Research Article

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Isolation and Quantification of Nanovesicles in Isoproterenol-Induced Myocardial Infarcted Rats

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Abstract

Introduction and Aim: *Myocardial infarction (MI) is one of the leading causes of death worldwide. The pathogenesis and aetiology of MI is still unclear and there is currently no cure for MI. Cardiac troponin is the only well-known cardiac-specific marker for the diagnosis of MI but due to the delayed release of troponin in the circulation, a novel cardiac biomarker is needed in the early stages of development of MI to reduce MI mortality. Recently, it has been reported that exosome concentration is highly regulated by stress and cardiovascular diseases (CVDs). We assessed the hypothesis that exosome secretion by the injured cardiomyocytes is increased during MI and thereby serve as biomarker for MI. The aim of this study was to quantify exosomes in an isoproterenol (ISO)-induced MI rats.*

Method: *Twelve rats were divided into two groups (group A and B). Group-A (n=6) was the normal control rats and group-B (n=6) was the ISO-treated group. Group-B animals were injected with isoproterenol (85mg/kg/bw) for two consecutive days to induce MI. Blood pressure (BP), heart rate (HR) and body weight were monitored for 7 days in all animals prior the ISO injection and throughout the experiment. After second ISO-injection, all animals were sacrificed and blood, heart tissues were obtained. Histopathological analysis was performed in heart tissue samples and levels of cardiac markers (creatine kinase-MB, lactate dehydrogenase and troponin T) were measured from the serum. Exosomes were isolated from the plasma by differential ultracentrifugation. Exosomes were quantified and characterized using nanoparticle tracking analysis (NTA), transmission electron microscope (TEM) study and ELISA for the quantification of circulating exosomal protein (CD63).*

Result and Discussion: *ISO injection caused the development of MI and was confirmed by the increase in BP, Cardiac markers (cardiac troponin T, lactate dehydrogenase and creatine kinase). NTA together with TEM analysis revealed particle sizes of the exosome and showed an elevated number of the exosome in ISO-treated animals. Levels of exosomal protein (CD63) were also increased in ISO-treated animals.*

Conclusion: *The concentration of exosomes was increased in MI rats indicating that circulating exosomes may be used as a novel diagnostic marker for MI.*

Keywords: Isoproterenol, Myocardial infarction, Exosome, Nanoparticle tracking analysis, Cardiac markers

Introduction

Cardiovascular diseases (CVD) and their thrombotic complications are the leading cause of morbidity and mortality in the world and mostly in developed countries [1]. The World Health Organisation (WHO) estimated that approximately 17.3 million people die from CVD and its complications worldwide and in African nations CVD constitute 8.8% of all deaths [2,3]. MI is one of the complications of CVD [4]. MI is commonly known as a heart attack and occurs when there is an imbalance in the oxygen supply and demand, which is caused by injury to the coronary artery, blocking blood flow to the heart thus myocardial cell death [5]. Early diagnosis of myocardial infarcted patients is required to prevent or reduce ischemic injury to the myocardium and subsequently prevent cardiac remodelling.

Creatine kinase (CK) and lactate dehydrogenase (LDH) were previously used for diagnosis of myocardial infarcted patients [6]. However, these markers are not cardiac specific markers as their levels increase in the circulation as result of various tissue damage. Thus they were replaced with cardiac troponin T and troponin I which are cardiac specific markers, these biomarkers have led to early diagnosis of MI. However there is a relative delay of the

release of troponin as in AMI patients, troponin levels raise around 3.5 hours after the onset of chest pain [7]. Earlier biomarkers with high specificity and sensitivity are in high demands to reduce MI mortality. In addition additional alternative markers are needed to reduce MI mortality. Recent studies have focused on exosomes as both potential biomarkers and therapeutic agent [8].

