REVIEW

Extracellular vesicles in diabetic cardiac and cerebro-vascular pathology
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Abstract: Individuals with type 2 diabetes mellitus develop more frequently than healthy controls cardio- and cerebro-vascular disorders. It is important to understand the mechanisms through which diabetes contributes to the development and severity of these complications. Extracellular vesicles (EV) are important mediators of cell to cell communication in several diseases, including diabetes mellitus and vascular disease. Three populations of EVs are described presently: exosomes, microvesicles and apoptotic bodies. Recent studies have shown that atherosclerotic lesions of all stages contain microvesicles. Higher levels of circulating EVs have been discovered in individuals with cardiovascular risk factors, with both pro-atherogenic and anti-atherogenic effects. Regarding cerebrovascular disease, studies have shown that exosomes, especially those derived from stem cells, play an important role, preventing post-ischemic suppression. Different cell types in the heart contribute to the pathogenesis of diabetic cardiomyopathy and EV seem to be essential in the intercellular crosstalk between heart cells. According to recent research EVs could play an important role in different cardiac and cerebral regenerative therapies and could also be used as therapeutic vectors in cardiovascular medicine. Further large animal and human studies are necessary to validate EVs as diagnostic and therapeutic tools.

Keywords: Diabetes, cardiovascular disease, cerebrovascular disease, cardiomyopathy.

INTRODUCTION
Diabetes mellitus is a common chronic disease all over the world, with increasing incidence and prevalence, due to contemporary lifestyle with reduced physical activity, processed food and increased obesity. A meta-analysis published in 2009, including studies from 91 countries, reported an estimate for 2010 of 285 million people with diabetes worldwide, with significant differences between populations and regions, and a predicted increase from 2010 to 2030 of 54%, corresponding to an annual growth of 2.2%, nearly twice as high as the annual growth of the total world adult population1.

Individuals with type 2 diabetes mellitus develop more frequently than healthy controls cardiovascular disorders, including coronary heart disease, stroke,
Peripheral arterial disease, and diabetic cardiomyopathy, mainly through the chronic, damaging exposure of the vascular system to hyperglycemia. Therefore, it is important to understand the exact mechanisms through which diabetes contributes to the development and severity of these complications.

Extracellular vesicles (EV) represent small cell-secreted structures naturally released into the extracellular space by all eukaryotes and many prokaryotes, containing proteins, lipids, nuclear material and noncoding RNAs. They were first described in the late 1960s and are important mediators of cell to cell communication in several diseases, including diabetes mellitus and cardiovascular disease. Most cell types can release vesicles into the interstitial space. These vesicles can be found in body fluids, both human and animal, such as blood, urine, tears and saliva, as well as in cell cultures.

Three populations of extracellular vesicles are described presently: 1. Exosomes are the smallest, with a diameter around 30-150 nm in diameter. They are released through exocytosis after fusion of multivesicular bodies with the plasma membrane; 2. Microvesicles (microparticles/ectosomes) are larger vesicles, with a diameter of 100-1000 nm, which are formed by the outward budding and scission of the extracellular membrane; 3. Apoptotic bodies are the largest subtype of microvesicles with a diameter of 1-5 μm, generated by the plasma membrane of apoptotic cells.

Several strategies are currently available for the quantification of extracellular vesicles, the most popular being ultracentrifugation, among others, such as: density gradient, precipitation, field flow, chromatography and affinity based capture and microfluidic techniques. All available isolation methods are time intensive, require expensive equipment, and are limited by the fact that they do not purify specific populations of vesicles, probably due to lack of standardization of the techniques and methods. In the last 8 years, the International Society of Extracellular Vesicles has constantly tried to update the topics of nomenclature, separation, characterization and functional analysis of EVs.

**EV AND CORONARY ARTERY DISEASE**

Recent studies have shown that atherosclerotic lesions of all stages contain microvesicles. Higher levels of circulating microvesicles have been discovered in individuals with cardiovascular risk factors, such as smoking, dyslipidemia, diabetes mellitus and arterial hypertension, probably through activation or from apoptosis of different cells being exposed to a damaging stimulus.

Data extracted from *in vitro* studies suggest that microvesicles can have both pro-inflammatory and anti-inflammatory effects, depending on different situations. Microvesicles increase the release of pro-inflammatory cytokines (mainly interleukin 6 and 8) from endothelial cells and leukocytes, promoting the adhesion of monocytes to the endothelium and their migration to the atherosclerotic plaque. Also, endothelial microvesicles can activate monocytes. Another effect of microvesicles is their interaction with the vascular endothelium and decreasing the NO production by endothelial cells—consequently impairing endothelial properties. Endothelial microvesicles and platelet derived microvesicles increase endothelial permeability. Microvesicles promote adhesion of monocytes to the endothelium by increasing endothelial expression of adhesion molecules.

