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Oxidative Properties of Blood-Derived Extracellular Vesicles in 15 Patients After Myocardial Infarction

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Background: In this study, we investigated the yield and composition of extracellular vesicles (EVs) derived from 40- to 60-year-old healthy male controls and post-myocardial infarction (post-MI) patients' blood samples and assessed their pro-inflammatory and oxidative-related properties. Our study aimed to determine the EV yield and composition differences between both groups and to find out if there were differences between EV-mediated oxidative stress reactions.

Material/Methods: Fifteen post-MI patients and 25 healthy individuals were included. EVs were isolated by ultracentrifugation and analyzed using nanotracking analysis (NTA), western blotting and fluorescent flow cytometry (FFC). Oxidative stress (OS) in blood samples was identified by measuring malondialdehyde concentration from serum, while EVs-induced OS was measured in the human vein endothelium cells (HUVEC) using H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) fluorescence as a marker.

Results: We found higher EVs concentration in healthy controls than in the post-MI group (7.07±3.1 E+10 ml vs 3.1±1.9 E+10 ml, P<0.001) and a higher level of CD9-positive exosomes (MFI 275±39.5 vs 252±13, P<0.001). Post-MI patients' EVs carry pro-oxidative nicotinamide adenine dinucleotide phosphate (NADPH) oxidases isoforms NOX1 (NADPH oxidase 1), NOX5 (NADPH oxidase 5) and NOX2 (NADPH oxidase 2) and anti-oxidative thioredoxin, extracellular signal-regulated kinases 1/2 (ERK1/2), and protein kinase B (Akt B). In the post-MI EVs, there was a higher predominance of enzymes with anti-oxidative effects, leading to weaker OS-inducing properties in the HUVEC cells.

Conclusions: We conclude that post-MI patient blood sample EVs have stronger anti- than pro-oxidative properties and these could help fight against post-MI consequences.

Keywords: Atherosclerosis • Blotting, Western • Extracellular Vesicles • Flow Cytometry • Oxidative Stress

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Background

According to the American Heart Association, cardiovascular diseases (CVDs) remain one of the main causes of death worldwide [1]. CVDs comprise multiple maladies such as coronary artery disease, hypertension, stroke, and peripheral vascular disease. Atherosclerosis (AS) is the main cause of morbidity and mortality in CVD patients [1]. AS is a lipid-driven inflammatory disease of arterial intima in which the balance of pro- and anti-inflammatory mechanisms dictate the final clinical outcome [2]. AS is considered one of the most important pathologies in the modern world but the detection of early changes in the vasculature and prognosis of this disease is still lacking [3]. Oxidative stress plays an important role in atherosclerosis by causing the production of reactive oxygen species (ROS), which are important for the pathogenesis of myocardial infarction (MI) and post-stroke survival [4]. In subclinical AS, oxidative stress can induce endothelial cell activation, permeability changes, recruitment of various inflammatory cells, and other important processes [5]. Combined, these processes eventually facilitate damage to the endothelium and induce the progression of AS [5]. EVs are closely associated with damaging ROS signaling to the endothelium [6]. In CVD patients' blood, EVs can be released by blood cells, such as platelets, erythrocytes, and leukocytes; and by heart and blood vessel cells such as cardiomyocytes, cardiac fibroblasts, and endothelial cells [7]. In general, EVs are particles released from various types of cells, have a membrane composed of lipid bilayer transmembrane proteins, carry soluble proteins, and do not replicate [7]. EVs have been identified in a diverse range of human biofluids including serum and plasma as well as urine, saliva, breast milk, amniotic fluid, ascites fluid, cerebrospinal fluid, and bile [8-10]. EVs can be classified as exosomes or microvesicles. The term 'exosome' is used for vesicles 40-100 nm in size that are released as a consequence of multivesicular endosome fusion with the plasma membrane, while 'microvesicle' refers to EVs that are generally up to ~1000 nm in diameter and are shed from the plasma membrane [8,11,12]. Small vesicles of 100 nm may also bud from the cell surface [13]. Membrane vesicles originate from endosomal and plasma membranes and are released into the extracellular environment by various cells [8]. They have an important role in intercellular communication, carrying cytosolic proteins, lipids, and RNA [8]. A systematic review compared studies on the cardioprotection effect of EVs [14], showing that EVs from the investigated sources mediated the protection using different pathways by carrying proteins or nucleic acids responsible for cell survival. For instance, in an oxidative stress environment, EVs from cardiac progenitor cells were found in greater quantity and at a higher count and carrying different cargo than control [14]. When researching the effects of EVs on cardiomyocytes from MI patients and donors, only donor EVs had positive protective effects in preventing cell death. The authors of the aforementioned systemic review showed that EVs cardioprotective effects observed in different studies from different sources are inconsistent. It would seem that EVs, in general, are protective, but it is nearly impossible to properly compare the studies because of differences in purification methods and the EVs experiments themselves [14], which is a problem that calls for the standardization of research techniques. The ultimate goal would be to use the EVs as a therapeutic or diagnostic tool in routine clinical work. Serum and plasma are attractive sources for EV isolation since blood sampling is a minimally invasive procedure [15]. However, the isolation and purification of EVs from human blood are complicated due to high serum viscosity, high abundance of various proteins, and non-EV lipid particles [10]. All of these serum features significantly impact the yield of EVs from human serum [10]. Currently, available purification methods do not allow full discrimination between exosomes and microvesicles, and, as such, purified EVs might include both fractions [16]. However, there are some cell surface markers, such as CD9, CD63, and CD81, which are thought to ensure the recognition of exosomes [17].