Exosomes are membrane vesicles that differ from others by their size (30-140nm), density and specific composition of molecules [9]. These are the smallest extracellular vesicles, originating from cellular endosomes and are generated in endosomal vesicles called multivesicular bodies (Figure 1) [10]. Exosomes have emerged as important biological signalling entities because they act as a vehicle to transport important molecules such as proteins, lipids and nucleic acids to distant cells. They are secreted by multiple cell types, including smooth muscle cells, endothelial cells, cardiomyocytes and stem cells [11]. The exosomes have unique protein markers which include CD9, CD63 and CD81 amongst others and are important in differentiating exosomes from other extracellular vesicles. Exosomes can be found in most body fluids such as saliva, blood and urine since they are secreted by multiple cells. Studies have shown that exosomal content is regulated by stress and disease conditions [12]. Although research on cardiac exosomes is new, a few publications provide strong evidence that exosomes can exert pathological effects during the cardiac response to stress and different myocardial diseases [13].

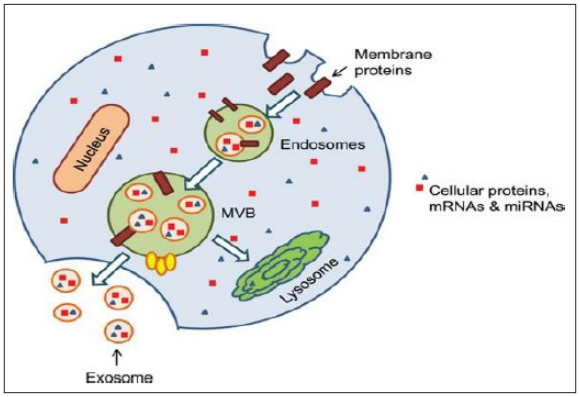


Figure 1: Biogenesis of exosomes from the cellular endosomes

A recent study reported that peripartum cardiomyopathy (PPCM) result to the release of miR-146-enriched exosomes by endothelial cells. These exosomes can be used as a vehicle by cardiomyocytes where miR-146a impedes with the physiological metabolism and contractile ability of the cell, resulting in hypertrophy. Moreover, levels of exosomal miR-146a are higher in plasma of acute PPCM patients as compared to patients with dilated cardiomyopathy and healthy postpartum controls. Furthermore, heart failure therapy in PPCM patients shows decreased circulating exosomal miR-146a to normal levels, suggesting that miR-146a is a strong potential biomarker for diagnosis PPCM [14]. The type of stress to which myocardial tissue is exposed may determine the level of exosome secretion. During MI where the heart is exposed to ischemic stress signals such as inflammation, hypoxia and injury, cardiomyocytes may elevate the secretion of exosomes [12]. miR-1 together with miR-208 are increased in the urine of acute MI patients suggesting that circulating miRNAs released from the injured myocardium can go to distant organs through exosomes as they are stable and protected from degradation by RNases present in the different body fluids (Figure 2) [12,15].

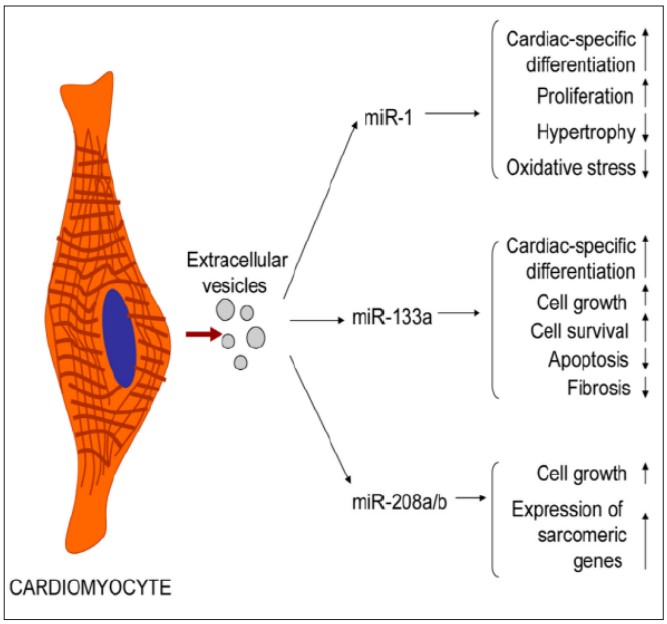


Figure 2: Exosomes secreted by cardiomyocyte transport miRNAs

The present study aimed to isolate and quantify exosomes in isoproterenol-induced MI rats. In this study we assessed the hypothesis that exosome secretion by the injured cardiomyocytes is increased during MI and thereby serve as potential biomarker for MI. We used isoproterenol-induced myocardial infarcted Wistar rats model to quantify exosomes in MI rats. We monitored blood pressure and cardiac marker's level to confirm the development of MI.

