): To assay the TAC level in cardiomyocytes, a commercial kit (ZellBio GmbH, Germany) was used according to the manufacturer's protocol. TAC method as the oxidation - reduction colorimetric assay was performed to measure the amount of antioxidant in each sample in comparison with ascorbic acid action as the standard. In brief, 20 µl of either samples or standards were added into their special wells and 20 µL of Assay Buffer was used as the zero standard. 20 µL of Ascorbic Acid Working Solution was added into wells as an optional control. Then 75 µL of FRAP Color Solution was added to each well and incubated at room temperature for 30 min. Finally, the optical density of each well was recorded at a wavelength of 560 nm.

CAT (Catalase activity): the activity level of the catalase enzyme was assayed according to the protocol of ZellBio Catalase Activity assay kit. This colorimetric assay evaluated the catalase activity for decomposition of H_2O_2 to H_2O and O_2 in one minute. Briefly, 100µL of R1 reagent then 10µL of R2 (containing H_2O_2 as substrate) reagent were added to each well, mixed, and incubated for 1 min at 37°C. 100µL of R3 reagent (containing chromogen) then 10µL R4 reagent (containing clearing agent) were added, mixed, and read the absorbance at the wavelength of 405 nm. To calculate the catalase activity for each sample, the following formula was used:

Catalase activity (U/ml) = $(OD_{blank}-OD_{sample})\times 271\times(\frac{1}{60}\times \text{Sample Volume})$

SOD (Superoxide Dismutase): A colorimetric method for evaluation of SOD activity was performed using the commercial ZellBio SOD assay kit

according to the manufacturer's recommendations. The SOD enzyme detoxifies O_2^- as a superoxide anion and converts it into two less damaging species, including O_2 and H_2O_2. First, 10 µl of either samples or standards were added into their special wells and 10 µL of Assay Buffer was used as the zero standard. Then 50 µL of Substrate Working Solution was added into wells. 25 µL of Xanthine Oxidase Solution was added to each well and incubated at room temperature for 20 min. Finally, the optical density of each well was recorded at a wavelength of 450 nm.

GPX (Glutathione Peroxidase). The colorimetric base ZellBio assay kit was used for the quantitative assessment of glutathione peroxidase activity, according to the manufacturer's standard protocol. The GPX enzyme catalyzes the reduction of glutathione and has a role in the protection of the cell from oxidative damage. In brief, 50 µL of calibrator, diluted sample, and diluted control (buffer assay was used for dilution) were poured into their special wells. Then 50 µL of DTNB -5-5′-Dithiobis 2-nitrobenzoic acid- and then 50 µL of GR (Glutathione reductase) was added to each well and was incubated for 3-5 min at room temperature. 50 µL of reconstituted NADPH -β-Nicotinamide adenine dinucleotide phosphate- was added to each well. The absorbance was recorded at the wave-length of 412 nm.

Molecular study on apoptotic and anti-apoptotic factors

The quantitative Real -Time PCR (qRT-PCR) was carried out to evaluate the expression level of Cox8, Caspase3, and miR-24 as apoptotic and anti-apoptotic factors, as follows:

Total RNA extraction and cDNA synthesis. The RNX-Plus™ reagent (Cinnagen, Iran) was used for total RNA extraction from 2D and 3D cultured cells (on day 14) based on the standard protocol. Then, DNA contamination was removed from extracted RNA using DNase I, RNase-free Kit (Thermo Scientific, US). cDNA synthesis was immediately carried out by Revert Aid™ first-strand cDNA synthesis kit (Fermentase, Lithuania), according to the manufacturer's protocol. NCBI BLAST (https://blast.ncbi.nlm.nih.gov), as well as Gene runner (https://gene-runner.software.informer.com) and miRprimer (https://tools4mirs.org) software were administered to design and validate primers sets. The β-actin gene was used as an internal control against which mRNA expression of the Cox8 and Caspase3 genes were normalized. As well, the U6 internal control was considered for expression of mir-24. The list of primer sets and their sequences are illustrated in table 1.