Various microvesicles contribute to foam cell formation in the atherosclerotic plaque by stimulating lipid and cholesterol formation in macrophages. Macrophages and foam cell undergo afterwards apoptosis, forming a core of extracellular lipids. Increased monocytes and macrophage apoptosis contributes to increased microvesicle release in the plaque. Microvesicles of monocyte and macrophage origin are the largest population of microvesicles in human atherosclerotic lesions.

Infiltration of LDL particles in the vascular wall during the atherosclerotic process can induce the formation and release of tissue factor enriched microvesicles from the smooth muscle cell, microvesicles which in their turn influence smooth muscle cell proliferation and migration.

Extracellular vesicles of different origins, with different microRNA content, contribute to smooth muscle cell proliferation.

Several studies of patients with stable coronary artery disease have reported increased levels of circulating microvesicles. Specific microvesicle subpopulations, especially those of endothelial origin, characterized by CD 144+, CD 131+/annexin A5+, or microvesicles containing miR-199a and miR-126, are currently researched as interesting biomarkers for cardiovascular risk and mortality in these patients.

Calcifications present in the atherosclerotic plaque have destabilizing effect in early lesions, favoring rupture, but gain a potential protective effect in advanced...
lesions with heavy calcium deposits\textsuperscript{23}. The calcification process is based on three mechanisms: 1. Cell apoptosis, which releases microvesicles and necrotic debris, leading to nucleate apatite; 2. Deficiency of mineralization inhibitors (both tissue derived and circulating), leading to random apatite deposits; 3. An altered differentiation of vascular smooth muscle cells and stem cells, leading to bone formation\textsuperscript{24}. As several studies have shown, atherosclerotic plaque calcifications are associated with extracellular vesicles of endothelial, smooth muscle cell and macrophage origin.

Exposure of vascular smooth muscle cells to pro-inflammatory cytokines can stimulate the release of exosomes which can mineralize when inhibitors of calcification are missing or not functioning\textsuperscript{25}. Also, endothelial cells exposed to proinflammatory stimuli can release microvesicles rich in bone morphogenetic protein 2, promoting calcification in vascular smooth muscle cells\textsuperscript{26}. Alterations in local homeostasis of calcium and phosphate lead to the formation of macrophage derived exosomes which stimulate mineralization\textsuperscript{27}.

Recent studies have also noted that in humans, advanced atherosclerotic plaques have a high content of procoagulant microvesicles, originating form leukocytes, erythrocytes and smooth muscle cells. These microvesicles can actively initiate the coagulation cascade, either through the presence of tissue factor on their surface, or by exposing phosphatidylserine, which concentrates factors VII and VIIa on their outer membrane\textsuperscript{16,28}.

Circulating leukocyte and platelet derived microvesicles can affect the clotting\textsuperscript{29}, but the actual magnitude of the prothrombotic effects of microvesicles in acute coronary syndromes is still under evaluation\textsuperscript{3,30}.

In opposition to microvesicles, exosomes have shown antithrombotic effects. In animal studies platelet-derived exosomes suppressed platelet aggregation and occlusive thrombosis\textsuperscript{31}.

Microvesicles influence different mechanisms that lead to plaque destabilization and rupture. Intraplaque hemorrhages are produced by neovascularization originating from adventitial tissue, stimulated by plaque microvesicles, such as CD40\textsuperscript{+} vesicles of macrophage origin. Hemorrhages are also favored by leukocyte and endothelial microvesicles with fibrinolytic activity\textsuperscript{32,33}.

Microvesicles can rise endothelial permeability\textsuperscript{34} and also modulate inflammation in the plaque\textsuperscript{35}, promoting fibrous cap rupture. Fibrous cap weakening is associated with smooth muscle cell apoptosis, induced by the presence of microvesicles and exosomes, released in some pathological conditions\textsuperscript{36}. Moreover microvesicles can influence breakdown of matrix structural proteins through metalloproteinase (MMP) interactions\textsuperscript{3}.

The final evolution of the atherosclerotic plaque is represented by plaque erosion or rupture with in situ thrombosis, clinically expressed as acute coronary syndrome\textsuperscript{3}.