Materials and Methods

Ethics

UKZN Animal Ethics Sub-committee (Clearance Number 082/016PD) approved the experimental protocol used in this study.

Experimental Animals

All the experiments were carried out with male albino Wistar rats weighing 250-300g, obtained from Biomedical Research Unit, University of KwaZulu-Natal, Westville, Durban, South Africa. They were housed in polypropylene cages (47cm×34cm×20cm) lined with husk and renewed every 24h under a 12:12h light/dark cycle at around 22°C. The rats had free access to tap water and food. The rats were fed on a standard pellet diet. The experiment was carried out according to the Guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Durban, South Africa.

Drug and Chemicals

Isoproterenol (ISO) was purchased from Sigma Chemical Co., St. Louis, MO, USA. All other chemicals used were of analytical grade.

Experimental Design

Twelve animals were used in this experiment. They were grouped into two groups, group A (n=6) were the normal control rats and group B (n=6) were the test (MI) rats. The body weight and blood pressure of the both groups were measured every day. On the 8th day and 9th day Isoproterenol (85mg/kg body weight) was dissolved in ice-cold saline and injected subcutaneously into group B at an interval of 24h for two consecutive days to induce MI [16]. At the end of

the experimental period, after 12h of second isoproterenol injection (i.e. on 9th day), all the rats were anesthetized with the high dose of Isofar and were then sacrificed by cervical decapitation. Blood was collected in dry test tubes without anticoagulant for serum and plasma. Heart tissues were excised immediately, rinsed with saline and tissue homogenates were prepared phosphate saline buffer for the estimations/assays of various biochemical parameters. All the enzyme assays were done immediately.

Estimation of Cardiac Markers

The level of serum cardiac troponin-T, CK and CK-MB and LDH were estimated by electro chemiluminescence immunoassay using a standard kit (Roche Diagnostics, Switzerland).

Histopathological Examination

Immediately after the heart tissues were dissected from experimental animals, they were washed with saline and cut across the left ventricle into two slices. The heart tissue slices were then fixed in 10% buffered formalin and were embedded in paraffin wax, sectioned at 5µm and stained with haematoxylin and eosin (H&E). After fixation, the heart tissues were dehydrated by immersion into 70% ethanol overnight, followed by 90%, 100% and 100% for an hour. The tissues were then immersed into xylene two times for an hour. Finally, the tissues were put in molten wax (55-60°C) and moved into the pre-heated oven overnight to solidify before sectioning. The heart tissues were sectioned and mounted onto the slides. The slides were cleared using xylene solution and then dehydrated using ethanol (100, 90, 70 and 50%) for 2 minutes in each percentage. The slides were rehydrated using distilled water until ready for staining. The slides were stained with haematoxylin and rinsed off with distilled water, and stained again with eosin for 2-3 minutes. The residual eosin was rinsed-off with distilled water and slides were dipped into 90% ethanol to remove water. The slides were finally immersed into xylene solution and then left out to dry. They were covered with the coverslips and left overnight. Finally, they were examined under high power microscope (100X) and photomicrographs were taken.

Exosome Isolation and Quantification

Exosomes were isolated according to the method as described. Plasma (1ml) was diluted with an equal volume of phosphate buffered saline (PBS; pH 7.4). Exosomes were isolated and purified by differential ultracentrifugation using a 30% sucrose cushion. In brief, to remove cells, centrifugation was initially performed at 2000Xg at 4°C for 30 min, followed by 12 000Xg at 4°C for 45 min. To remove the remaining debris, the supernatant was transferred to other centrifuge tubes and centrifuged at 110 000Xg at 4°C for 120 min (Optima™ MAX-XP Ultracentrifuge, fixed angle MLA-55 rotor, Beckman Coulter Inc., Brea, CA, USA). To remove particles that are bigger than 200nm, the pellet was suspended in 2ml of PBS and filtered through a 0.20µm pore filter (Cellulose acetate, GVS™, Europe). The filtrate was centrifuged at 110 000Xg at 4°C for 70 min, the pellet was re-suspended in 2ml PBS (pH 7.4) and centrifuged at 110 000Xg for 70 min at 4°C. To purify the exosomes, the exosome pellet was suspended in 2ml of PBS and subsequently purified using a 30% sucrose cushion and centrifuged at 110 000Xg at 4°C for 75 min. The final pellet was re-suspended in 200µl of PBS and stored at -80°C.