Ultracentrifugation (UC) is a classical method for EV purification yielding a large amount of EVs [10]. The most common methods for distinguishing EVs based on their size are scanning and transmission electron microscopy; atomic force microscopy, which is based on EV surfaces imaging; dynamic light scattering, which is based on fluctuations of scattered light intensity; and nanoparticle tracking analysis (NTA), which directly visualizes individual particles by scattering of illuminated laser beam [16]. EV detection methods based on their surface markers, such as immunoaffinity-capture by beads, enzyme-linked immunosorbent assay (ELISA), and conventional flow cytometry, can be also used to analyze EV subpopulations expressing specific antigens [18].

In this study, we isolated EVs, mainly exosomes, from blood samples from 40- to 60-year-old men who were either healthy (healthy controls) or had previously had a myocardial infarction (post-MI group). We investigated the EV quantity, composition, and oxidative stress-related properties in human endothelial cell lines (HUVEC) for both isolated EV groups.

Our study aimed to determine the differences in the quantity and composition of EV between healthy and post-MI patients and to investigate possible differences in EV-mediated oxidative stress reactions between the 2 groups.

Material and Methods

Patients

The study protocol was approved by the local Vilnius University Bioethics Committee (No.: 158200-18-990-495 and 158200-15-807-319). All procedures followed were in accordance with the

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ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1964 and its later amendments. Informed consent was obtained from all patients for inclusion in the study.

Healthy Subjects

Healthy 40- to 60-year-old men were examined between December 2015 and July 2017. Healthy subjects had no prior history of acute cardiovascular disease and were not under any treatment for cardiovascular diseases.

Post-MI Group

The test group included 15 men who had been treated for MI either at the Cardiac Intensive Care or at the Intensive Care hospital units, or the first cardiology ward of Vilnius University Santaros Klinikos Hospital 3 months prior to inclusion. Patients were enrolled between February and May of 2018 and were 40 to 60 years old, had had MI for the first time in their life, and coronary angiography confirmed coronary occlusion of more than 50% in at least 2 arteries. Coronary catheterization and coronary angioplasty were performed on all patients. At the time of blood collection, patients were under antihypertensive treatment and received aspirin and statins. Twelve patients had primary arterial hypertension. Altogether, only 1 patient had systemic inflammation, diabetes mellitus, and primary arterial hypertension at the same time.

Blood Collection

We drew 4 ml of blood into plasma sodium citrate tubes. To produce platelet-free plasma, the plasma tubes were then subjected to differential centrifugation. Plasma tubes were centrifuged at 3000 g for 10 min, and three-quarters of recovered plasma was transferred into a sterile tube and centrifuged again at 10 000 g for 30 min. After centrifuging, the plasma was transferred to separate tubes and kept at -80°C until ultracentrifugation. The post-MI patients' blood was collected 3 months after the MI, which is a regular follow-up period.

