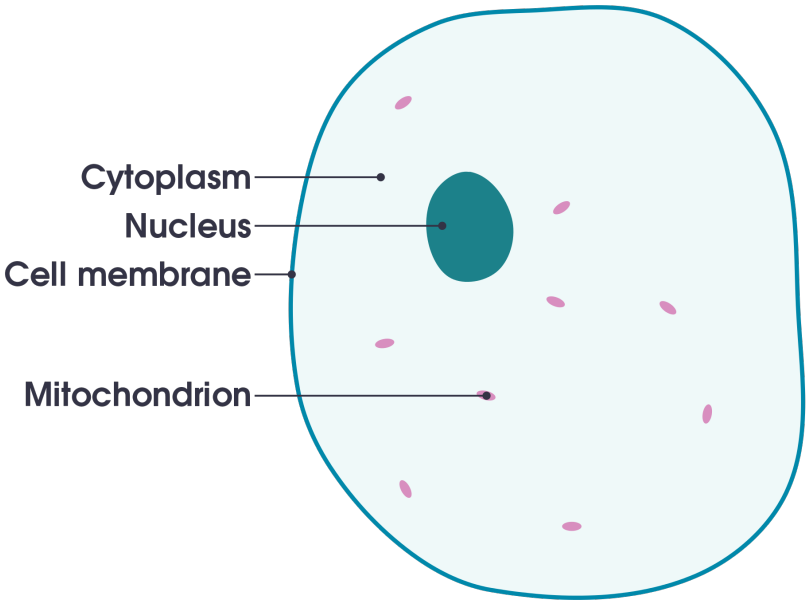
ISSN: 2198-4093

www.bmrat.org

Volume 4
Issue 01
January 2017

BIOMEDICAL RESEARCH AND THERAPY



BioMedPress
The Open Access Publisher

Editorial Team

Contact us

Journal Contact

BIOMEDPRESS (BMP)
Laboratory of Stem Cell Research and Application
University of Science, Vietnam National University, Ho Chi Minh city
227 Nguyen Van Cu
District 5, Ho Chi Minh city
Vietnam
Email: contact@bmrat.org

PRINCIPAL CONTACT
Lili Hami
BIOMEDPRESS (BMP)
Laboratory of Stem Cell Research and Application
University of Science, Vietnam National University, Ho Chi Minh city
227 Nguyen Van Cu
District 5, Ho Chi Minh city
Vietnam
Email: managingeditor@bmrat.org

SUPPORT CONTACT
Support Team
Email: support@bmrat.org

EDITOR-IN-CHIEF
Phuc Van Pham
Email: pvphuc@bmrat.org

Table of Contents

Vol 4 No 1 (2017): 1062 - 1128

Reviews

Research articles

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Review

Mesenchymal stem cells for diabetes mellitus treatment: new advances

Loan Thi-Tung Dang*, Ngoc Kim Phan, Kiet Dinh Truong

Laboratory of Stem Cell Research and Application, University of Science, Vietnam National University, Ho Chi Minh city, Viet Nam

Abstract

Mesenchymal stem cells (MSCs) are the most widely used stem cells of the human body due to ease of successful isolation and expansion for many years. In particular, from 2012 until now, MSCs have been widely clinically used to treat various diseases, including graft versus host disease (GVHD), Crohn's disease, and knee osteoarthritis. In this review, the applications of MSCs in diabetes will be reviewed and discussed. Diabetes mellitus type 1, also known as Type 1 diabetes (T1DM), is an autoimmune disease in which immune cells attack the beta cells in islets of Langerhans (pancreatic islets). Although type 2 diabetes (T2DM) is considered to be a disease related to insulin resistance, several recent studies have shown some relation of immune dysfunction in this disease. Therefore, MSC transplantation may be a beneficial treatment for both T1DM and T2DM. MSC transplantation in preclinical trials and clinical trials for T1DM and T2DM have shown a moderate to significant improvement in diabetes without adverse side effects. In this review, we will discuss some of the updates from preclinical and clinical trials of MSC transplantation for diabetes.