The concentration of total exosomes in the circulation was determined by the quantification of total immune-reactive CD63 enzyme-linked immune absorbency assay (ExoELISA™, System Biosciences, Mountain View, CA), as described by Salomon et al. (2014). CD63 is not an exosome specific marker but is commonly bound to the exosomal membrane and hence the method employed uses isolated and purified exosomes, which contain the CD63 marker. The kit used consists of an exosome specific primary CD63 antibody developed by the manufacturer. Briefly, exosomes were immobilized on microtiter plates for overnight at 37°C using exosome binding buffer supplied the manufacturer (System Biosciences). Plates were washed and incubated at room temperature for 1h with exosome specific primary antibody (CD63), followed by a wash step and incubation with secondary antibody (1:5000) at RT for 1h with agitation. Plates were thereafter washed and incubated with Super-sensitive TMB ELISA substrate at RT for 45 min with agitation. The reaction was terminated using Stop Buffer solution. Absorbance was measured at 450nm. The number of exosomes/ml, (ExoELISA™ kit) was obtained using an exosomal CD63 standard curve that was generated using the calibrated exosome standard that was supplied.

Nanoparticle Tracking Analysis

The size distribution of exosomes and their concentration were determined using the NS500 equipped with a 405nm laser and sCMOS camera (NanoSight NTA 3.0 Nanoparticle Tracking and Analysis Release, Version Build 0069). Plasma samples were diluted with PBS (1:100) prior to analysis in order to obtain particle distribution of 10 and 100 particles per image (optimal, 50 particles per image) before the analysis with NTA system. Samples were introduced into the sample chamber using the following script: PUMPLOAD, REPEATSTART, PRIME, DELAY 10, CAPTURE 60 and REPEAT 5. Videos were recorded at a camera level of 10, camera shutter speed of 20ms and camera gain of 600, these settings were kept constant between samples. Each video was then analysed to give the mean particle size together with the concentration of particles. The size of the exosomes was represented as the mean particle size ± SD.

Transmission Electron Microscopy

Exosomes were applied to a continuous carbon grid and negatively stained with 2% uranyl acetate. The size and morphology of the particles were examined using a JEOL 1010 transmission electron microscope (JEOL, Peabody, MA, USA) at the Electron Microscopy Unit, University of KwaZulu-Natal.

Statistical Analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) by SPSS software 14.0 followed by Duncan's multiple range test (DMRT). Results were expressed as mean±S.D. from six rats in each group. P values <0.05 were considered as significant.

Results

Blood Pressure, Body Weight and Heart Weight

A significant increase in blood pressure and heart rate ($P<0.05$) in ISO-treated animals when compared to control group (Table 1). At the end of the experiment, the body weight of group B (ISO-treated animals) was considerable decreased ($P<0.05$) when compared to control group. The heart weight of ISO-treated animals was significantly increased ($P<0.05$) when compared to the control group (Table 1).

Table 1: Showing the effects of isoproterenol (ISO) on blood pressure, body weight, heart rate and heart weight of ISO-induced rats. Each column is mean ± standard deviation for six rats in each group; *as compared to normal control ($p < 0.05$ as compared to ISO treated, (DMRT)

Groups	Blood pressure (mmHg)	Heart rate (beats/min)	Body weight (g)	Heart weight (g)
Control	101.34 ±9.08	360 ±23.42	264.33 ±19.36	0.94 ±0.028
ISO-treated	178.66* ±10.13	497* ±32.04	201.45* ±18.26	1.47* ±0.033

Cardiac Markers

Rats treated with ISO showed significant ($P<0.05$) increase in the activities of cardiac marker enzymes in serum compared to normal control rats. Figure 3A shows a considerable ($P<0.05$) increase in the levels of serum troponin T (cTnT) in ISO-induced rats as compared to control rats. Figure 3B, 3C and 3D shows a significant increase in the activities of creatine kinase, creatine kinase-MB and lactate dehydrogenase (LDH) ($P<0.05$) in ISO- treated rats when compared to control rats.