Circulating levels of procoagulant microvesicles are higher in patients with acute coronary syndromes compared to healthy controls or patients with stable coronary artery disease\textsuperscript{37}, the origin of those microvesicles being mostly endothelial cells, leukocytes, erythrocytes and platelets\textsuperscript{22,37}. Circulating microvesicles alterate endothelium dependent NO mediated vasodilation and endothelial microvesicles increase endothelial thrombogenicity\textsuperscript{14,38}.

Circulating microvesicles have been also investigated as prognostic markers in secondary prevention, in order to identify patients at high cardiovascular risk\textsuperscript{21,22}. Increased levels of CD11b\textsuperscript{+}/CD66\textsuperscript{+} leukocyte derived microvesicles could be useful in identifying asymptomatic patients at high risk for plaque rupture\textsuperscript{39}, while CD3\textsuperscript{+}/CD45\textsuperscript{+} microvesicles could identify individuals who will develop a major cardiovascular event\textsuperscript{40}.

In patients with acute ST elevation myocardial infarction, circulating microvesicles from the coronary arteries contain higher levels of oxidation specific epitopes, linked to inflammatory responses involved in atherosclerosis, than microvesicles from the peripheral circulation\textsuperscript{41}.

Circulating exosomes and microvesicles with specific cardiac microRNAs, increase after coronary artery by-pass. Expression of miR-208a in circulating exosomes increases in patients with acute coronary syndromes\textsuperscript{42} and specific p-53 responsive microRNAs from plasma exosomes are predictive indicators for heart failure after myocardial infarction\textsuperscript{43}.

**EV AND CEREBROVASCULAR DISEASE**

Regarding cerebrovascular disease, especially stroke, studies have shown that exosomes, especially those derived from stem cells, play an important role in neurological disease, preventing post-ischemic suppression. Also, exosomes might be an interesting therapeutic resource in the field of regenerative medicine after stroke\textsuperscript{44}.

After stroke, exosomes are released from brain cells and can be detected in the peripheral blood or
the cerebro-spinal fluid\textsuperscript{45,46}. As a response to stroke, exosomes are released also from blood cells and endothelial cells\textsuperscript{57}. Circulating exosomes could therefore be useful biomarkers for stroke progression and recovery\textsuperscript{44}.

Exosome levels of cystatin C and CD 14 have been good predictors in studies of vascular risk in patients with coronary artery disease and also they have been associated to the progression of cerebral atrophy in patients with vascular disease\textsuperscript{48}.

Circulating exosomes can express different microRNAs in various types of cerebrovascular disease. Serum exosomal miR-9 and miR-124 levels are higher in patients with stroke compared to controls\textsuperscript{49}. Another study has reported higher levels of miR-223 in acute ischemic stroke, correlated to stroke severity and short term outcomes\textsuperscript{50}. Finally miR-199b-3p, miR 27b-3p, miR-130a-3p, miR 221-3- and miR-24-3p are more expressed in patients with asymptomatic carotid artery stenosis progression\textsuperscript{51}.

Exosomes derived from mesenchymal stem cells have enhanced in animal studies the restorative effects in the brain after stroke, reducing neurological impairment, promoting grey matter repair and white matter repair, as well as neurogenesis and reversing stroke-induced peripheral immunosuppression\textsuperscript{52-56}.

Cardiovascular dysfunction has been proposed as one of the main causes of cognitive impairment in the elderly, this association being stronger in patients with diabetes mellitus\textsuperscript{57,58}. In vitro studies have shown that extracellular vesicles in diabetic microvascular disease may increase the haemato-encephalic barrier permeability\textsuperscript{59,60}.

**EV AND DIABETIC CARDIOMYOPATHY (DCM)**

Diabetic cardiomyopathy can be clinically defined by the presence of abnormal myocardial performance or structure in the absence of epicardial coronary artery disease, hypertension, and significant valvular disease. Hyperglycemia is the cornerstone of the pathogenesis, inducing stimuli that result in myocardial fibrosis and collagen deposition. These processes are generating altered myocardial relaxation and determine diastolic dysfunction on ultrasound imaging\textsuperscript{61}. Over time, the progression of diabetic cardiomyopathy can lead to clinically manifest heart failure. Different cell types in the heart (such as cardiomyocytes, endothelial cells, smooth muscle cells, hematopoietic derived cells and fibroblast cells) contribute to the pathogenesis of DCM and several studies have shown that EV are essential in the intercellular crosstalk between heart cells\textsuperscript{3,62}.

Cardiomyocyte’s derived EV are implicated in diabetic cardiomyocyte steatosis. Higher levels of miR-1 and miR-133a were noted in EV derived from lipid loaded cardiomyocytes, in the serum of mice fed with a high fat diet and in the circulation of diabetic patients\textsuperscript{63}.