Ultracentrifugation

The frozen plasma was thawed and up to a 100 µl of each blood plasma sample was diluted to a total volume of 1 ml with PBS (phosphate-buffered saline, pH=7.4) and samples were centrifuged at 1200 g for 30 min at 4°C (Pico 21, Thermo Scientific) to remove cells and debris. Then, 3 ml of PBS was added to the supernatant and it was further centrifuged at 120 000 g for 1.5 h at 4°C using the Optima™ L-90K Ultracentrifuge with installed rotor SW55Ti. The EVs were re-suspended in 300 µl of Dulbecco's PBS (DPBS) (Sigma-Aldrich). During the purification of exosome samples, some material could be lost, and there was no procedure in place to assure compensation of the loss of material during purification. The Bradford protein assay (Bio-Rad Laboratories; USA) was used to measure the concentration of EVs in each sample. BSA (bovine serum albumin) was used as a standard.

Clinical Chemistry Analysis

The concentrations of C reactive protein (CRP), glucose, total cholesterol, triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) were measured from patients' and healthy subjects' blood using routine techniques of a clinical chemistry laboratory (Architect ci8200, Abbott, Chicago, Illinois).

Malondialdehyde

Malondialdehyde (MDA) is a marker of lipid peroxidation and is often used to represent the level of oxidative stress in biological samples [19]. We mixed 50 µl of thawed serum with 750 µl of 0.44 M phosphoric acid solution, 250 µl of 42 mM thiobarbituric acid solution, and 450 µl of deionized water. The prepared samples were incubated at 100°C for 60 min. After incubation, the samples were rapidly cooled in an ice bath, and 500 µl of the sample was diluted in a methanol solution (1: 1). The blood serum sample was mixed by shaking and then centrifuged for 3 min at 10 000 g. After centrifugation, 500 µl of the centrifuged serum sample was added to the chromatographic vial, and serum sample was analyzed via the high-performance liquid chromatography (HPLC) method. Malondialdehyde concentration was measured by a Shimadzu Nexera X2 UHPLC system (Shimadzu). Data were collected and processed using LabSolutions software (Shimadzu).

Nanoparticle Tracking Analysis

Nanoparticle tracking analysis (NTA) was performed with a ZetaView nanoparticle analyzer (Particle Metrix GmbH; Germany). The 102-nm polystyrene beads were used to calibrate the apparatus before each session. We recorded 11 measurements for each case twice and the results were averaged in at least 1 dilution in Dulbecco's PBS (Corning, Inc., NY, USA). ZetaView Software 8.04.02 (Particle Metrix GmbH) was used to analyze images, settings were set to default image evaluation, and we used the following camera acquisition parameters: sensitivity set to 85, shutter set to 70, and frame rate set to 30. Each sample was measured 3 times. Concentrations of the samples of extracellular vesicles were expressed as the number of EVs per 1 ml of diluents; in this case, PBS.

Fluorescent Flow Cytometry

For the identification of EVs with special surface antibodies by fluorescent flow cytometry (FFC) analysis, we used the method

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described by Mellisho et al [20]. EV sample volume was adjusted to the total protein concentration. A vial containing only PBS was used as a negative control. To all EV samples and the negative control, 5 µl of latex beads was added and left overnight at 4°C. In the morning, 1 µl of 2M glycine was added to each EV sample and the negative control vial and kept for 45 min at room temperature. Then, 2 washing steps at 4000 rpm for 5 min at 4°C were performed using a 0.5% PBS/BSA blocking solution, and EV samples were incubated for 20 min at room temperature. EV samples were incubated with antibodies for 1 h at room temperature and centrifuged once at 4000 rpm for 5 min at 4°C. The supernatant was removed until 100 µl was left in the tubes; then, 400 µl of PBS was added and the pellet was carefully re-suspended. This solution was tested on an LSRII flow cytometer (BD, San Jose, California).