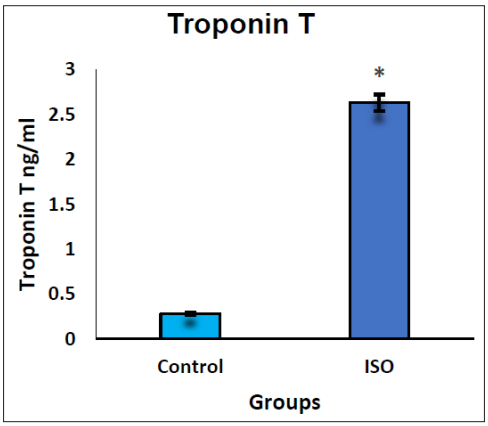


Figure 3A: Graph showing changes in the levels of serum cardiac troponin T (cTnT; in ng/ml) in rats following the treatment with isoproterenol (ISO). Each column is mean ± standard deviation for six rats in each group; *as compared to normal control; $p < 0.05$ as compared to ISO treated, (DMRT)

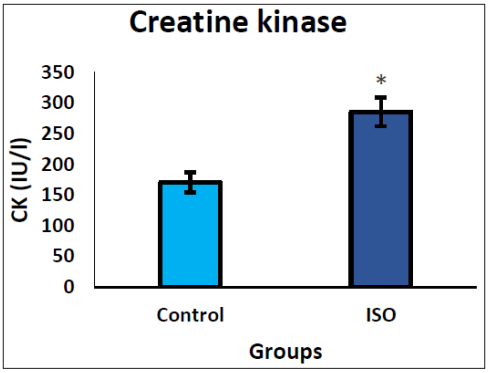


Figure 3B: Graph showing changes in the levels of serum creatine kinase, each column is mean ± standard deviation for six rats in each group; *as compared to normal control; $p < 0.05$ as compared to ISO treated, (DMRT)

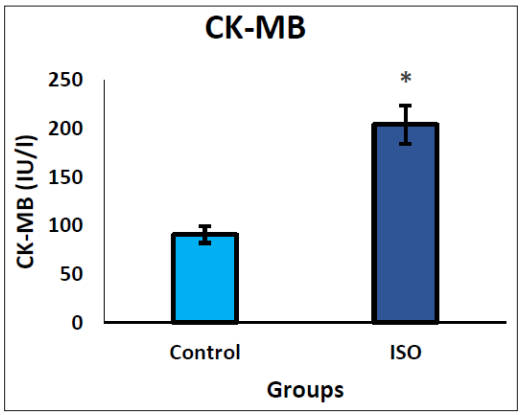


Figure 3C: Graph showing changes in the levels of serum Creatine kinase-MB (CK-MB, in rats following treatment with ISO. Each column is mean ± standard deviation for six rats in each group; *as compared to normal control; $p < 0.05$ as compared to ISO treated, (DMRT)

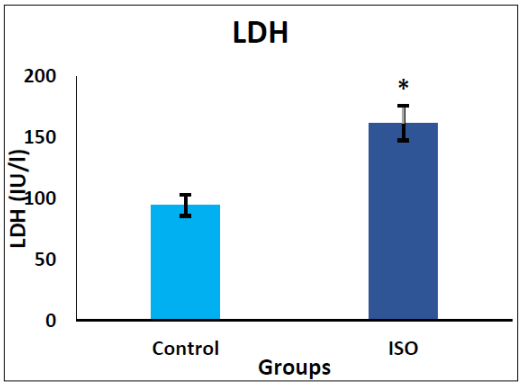
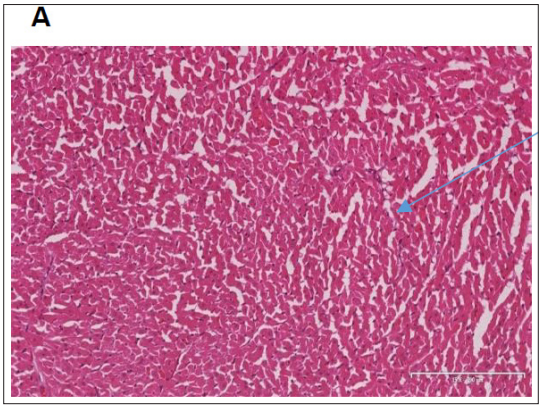