Table 1: List of primers and their sequences used for qRT-PCR

Gene	Primer sequence
Cox8	Forward-5' *ACAAGAAGCGGGAGTGAAGGGA* 3' Reverse-5' *TGCAGAAGAGGTGACTGGAATTG* 3'
Caspase3	Forward-5' *AGTGGGATTGATGAGGAGATGG* 3' Reverse-5' *AGTGGAGTGTAGGGAGAAGGA* 3'
β-actin	Forward-5' *TCAGAGCAAGAGAGGCATCC* 3' Reverse-5' *GGTCATCTTCTCACGGTTGG* 3'
miR-24	Forward- *tggctcagttcagcagg* STEM LOOP- *ctcaactggtgtcgtggagtcggcaattcagttgacnnnnnnnn* STEM LOOP Reverse- *aactggtgtcgtggagtc*
U6	Forward-5' *CGCTTCGGCAGCACATATAC* 3' Reverse-5' *AAATATGGAACGCTTCACGA* 3'

Quantitative RT- PCR. The StepOne™ Master Mix and SYBR® Green (Applied Biosystems, UK) according to the manufacturer's recommendations, were used for qRT-PCR, and reactions were prepared in a total volume of 23 µl.

Thermocycler conditions for qRT-PCR program were adjusted as follows: The initial step at 95°C (15 min), followed by 40 cycles at 94°C (20 s) for denaturation, then 58-60°C (40 s) for annealing and extension, and finally 72°C (30 s) for final extension. Eventually, melting curve analysis was performed to confirm the primers' efficiency and specificity as well as nonoccurrence of primer-dimer formation. All reactions were carried out in triplicate and data were analyzed by threshold cycle (Ct) values.

All measurements were carried out in triplicate and GraphPad Prism 8.0 was employed for data analysis and drawing the charts. ANOVA test followed by the post hoc (Tukey) test was employed to compare differences among all groups. The mean ± SD -standard deviation- was calculated and used to present the numerical data. The statistically significant p values were indicated as $p<0.05$, $p<0.01$, and $p<0.001$.

Results

Serum levels of CK-MB and cTnI in healthy and MI groups

Induction of the infarction model was confirmed by evaluation of the serum levels of cTnI and CK-MB in both MI and control groups. The activity of CK-MB (p= 0.05) and cTnI (p= 0.003) showed a significant difference between healthy and MI groups. The mean value of CK-MB and cTnI are represented in table 2.

Table 2: Median values of cardiac markers (CK-MB and cTnI) in normal and MI groups

Analyte		Lower quartile	Upper quartile	(Mean±SD)	*p* value
CK-MB (U/L)	Normal	111.12	138.33	128.54±6.14	0.05
	MI	238.70	281.50	256.03±7.55	
cTnI(ng/ml)	Normal	1.61	2.36	2.03±0.14	0.003
	MI	18.23	25.61	21.77±1.38	
MI: Myocardial infarction					

Cell viability rate in healthy and MI cardiomyocytes

The infarcted and healthy cardiomyocytes, cultured in 2D and 3D (acellular aortic scaffold) systems were examined by MTT assay to identify the effect of acellular aortic scaffold on viability rate of healthy and MI cells. The results showed that MI cells have significant low viability rate compared to healthy cardiomyocytes, both in 2D and 3D culture systems ($P<0.05$); demonstrating negative effect of myocardial infarction on viability and proliferation of cardiomyocytes. However, comparison between MI cells cultured in 2D and 3D systems showed that MI cell viability in 3D culture was significantly higher than 2D culture ($P<0.01$). Similarly, healthy cardiomyocytes showed a high viability rate in 3D culture compared to 2D culture ($P<0.01$). The results of the MTT assay for each group are represented in figure 2.

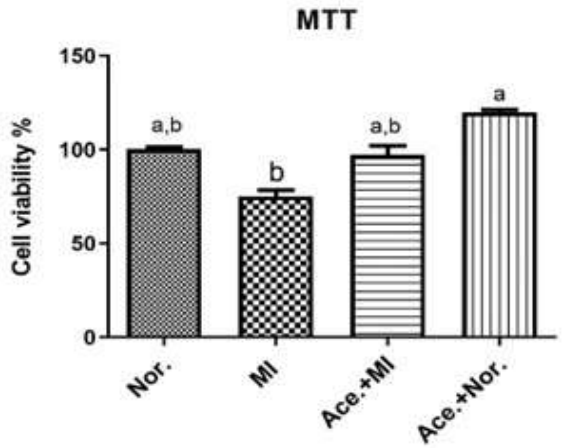

Figure 2. The MTT assay for viability rate evaluation in healthy and MI cardiomyocytes, cultured in 2D and 3D systems. The difference superscript letters (a and b) indicate statistically significant differences between the mean values ($P<0.05$). The values are displayed as mean ± SD. Nor: normal cell, MI: myocardial infarcted cell, Ace+MI: MI cell, cultured on acellular aortic scaffold (3D culture system). Ace+Nor: normal cell, cultured on acellular aortic scaffold (3D culture system).