Western Blotting

For immunoblot analysis, all samples were first lysed in sodium dodecyl sulfate (SDS)-sample buffer, heated at 100°C for 5 min, and loaded onto 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE). After electrophoresis, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corp.) and blocked for 30 min overnight with 2% nonfat dry milk or 3% bovine serum albumin (BSA) in TBST (Tris-Buffered Saline Tween 20) buffer (20 mM Tris pH 7.5, 0.15 M NaCl, 0.01% Tween20). The primary antibodies used were anti-TSG101 (tumor susceptibility gene 101) (Abcam, Germany) and anti-thioredoxin (Abcam, Germany). The concentration of primary antibody was always 1: 1000. Secondary goat anti-rabbit antibody (LabAS, Tartu, Estonia) was used at the dilution of 1: 10 000. The signal was visualized by adding the enhanced chemiluminescence (ECL) substrate (Amersham ECL Western Blotting Detection Reagents) for 1 min, and the film was exposed for 10 min, after which it was scanned in using Epson Expressions 1680 software. Two healthy control samples and 2 post-MI samples were chosen for further analysis to detect NADPH (nicotinamide adenine dinucleotide phosphate) oxidases (NOX), protein kinase B (Akt B), and extracellular signal-regulated kinases 1/2 (ERK 1/2) with the primary antibody being anti-NOX2 (Abcam, Germany), anti-NOX5 (ThermoFisher, USA), anti-NOX1 (ThermoFisher, USA), anti-Akt B (ThermoFisher, USA), and anti-ERK 1/2 (ThermoFisher, USA). All other western blot steps were done in the same manner as before.

Oxidative Stress in Cell Culture

To determine the oxidative stress in the cell culture, we used the method described by Wei et al [21]. EVs were added to the pooled HUVEC cells, which were at up to the 5th passage (Corning, USA, catalog number: 354151) and incubated for 20 h. EV concentration was equalized in each sample of HUVEC cells by using NTA data. After the incubation, every sample was stained with 5 µM H2DCFDA (2',7'-dichlorodihydrofluorescein diacetate) in the dark for 30 min. After staining, cells were lifted with trypsin and washed once with complete medium EGM-2 BulletKit (Lonza, Germany) and centrifuged at 180 g for 7 min. After washing, samples were measured with flow cytometer FACS Canto II (BD, USA) using FITC (fluorescein isothiocyanate) channel at excitation 490 nm and emission 520 nm. Collected data was analyzed using FACS Diva software. Data showed that post-MI EVs on average gave less H2DCFDA fluorescence intensity in the sample when compared to EVs of the healthy group.

Statistical Analysis

Variables were tested by the paired non-parametric Wilcoxon test and Spearman test for the correlation coefficient. The confidence level for the test was set at 0.05; all *P* values were two-sided. Statistical analysis was carried out using R statistical software version 1.0.136 and SPSS version 21 (IBM, USA).

Results

Post-MI patients had a significantly elevated concentration of blood serum MDA (106±26.5 vs 154.7±30.7, P<0.001) and had lower levels of low-density lipoprotein cholesterol (3.4±0.9 vs 2.1±0.6, P<0.001), high-density lipoprotein cholesterol (1.2±0.2 vs 1.1±0.3, P=0.03), and total cholesterol concentrations (5.5±1.1 vs 3.8±0.7, P<0.001) compared to healthy controls (**Table 1**).

According to nanoparticle tracking analysis (Zeta View, Particle Metrix, Germany), there was a statistically significant difference in blood particle concentration between the healthy control group and the post-MI group ($7.07\pm3.1\times10^{10}$/ml vs $3.1\pm1.9\times10^{10}$/ml, *P*<0.001, **Figure 1A**). However, the total protein level measured by the Bradford method was higher in post-MI patient samples (1.01±0.49 mg/ml vs 1.95± 1.07 mg/ml, P=0.001, **Figure 1B**), which might be related to the higher level of protein cargo carried in the post-MI blood EVs. In addition, healthy groups' blood EVs showed a positive correlation (R=0.55) between Bradford measurements and NTA, while post-MI EVs did not (R=-0.15). This suggests that both methods cannot be equally used to evaluate EVs, as healthy and post-MI EVs can carry different levels of proteins. For further western blotting studies, the NTA method was chosen.