Figure 3D: Graph showing changes in the levels of serum lactate dehydrogenase (LDH, in IU/L) in rats following treatment with isoproterenol. Each column is mean ± standard deviation for six rats in each group; *as compared to normal control; $p < 0.05$ as compared to ISO treated, (DMRT)

Histopathological Analysis

Figure 4 (A and B) shows histology of heart in normal control and ISO-induced MI rats. Normal untreated rats showed normal cardiac fibres without any infarction (Figure 4A). Figure 4B shows the myocardial infarcted rat myocardium with massive necrosis of cardiac muscle fibres and inflammatory cells.



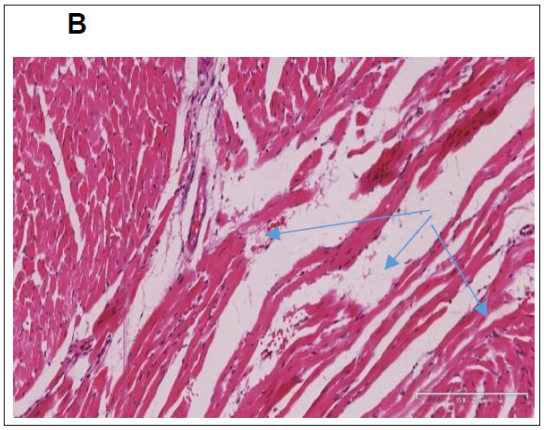


Figure 4A and B: shows a photomicrograph of control rat heart with normal cardiac muscle fibres. B shows a photomicrograph of ISO treated rat heart with swollen cardiac myocyte and massive necrosis of muscle fibres and inflammatory cells are visible

Exosome Quantification and Characterization

Figure 5 (A and B) shows electron micrograph of isolated exosomes, scale bar of 200nm with a particle diameter of ~100nm.

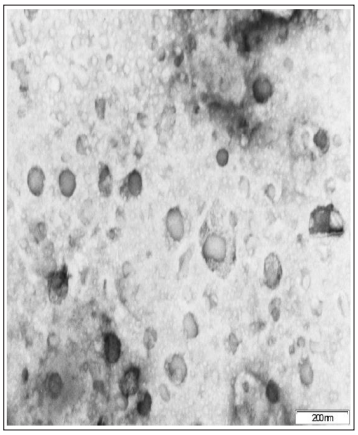
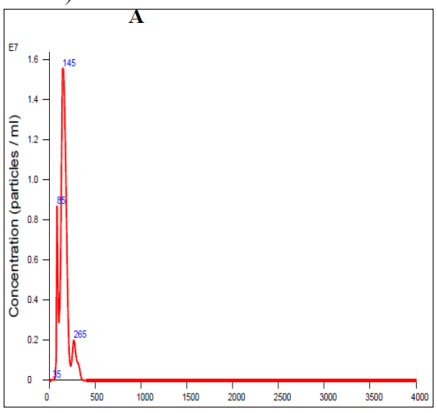


Figure 5: characterization of exosomes from plasma. Figure 5 shows electron micrographs of isolated exosomes, scale bar 200 nm

Figure 6 and Figure 7 show NTA results of the size distribution and exosome concentration of both groups. NTA of exosomes showed a significant increase in total exosome concentration of the ISO-treated rat plasma (2.88×10^8 total exosomes/ml) as compared to the total exosome concentration of control rat plasma (1.54×10^8 total exosomes/ml).