Effect of acellular aortic scaffold on MI cardiomyocytes' antioxidant and catalytic activities

The results of ROS, TAC, SOD, GPX, and CAT assays showed a significant change in SOD and GPX in MI cells, cultured on the acellular aortic scaffold (3D culture) rather than MI cells in the 2D culture. Indeed, all these catalytic factors in MI cells were different from normal cells, both in 2D and 3D cultures. However, only a significant increase (P<0.001) in GPX and SOD was observed in MI cardiomyocytes, cultured on acellular aortic scaffold compared to these cells in 2D culture, demonstrating the effect of acellular aortic scaffold on GPX and SOD levels. The results of antioxidant and catalytic activities assays for each group are represented in figure 3.

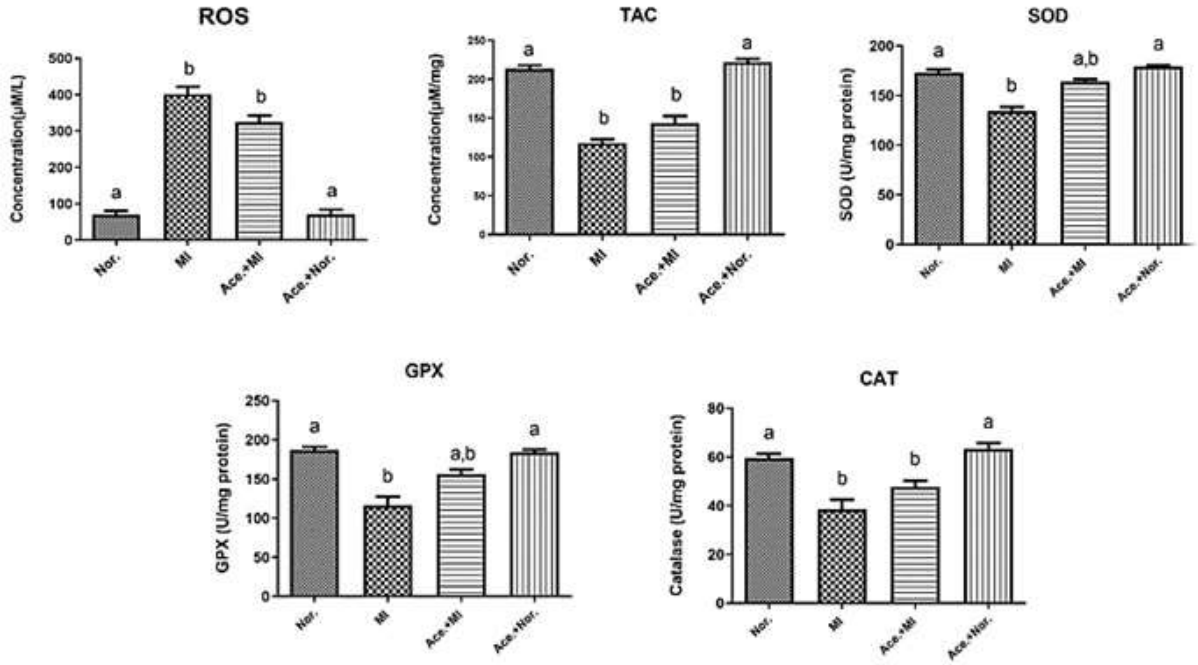

Figure 3. The Reactive Oxygen Species (ROS), Total Antioxidant Capacity (TAC), Catalase activity (CAT), Superoxide Dismutase (SOD), and Glutathione Peroxidase (GPX) levels in healthy and MI cardiomyocytes, cultured in 2D and 3D systems. The difference superscript letters (a and b) indicate statistically significant differences between the mean values (P< 0.05). The values are displayed as mean ± SD. Nor: normal cell, MI: myocardial infarcted cell, Ace+MI: MI cell, cultured on acellular aortic scaffold (3D culture system). Ace+Nor: normal cell, cultured on acellular aortic scaffold (3D culture system).