The CD9 level on exosome membranes was tested by FFC (**Figure 1C**). Comparing CD9 median fluorescence intensity (MFI) and the percentage of positive events revealed that healthy human blood samples had a higher number of CD9-positive exosomes than post-MI group individuals (MFI 275±39.5 vs 252±13, *P*<0.001, percentage of events 23.01%±17.4 vs 1.3%±0.6,

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Table 1. Clinical characteristics and laboratory and extracellular vesicles analysis results of healthy and post-MI (myocardial infarction) patients.

	Variable	Controls (n=25)	Post MI group (n=15)	Statistical significance
Clinical characteristics	**Systolic blood pressure**			
	Mean (range)	128 (93-175)	122.9 (100-170)	No
	Median	122	120	
	SD	18.1	15.7	
	Diastolic blood pressure			
	Mean (range)	79.4 (61-100)	77.7 (60-99)	No
	Median	79	80	
	SD	10.4	9.1	
	Heart rate			
	Mean (range)	64.2 (47-88)	67.1 (55-87)	No
	Median	61	64	
	SD	10.8	8.8	
Laboratory analysis	**CRP (mg/l)**			
	Mean (range)	5.6 (4.2-8.1)	2.6 (0.15-11.2)	No
	Median	5.4	1.8	
	SD	0.8	2.9	
	LDL-C (mmol/l)			
	Mean (range)	3.4 (1.7-5.9)	2.1 (1-3.2)	2.5×10^{-5}
	Median	3.2	2	
	SD	0.9	0.6	
	HDL-C (mmol/l)			
	Mean (range)	1.2 (0.6-1.7)	1.1 (0.7-1.8)	0.03
	Median	1.2	1	
	SD	0.2	0.3	
	Triglyceride concentration (mmol/l)			
	Mean (range)	2.1 (0.7-9.9)	1.3 (0.7-1.9)	No
	Median	1.4	1.4	
	SD	1.9	0.3	
	Total cholesterol (mmol/l)			
	Mean (range)	5.5 (4.1-8.7)	3.8 (2.6-5)	6.4×10^{-6}
	Median	5.17	3.7	
	SD	1.1	0.7	

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Table 1 continued. Clinical characteristics and laboratory and extracellular vesicles analysis results of healthy and post-MI (myocardial infarction) patients.

	Variable	Controls (n=25)	Post MI group (n=15)	Statistical significance
Laboratory analysis	**Glucose (mmol/l)**			
	Mean (range)	5.6 (4.2-8.1)	7.4 (4-27.1)	No
	Median	5.4	5.8	
	SD	0.8	5.4	
	Malondialdehyde (MDA) concentration			
	Mean (range)	106 (69.6-177.1)	154.7 (107.6-208)	9×10^{-6}
	Median	101	154.3	
	SD	26.5	30.7	
Extracellular vesicle analysis	**Particle concentration ($\times10^9$/ml)**			
	Mean (range)	7.1 (5.04-9.15)	3.53 (2.84-4.22)	1.1×10^{-4}
	Median	5	3.1	
	SD	5	1.2	
	Protein concentration (mg/ml)			
	Mean (range)	1.08 (0.94-1.22)	2 (1.54-2.47)	6.1×10^{-4}
	Median	1	2	
	SD	0.3	0.8	
	CD9 mean fluorence intensivity (MFI)			
	Mean (range)	285.8 (270.75-300.85)	252.93 (247.17-258.7)	2.9×10^{-5}
	Median	275	252	
	SD	36.5	10.4	
	DFCDA mean fluorence intensivity (MFI)			
	Mean (range)	1866.04 (822.87-2909.21)	291.07 (232.6-349.53)	5.4×10^{-5}
	Median	791	271	
	SD	2527.18	105.57	

P<0.001). There was quite a strong correlation in both groups between CD9 MFI and the percentage of CD9-positive events (R=0.74 and R=0.6, respectively). The strong correlation between EV CD9 MFI and positive events percentage can be explained by the fact that a specific CD9 marker for EV investigation was used instead of Bradford or NTA methods. CD9 levels found in the isolated EV fraction confirmed the presence of exosomes in the EV samples. The presence of exosomes was also confirmed by TSG101 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in both healthy and post-MI EVs samples. GAPDH testing show those EVs from the patients had more proteins when compared to controls.