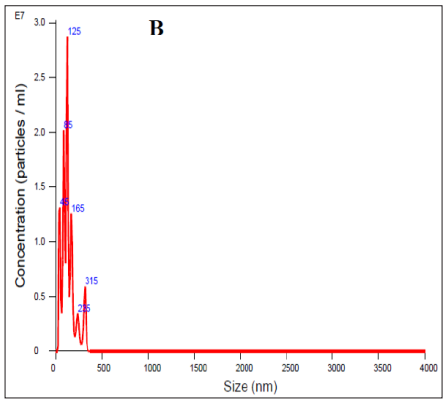


Figure 6: Characterisation and Quantification of Exosomes from Circulation. Exosomes from control and ISO treated rats were characterised and quantified using Nanoparticle Tracking Analysis. (A) Representative vesicle size distribution (145nm) and concentration (1.54×10^8 total exosomes/ml) of normal control rats. (B) ISO treated rats (125nm) and concentration (2.88×10^8 total exosomes/ml). Values are represented by mean

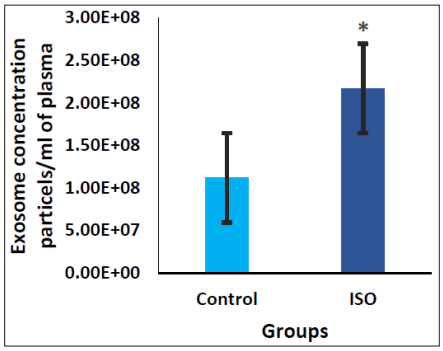


Figure 7: Graph showing the quantity of isolated exosomes from both control and ISO treated plasma. Exosomes were quantified using NTA. Values are mean ± SD

Quantification of Exosomes Using ELISA

ELISA kit was used for the quantification of total exosomal protein. ISO-treated circulatory exosomal protein concentration was significantly increased (476mg protein/ml plasma) as compared with control (350mg protein/ml plasma) (Figure 8). ELISA was used to quantify exosomal marker CD63, circulating exosomal protein (CD63) was significantly increased in ISO-treated rat's plasma (2.15×10^8 CD63/ml) as compared to normal control rat's plasma (1.10×10^8 CD63/ml) (Figure 9).

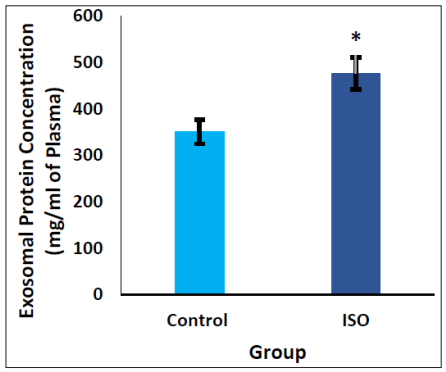


Figure 8: Graph showing total circulatory exosomal protein concentration (mg protein/ml plasma). There is a significant increase

in the total circulatory exosomes in ISO-treated animals as compared with Control; each column is mean ± standard deviation for six rats in each group; *as compared to normal control; $p < 0.05$ as compared to ISO control, (DMRT)

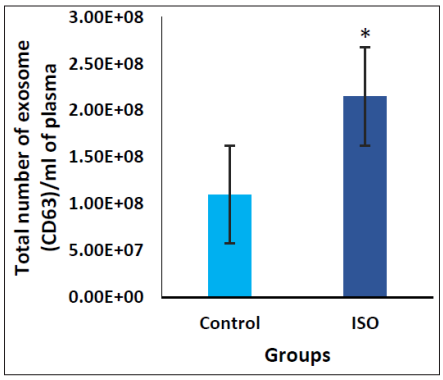


Figure 9: Graph showing changes in the levels of exosomal marker CD63 following treatment with ISO. Total exosomes were determined by the quantification of exosomal CD63 marker between control and ISO treated animals. There was a significant increase in CD63 in ISO-treated rats. Values are represented as mean ± SEM