An animal study performed in 2014 has shown that the communication between cardiomyocyte derived EV and endothelium is altered in diabetes, inducing an altered angiogenesis\textsuperscript{64}.

Endothelial cell death and dysfunctional angiogenesis are frequent in diabetes mellitus. Several microRNA based mechanisms have been studied in order to explain vascular dysfunction in diabetes\textsuperscript{6}. Hyperglycemia increases levels of miR-503 in the endothelium, leading to low endothelial cell proliferation and angiogenesis\textsuperscript{45}. Also, hyperglycemia reduces miR-126 expression in extracellular vesicles derived from endothelial cell, thus impairing endothelial cell repair\textsuperscript{64}. Reduced miR-126 expression in circulating EV and endothelial progenitor cells derived EV in patients with uncontrolled diabetes altered endothelial repair, increased apoptosis and the production of reactive oxygen species\textsuperscript{67}.

Cardiac fibroblasts are important components of the fibrotic response in diabetic cardiomyopathy. A potential mediator of the pro-fibrotic action induced by hyperglycemia in the cardiac fibroblasts is miR-21\textsuperscript{68}. Inhibition of miR-21\textsuperscript{69} in mice with cardiac hypertrophy suppressed the myocardial thickening\textsuperscript{68}. Also, a model including in vitro cellular stretch and in vivo pressure overload has induced the release of extracellular vesicles form cardiomyocytes enriched with angiotensin type I receptor\textsuperscript{69}.

**THERAPEUTIC POTENTIAL OF EV**

According to results from studies from the last 5 to 10 years, extracellular vesicles could play an important role in different cardiac regenerative therapies and could also be used as therapeutic vectors in cardiovascular medicine.

Platelet derived vesicles induce vascular endothelial growth factor (VEGF) dependent angiogenesis and stimulate post-ischemic revascularization after chronic ischemia\textsuperscript{70}. Also, plasma derived exosomes activate Toll like receptor 4 on cardiomyocytes and thus protect the myocardium from ischemia-reperfusion injury\textsuperscript{71}.

Mesenchymal stem cell derived extracellular vesicles could be an alternative to stem cell transplanta-
tion after myocardial ischemia, by transfer of specific microRNAs through embryonic stem cell extracellular vesicles\textsuperscript{72}.

The use of extracellular vesicles as therapeutic vectors could be done through bioengineering, either by modifying the cytosolic content of the vesicles which could be transferred to the target cell in order to influence cell metabolism; or by loading of extracellular vesicles with molecules to be delivered to target cells. Studies regarding the use of extracellular vesicles as therapeutic vectors in cardiovascular disease are few and are only on animal models. For example, administration of apoptotic bodies containing miR-126 decreased atherosclerotic plaque formation in mice\textsuperscript{73} and stimulate vascular endothelial cell repair after vascular injury\textsuperscript{48}.

Different cardiovascular medications can influence the level of circulating microvesicles. Antiplatelet agents (ticlopidine, abiciximab) inhibit platelet activation and also the release of platelet-derived microvesicles\textsuperscript{74,76}. Antihypertensive agents (such as angiotensin II receptor inhibitors, beta blockers and calcium channel blockers) lower the circulating levels of platelet and monocyte derived microvesicles\textsuperscript{77}. The effects of statin treatment on circulating microvesicles of platelet and endothelial origin are still under debate\textsuperscript{31,78}. Statins and antihypertensive medication are able to modify the properties of in vivo generated endothelial microvesicles and their effect on the expression of endothelial adhesion molecules, inhibiting the adhesion of monocytes to endothelial cells and improving endothelial function\textsuperscript{79}.

Exosomes from various cell types (such as embryonic stem cells, neural stem cells and mononuclear stem cells), have been tested as treatment for stroke in addition to mesenchymal stem cell derived exosomes and showed good results in animal models of stroke, with improvement in neurological scores and reduction in lesion volume and tissue loss\textsuperscript{64}, showing a promising clinical applicability regarding neurological restorative effects and meeting also important safety considerations.

CONCLUSIONS

Extracellular vesicles are vectors of biological information that could influence cardio- and cerebro-vascular disease in diabetic patients, by transferring beneficial or negative mediators/stimuli. Also, they have strong therapeutic potential, especially regarding regenerative medicine. The issue of correct isolation of extracellular vesicles from circulation, liquid biopsies and different tissue still limit current knowledge on this subject. Also, there is still little information about in vivo dynamics of extracellular vesicles. Further large cohort animal and human studies are necessary to validate extracellular vesicles as diagnostic and therapeutic tools.

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