To determine anti-oxidative EV properties, thioredoxin level was identified by western blotting. Thioredoxin was detected in almost all samples, but at a noticeably higher level in post-MI EV samples than in healthy individuals. Similar results were obtained with the cell survival-regulating enzymes: extracellular signal kinases 1/2 (ERK 1/2) and protein kinase B (Akt B) were detected in all EV samples, but were found at higher levels in post-MI EVs samples.

The investigation of pro-oxidative NADPH oxidases (NOX1, NOX2, and NOX5 isoforms) in EV samples showed a strong upregulation of all 3 NOX isoforms in post-MI EVs, but only NOX1 and NOX5 isoforms were obtained in the healthy EVs.

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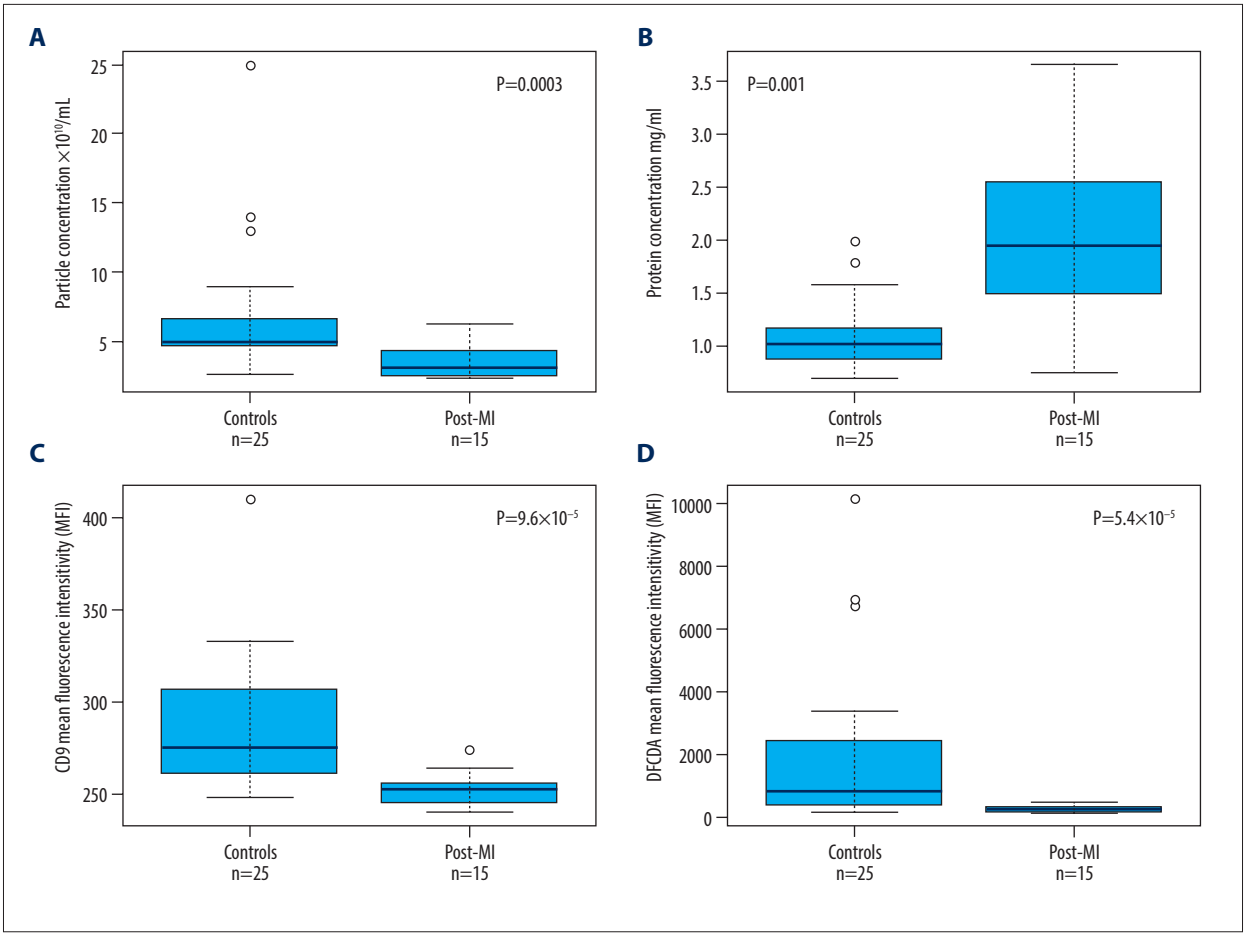