Effect of acellular aortic scaffold on MI cells gene expression

The RT-PCR quantification was used to evaluate the *Cox8*, *Caspase3*, and miR-24 expression levels in MI cells following the culture on acellular aortic scaffold. The results showed that not only *Cox8* and *Caspase3* expression levels in MI cells were different from normal ones in both 2D and 3D cultures, but also these expression levels were different between MI cells in 2D and 3D (acellular aortic scaffold) cultures. Indeed, in each group, *Cox8* and *Caspase3* expression levels showed a significant (P<0.001) increase in MI cardiomyocytes compared to normal ones. However, the expression levels of Cox8 and Caspase3 in MI cells, cultured in the 3D system were lower than MI cells in the 2D systems (P<0.01), demonstrating the effect of acellular aortic scaffold on *Cox8* and *Caspase3* expression levels in MI cells. The miR-24 expression levels showed statistically significant differences among

all groups (P<0.05). In MI cells, both in 2D and 3D cultures system, miR-24 expression levels were lower than normal cells; but the expression level in MI cells, cultured in the 3D system (acellular aortic scaffold) was increased in comparison with MI cells in the 2D culture system, indicating the positive effect of acellular aortic scaffold on miR-24 expression level in MI cells. The results for each group are represented in figure 4.

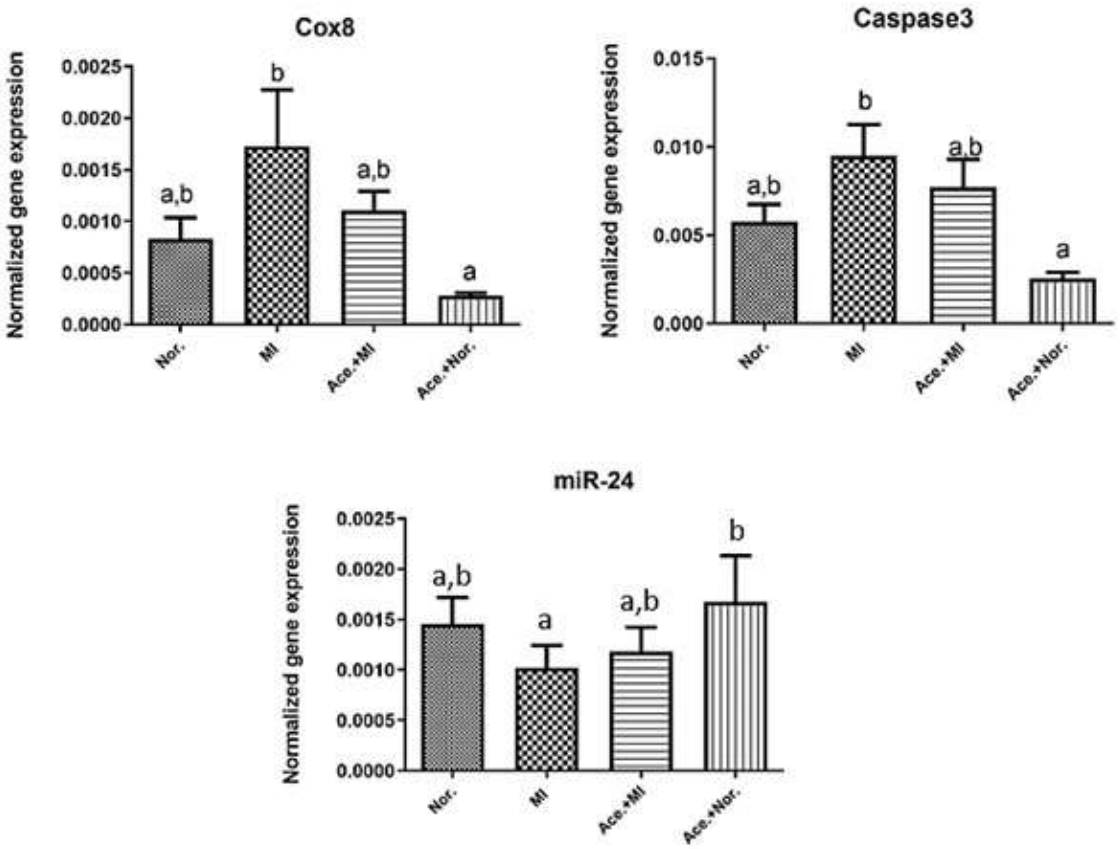

Figure 4. The relative expression of Cox8, Caspase 3, and miR-24 in healthy and MI cardiomyocytes, cultured on 2D and 3D systems. The difference signs (a and b) show statistically significant differences between the mean values (p < 0.05). The values are displayed as mean ± SD. Nor: normal cell, MI: myocardial infarcted cell, Ace+MI: MI cell, cultured on acellular aortic scaffold (3D culture system). Ace+Nor: normal cell, cultured on acellular aortic scaffold (3D culture system).