Discussion

Biogenesis, characterization and functions of exosomes are a novel and exciting field of research that has triggered significant interest over the past decade. Exosomes (as well as other types of extracellular vesicles) play a vital role in regulating a broad range of physiological and pathological cellular processes. They may be utilized for therapeutic purposes and act as candidate biomarkers in various diseases [17]. A vascular disease like MI affects a high proportion of the population. Based on the previously published studies we used an ISO model to induce MI in experimental rats in order to characterize and quantify exosomes secreted during MI. ISO is a potent synthetic catecholamine that causes severe stress in the myocardium, resulting in infarct-like necrosis of the heart muscle [18]. ISO induces oxidative stress and results in alterations of cardiac function and ultrastructure in experimental rats [19]. Experimental induction of MI by ISO in animals is a well-established model to study the protective role of various cardio protective agents. Studies have shown that animals develop MI when injected with high doses of catecholamines [20,21]. Evidence of MI was initially indicated by the significant increase in the blood pressure and heart rate of the ISO-treated rats. The ISO administration caused the leakage of cardiac marker enzymes such as cardiac troponin T, CK, CK-MB and LDH of heart tissues to the circulation as evidence was indicated by the increase in the levels of these markers in circulation of ISO-treated rats. Increased levels of these enzymes in the circulation may be due to myocardial necrosis, hypoxia and damage of cell membrane [22]. In addition, histological analysis of ISO-treated heart tissue revealed damages in the myocardium.

Hypoxia is a potent stimulator for exosome release by cardiomyocytes [23]. Studies have shown that exosomes secreted by primary cultured cardiomyocytes during hypoxia have a high content of tumor necrosis factor (TNF)-α, a proinflammatory cytokine whose cardiomyocyte expression is induced by hypoxia-inducible factor (HIF)-1α [24]. The type of stress to which myocardial tissue is exposed may determine the level of exosome secretion. During MI where the heart is exposed to ischemic stress signals such as inflammation, hypoxia and injury, cardiomyocytes are thought to elevate secretion of exosomes, but to the best of our knowledge, no study involve this model has provided evidence of the increased circulating exosome concentration secreted during MI [12]. Our study provided evidence of increased levels of the exosome in the circulation during MI. Exosomes with a mean particle size distribution of (125.4nm) and concentration of (1.54×10^8 total exosomes/ml plasma) were obtained from control rat plasma. However, in the ISO-treated rats there was a significant increase in the quantity of exosomes with a mean particle distribution of (123nm) and concentration of (2.88×10^8 total exosomes/ml plasma) compared to control rat's plasma. Exosomal protein (CD63) was significantly increased in ISO- treated rats as compared to normal control rats (Figure 9), indicating increased levels of exosomes in the circulation during MI. Although exosomes were quantified from the circulation, which may have several sources, we assumed that cardiomyocytes secrete elevated amount of exosomes during MI.

Exosomes are the smallest extracellular vesicles, therefore purification methods of exosomes are limited and have not been validated [25]. In our study, to confirm that the entities isolated from plasma were indeed exosomes, we used different techniques to verify the particle size. NTA and TEM were used to characterize the exosomes. All particles obtained from TEM analysis were spherical in shape with a particle diameter of about 40-90nm. A slightly different particle size was obtained from NTA, particle diameter of about 90-130nm. The difference in the particle size distribution obtained from NTA may be because NTA uses light scattering and Brownian motion in order to obtain particle size distribution in a solution and large particles contribute more strongly to the light scattering than small particles thus are more visible [26]. Hence, we concluded that the isolated extracellular vesicles were indeed exosomes.

Exosomes cannot only be viewed as potential biomarker candidate, are the key component of paracrine secretion in many cell types and are an important component in cell therapies [27]. Exosomes may have either harmful or beneficial effect, as exosomes secreted from neonatal rat cardiac fibroblasts were found to be enriched with miR-21 which promote cardiac cardiomyocyte hypertrophy and exosomes secreted by Goto-Kakizaki (GK) rat cardiomyocytes were found to be enriched with miR-320 which inhibits myocardial endothelial cell promotion, migration and tube formation [1,28]. In addition, exosomes secreted by platelets from patients with sepsis-induced endothelial cell apoptosis and cardiac dysfunction through NADPH, NOS and PDI [29]. Several studies have focused only on the pathological role of exosomes as the entities that spread diseases throughout the body, however that may not be the only case. Therefore, it is important to know the actual quantity of exosomes secreted during heart-related diseases. Our study showed evidence that there is a significant increase in the secretion of exosomes during MI. Nevertheless, further studies are still required to verify this study and quantify cardiac specific exosomes secreted during MI.

Conclusion

Our study has shown an increased exosome concentration secreted during MI. These findings indicate that exosomes are potential biomarkers for MI. However, exosomes were quantified in the circulation in this study, therefore the source is still unverified. Further studies are required to quantify cardiac specific exosomes secreted during MI.

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