Figure 1. Comparison between healthy controls and post-MI (myocardial infarction) patients. (**A**) Particle concentration measured by nanotracking analysis (NTA): number of EVs per 1 ml of diluents (PBS). (**B**) Protein concentration measured with Bradford assay. (**C**) CD9 mean fluorescent intensity (MFI) measured by fluorescent flow cytometry (FFC). (**D**) H2DFCDA (2',7'-dichlorodihydrofluorescein diacetate) mean intensity (MFI) measured by FFC. Picture created using SPSS version 21 (IBM, USA).

Oxidative stress testing of isolated EVs was done by adding them to the endothelial cell culture (HUVEC) and measuring the production of ROS through H2DCFDA fluorescence intensity. The negative (using cell growth medium) and positive controls (using 1M hydrogen peroxide) were used to define and normalize the results. Data showed that post-MI patient exosomes gave a lower oxidative stress response than the EVs of the healthy group, since post-MI exosomes on average gave less H2DCFDA fluorescence intensity in the sample when compared to the healthy group ($P<0.001$, **Figure 1D**).

Discussion

Atherosclerosis (AS) is the most common pathological process that leads to CVDs. AS is characterized by damaged large- and medium-sized arteries where atherosclerotic plaques consisting of necrotic cores, calcified regions, accumulated modified lipids, inflamed smooth muscle cells, endothelial cells, leukocytes, and foam cells have formed [22]. Oxidative stress is implicated in the pathogenesis of AS and is caused by the production of excess reactive oxygen species (ROS) [23]. MDA concentration in blood plasma is an effective oxidative stress biomarker and it has been shown to correlate with tissue damage in both acute and chronic diseases [24]. CRP is also used as a common inflammation marker [25]. For atherosclerotic events, CRP is measured with a high-sensitivity method, given the name 'hsCRP' and the range measured is between 1-3 mg/L [26]. Data from this study showed that post-MI patients have an underlying low-grade inflammation (identified by hsCRP) but a high level of oxidative stress as shown by the marker MDA when compared to control group. EVs from the post-MI group were of inflammatory origins as shown by their surface marker thioredoxin, yet they had low oxidative stress properties as shown in cell culture experiments. On the other hand, in the control group, the EVs had fewer inflammation

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markers, yet at the same time had a higher oxidative stress reaction in cell culture experiments. This might indicate that EVs from a lower inflammation origin mediate oxidative stress reactions before atherosclerotic events occur, and after their occurrence they are produced from a higher inflammation origin but are less involved in mediating oxidative stress reactions.

Looking at EVs counts, our data showed that post-MI patients' blood samples had a significantly lower EV yield when compared to the healthy group. Patients with atherothrombotic diseases and atherosclerotic lesions have been shown to have high levels of circulating EVs derived from endothelial cells, vascular smooth cells, platelets, and erythrocytes [27]. The release of platelet-derived EVs has been shown to be increased in conditions such as MI [28,29]. The level of circulating EVs starts to increase 1 h after myocardial infarction, with a significant increase occurring in 24 h after MI or ischemia/reperfusion [30,31]. Hydroxymethyl glutaryl coenzyme A reductase inhibitors, also known as statins, are some of the most commonly prescribed medications worldwide and statin therapy has significant benefits for both primary and secondary prevention of cardiovascular disease [32]. The decreased number of circulating EVs derived from the endothelium, platelets, and inflammatory cells after lipid-lowering therapies with statins has also been observed [33]. Simvastatin has been shown to reduce the secretion of EVs from various cell types [34], while atorvastatin, along with other hypertensive treatments, increases the number of endothelium-derived EVs [35,36]. These controversial data reveal that the effects of statins on EV release are not fully clear. Use of medications such as anti-atherogenic statins or the timing of blood collection (3 months post-MI) could have an impact on the post-MI EV yield.