Discussion

The present study was aimed to investigate the effect of acellular aortic scaffold on infarcted cardiomyocytes compared with healthy cardiomyocytes under the same conditions. The ROS, TAC, SOD, GPX, and CAT levels were evaluated to observe how the antioxidant and catalytic activities change, due to the culture of MI cells on AAS. Oxidative stress in an organism occurs as a result of imbalance between the reactive oxygen species (ROS) production and the total antioxidant capacity (TAC). The antioxidant defenses, especially, antioxidant enzymatic defenses such as superoxide dismutase (SOD), glutathione peroxidase (GPX), and catalase (CAT), are the main defense systems against ROS molecules (Pech et al, 2019). Since oxidative stress can trigger the inflammatory processes and endothelial dysfunction, as important factors in cardiovascular diseases (Vickers, 2017); we hypothesized that the ROS and TAC may alter in MI cells, and AAS may have a positive effect on the maintenance of their balance. We observed the decrement of

ROS and increment of TAC in MI cells, cultured on AAS compared with MI cells, cultured on the 2D system, demonstrating the positive effect of AAS on antioxidant activity of MI cells; but these results were not statistically significant (figure 3).

The first protection strategies against the potentially harmful ROS products are SOD, GPX, and CAT antioxidant enzymes activities (Pech et al, 2019); in which the SOD enzyme convert the O_2^- into H_2O_2 and ultimately initially detoxifies into H_2O as the end product, through the CAT and GPX pathways (Michałek et al, 2020). Among various isoforms of SOD encoding by different genes, SOD_3 has been observed in the cellular resources of the heart. This isoform, as the main enzymatic antioxidant defense, has an important role against cardiovascular diseases (Ighodaro and Akinloye, 2018; Michałek et al, 2020). Lebovitz et al, (1996) observed myocardial injury and perinatal death in mice, due to the mitochondrial SOD deficiency. GPX enzymes - especially GPX_1- are also implicated in the development and prevention of various diseases, including cardiovascular disease (Rayman, 2005). The presence and activity of GPX_3 isoform in the blood serum of cardiologic patients has been reported as well (Brigelius-Flohé and Maiorino, 2013).

We observed statistically significant differences of SOD and GPX levels ($p < 0.001$) in MI cells cultured on AAS compared with MI cells in the 2D culture (figure 3), indicating the positive effect of AAs on SOD and GPX as antioxidant agents. The increment of CAT level was also observed in MI cells, cultured on AAS in comparison with MI cells in the 2D culture (figure 3), but this difference was not statistically confirmed ($p= 0.2430$). Although the changes in the ROS and TAC levels in MI cells cultured on the acellular aortic scaffold were not statistically significant, but the considerable changes of SOD and GPX levels demonstrated that this acellular scaffold represented well biochemical properties to promote the antioxidant activity in MI cells.

The indispensable role of *Cox8*, *Caspase3*, and miR-24 in myocardium development, differentiation, and hemostasis has been previously reported (Cardona et al, 2015; Radford et al, 2002; Wang et al, 2020). miR-24 targets *TGF-β1* -transforming growth factor beta 1- and has effects on cellular function. Myocardial infarction affects the miR-24 expression level and consequently leads to the down-regulation of *TGF-β1* (Wang et al, 2020). COX (cytochrome c oxidase) is the terminal enzyme of the mitochondrial electron transport chain, which contributes to the establishment of electrochemical gradients across the inner membrane of mitochondria and provides the driving force for ATP synthesis. In mammals, VIa, VIIa, and VIII (Cox8A) subunits have been reported as liver, heart, and skeletal muscle isoforms that may modulate *COX* activity in various cellular metabolism. Cardiac dysfunction was observed by Radford et al. in mice lacking cytochrome c oxidase subunit VIaH (Radford et al, 2002). Caspases are a family of proteases, that regulates death signals propagation and genomic DNA degradation, consequently participating in apoptotic cell death (Bröker et al, 2005). Caspase3 expresses during heart development in rodents, and it progressively silences after the birth. Cardona et al, (2015) showed that lack of caspase 3 in mouse cardiomyocytes during development reduces the final number of cardiomyocytes; by proteomics study, they also showed the abnormally high abundance of glycolytic enzymes as well as the lower amount of oxidative phosphorylation proteins in neonatal caspase-deficient myocardium compared with wild type hearts.