Recent studies have indicated the positive effect of cell-released exosomes on injured tissues [14]. It has been shown that treatment using exosomes can reduce systemic inflammation in mice after myocardial ischemic/reperfusion [37]. Those exosomes were found to induce phosphoinositol-3-kinase/protein kinase B (PI3K/Akt B) signaling, which is known to reduce oxidative stress and help protect cells and tissues [38]. Results from our study show that exosomes from both post-MI patients and healthy individuals carry Akt B and ERK1/2 at higher levels compared to EVs from healthy controls. We also tested whether EVs carry enzymes that regulate anti-oxidative and pro-oxidative properties. It has been shown that exosome-like structures could transfer anti-oxidant thioredoxin, which is upregulated as a response to increased oxidative stress [39]. The same study also found an interesting association between pro-oxidant NOX2 and anti-oxidant thioredoxin levels: in macrophages, the downregulation of NOX2 also decreased thioredoxin levels. The link between NOX2 and thioredoxin was observed in our study as well; NOX2 was found only in post-MI patients' EVs, which also had a higher thioredoxin level according to western blot data. NADPH oxidases are usually described as enzymes responsible for increased atherogenesis except for NOX4 (NADPH oxidase 4) [40]. NOX2 isoform is undoubtedly an important enzyme in oxidative stress, yet its function in atherosclerosis remains enigmatic. NOX2 usually has a negative effect on the endothelium [41]. However, NOX2 deficiency has been shown to cause the loss of survival signaling through ERK and Akt B in neonatal rat myocytes [42]. Data from our study show that exosomes isolated from post-MI patients' blood carry both pro- and anti-oxidative enzymes, with a higher predominance of anti-oxidant effects. This finding was confirmed in the experiments with HUVEC cell culture, since exosomes isolated from the post-MI patients' blood induced lower oxidative stress when compared to the healthy group. It is an interesting finding, since MDA concentration, which indicates oxidative stress levels in post-MI patients, was higher when compared to the healthy ones. There is a possibility that the release of pro-oxidant NOX enzymes from the post-MI EVs might be more complicated and as such occurs less. Data from the present study suggest that exosomes in post-MI patients' blood might have anti-oxidative properties that could serve as an additional factor in controlling the post-MI condition.

Limitations of the Study

Since this is a pilot study, only a small sample of patients was collected. We plan to conduct further research based on the present study, but with a larger sample size and including both men and women, which could generate more accurate results. Another limitation is the implementation of the data collection method. There was no procedure to prevent material loss during the purification of exosomes. The ultracentrifugation method used here is known for losing sample material during purification. But we chose this method since the idea was to use the least expensive and most easily accessible method that could be employed in the clinical laboratory.

Conclusions

We compared biochemical markers (the yield and inflammatory and oxidative properties of EVs) isolated from both healthy controls and post-MI patients' blood samples. The isolated EV yield from post-MI patients was lower when compared to healthy controls. Despite the inflammatory origin of post-MI EVs, which was determined by showing a higher level of MDA in post-MI patients' blood, and as they carried pro-oxidant enzymes NOX1, NOX5, and NOX2, post-MI EVs caused less oxidative stress in endothelial cell culture experiments. Higher levels and better release of thioredoxin, ERK1/2, and Akt B might have strongly contributed to this effect. We conclude that there are quantitative and qualitative differences between

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the EVs of healthy vs post-MI individuals. Healthy controls EVs, as well as post-MI patient EVs, carry both pro-oxidant and anti-oxidant enzymes, but post-MI EVs have a stronger anti-oxidative effect on the endothelium, which could help improve the post-MI condition.

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Declaration of Figures' Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

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