We studied the expression level of *Cox8*, *Caspase3*, and miR-24 in healthy and MI cardiomyocytes to identify the effect of acellular aortic scaffold on these apoptotic and anti-apoptotic factors. The *Cox8* and

Caspase3 expression levels showed a significant (P<0.001) increase in MI compared with healthy cardiomyocytes although their expression levels in MI cardiomyocytes, cultured on AAS, were lower than MI cardiomyocytes in the 2D system (p<0.01) (figure 4). We observed statistically significant differences in miR-24 expression levels among all groups (P<0.05). In MI cells, miR-24 expression levels were lower than normal cells; but the expression level in MI cells, cultured on AAS, was increased in comparison with MI cells in the 2D culture system (figure 4).

In conclusion, myocardial infarction can lead to the dysregulation of Cox8, Caspase3, and miR-24, consequently affecting essential cellular metabolism. The biochemical properties and molecular composition of AAS may positively maintain the cellular homeostasis of MI cells. In summary, our study showed that acellular aortic scaffold can positively control the catalytic and antioxidant activities and cellular homeostasis of cardiomyocytes after myocardial infarction, demonstrating the potential of such natural biomaterials for cardiac tissue reconstruction. However, to reach favorable results and ideal therapeutic application, further studies are required.

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Conflict of Interest

The authors declare they have no conflict of interest.

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اثرات مثبت داربست آئورت بدون سلول بر فعالیت‌های آنتی‌اکسیدانی و هموستاز سلولی: یک مطالعه تجربی بر روی کاردیومیوسیت موش‌های دچار انفارکتوس

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چکیده

انفارکتوس میوکارد (MI) در سال‌های اخیر به دلیل افزایش سن و همچنین تغییر سبک زندگی در جمعیت افزایش یافته است. در دهه‌های اخیر، شاخه پزشکی بازساختی برای ارائه روش‌های مدرن و کارآمد برای درمان بیماران MI مورد توجه قرار گرفته است. مطالعه حاضر با هدف بررسی اثر داربست آئورت بدون سلول (AAS) بر سلول قلبی دچار MI برای شناسایی پتانسیل درمانی این ماده طبیعی برای بیماران MI انجام شد. این مطالعه در پنج مرحله اصلی انجام شد: تهیه مدل حیوانی MI، تهیه سلول‌های MI، کشت سلول‌های MI حیوانی بر روی AAS، ارزیابی زنده‌مانی سلول (آزمون MTT) و همچنین فعالیت‌های آنتی‌اکسیدانی و کاتالیزوری (سنجش ROS، TAC، SOD، GPX و CAT) و مطالعه مولکولی بر روی عوامل آپوپتوز و ضد آپوپتوز به روش qRT-PCR انجام شد. AAS به طور مثبتی بر میزان زنده ماندن سلول‌های MI تاثیرگذار بود، همچنین سطوح GPX و SOD به دلیل کشت روی AAS به طور معنی‌داری در سلول‌های MI افزایش یافت. آزمون RT-PCR کاهش سطح بیان ژن‌های Cox8 و Caspase3 را در سلول‌های MI که روی AAS کشت شده بودند، نشان داد. در حالی که افزایش سطح بیان miR-24 در سلول‌های MI در نتیجه کشت بر روی AAS مشاهده شد. نتایج مطالعه ما بیان کرد که داربست آئورت بدون سلول می‌تواند به طور فزاینده‌ای فعالیت‌های کاتالیزوری و آنتی‌اکسیدانی سلولی و همچنین زنده‌مانی سلولی کاردیومیوسیت‌ها را پس از انفارکتوس میوکارد، کنترل کند که نشان از پتانسیل چنین مواد زیستی طبیعی برای بازسازی بافت قلب است. با این حال، برای رسیدن به نتایج مطلوب و کاربرد درمانی ایده آل، مطالعات بیشتری مورد نیاز است.

کلمات کلیدی: انفارکتوس میوکارد، طب احیا کننده، داربست آئورت بدون سلول، فعالیت آنتی‌اکسیدانی

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