Keywords

Diabetes mellitus, Mesenchymal stem cells, Stem cell therapy, Stem cell transplantation

***For correspondence:**

dttloan@hcmus.edu.vn

Competing interests: The authors declare that no competing interests exist.

Received: 01 December 2016
Accepted: 10 January 2017
Published: 15 January 2017

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Introduction

Diabetes mellitus (DM) is a metabolic disorder caused by deficient insulin secretion or insulin dysfunction leading to hyperglycemia as well as chronic metabolic change of carbohydrates, protein and fat (Bastaki, 2005). Chronic hyperglycemia leads to many serious complications, namely kidney failure, heart issue, and eye diseases. Thus, it considered a costly disease because a majority of countries spend 5% to 20% of their health expense on diabetes, according to the International Diabetes Federation (IDF) (IDF, 2015). Moreover, diabetes is a high-incidence disease. In 2004, there were more than 200 million DM patients; the predicted prevalence of DM is expected to double by 2025 (Halban, 2004). Also according to the IDF, of the approximate 7 billion people worldwide, about 415 million adults (aged 20-79) suffered from diabetes in 2015 and that number is expected to rise to 642 million people in 2040 (IDF, 2015). The Western Pacific region of the world has the highest prevalence of diabetes, with 153 million cases which account for about 37% of total worldwide cases.

Diabetes is classified into two main types (type 1 and type 2) which both lead to hyperglycemia (Moorefield, 2012). Diabetes mellitus type 1, or Type 1 diabetes (T1DM), is described as a genetic autoimmune disease. It is caused by a severe deficiency of insulin due to damage of pancreatic islet beta cells (Zhang et al., 2008). T1DM patients have to depend on exogenous insulin injection to stabilize their blood glucose. Diabetes mellitus type 2 (T2DM) accounts for 90-95% of diabetes cases, and is caused by insulin resistance in peripheral tissues. T2DM is related to many risk factors, such as obesity, hypertension, lifestyle, age, family history, past history of impaired glucose tolerance (IGT) and impaired fasting glucose (IFG), and gestational diabetes (GDM). Many of the risk factors can be preventable.

Diabetic specialists have been studying ways to optimize therapy for diabetes since current therapies have two major limitations. The first is the challenge of insulin injection; there is long-term dependency, difficulty in adjusting the exact amount of exogenous insulin appropriate for each moment, and potential of insulin resistance when used long-term. The second limitation is pancreas/ islet/ islet cell transplantation; there is the problem of donor shortage and non-availability, potential of transplant rejection, and difficulty to functionally activate and prolong grafted materials. Therefore, improved treatments for diabetes which can address the aforementioned limitations will have valuable application to both diabetes research and management.

Recently, stem cell therapy has achieved positive results in medicine related to the 3Rs (Replacement, Repair and Regeneration). Of note, 3R therapy uses stem cells and progenitor cells as effective tools to prevent, repair, replace and treat damaged organs. In fact, stem cell therapy has been applied successfully in diabetes, from preclinical to clinical studies (Dave et al., 2014; Dong et al., 2008;

ISSN: 2198-4093
www.bmrat.org

Gao et al., 2014; Le et al., 2016; Si et al., 2012; Thi-Tung Dang, 2015; Voltarelli et al., 2011; Wu et al., 2015; Wu et al., 2007; Xiao et al., 2013; Xie et al., 2016; Yang et al., 2010; Zhou et al., 2009). Stem cell transplantation is an excellent platform for diabetes therapy due to the fact that it can slow down the progression of diabetes and eliminate the complications of long-term blood glucose homeostatic effect.

The most popular stem cell type that has been evaluated in diabetes mellitus treatments have been mesenchymal stem cells (MSCs). These cells have great potential and it is feasible to isolate them, there is an abundant source, and ethical concerns are minimal. MSCs are isolated from various tissues, including adipose tissue, bone marrow, umbilical cord blood, umbilical cord and dental pulp (Pham et al., 2014; Ren et al., 2016; Van Pham et al., 2016).

Mesenchymal stem cells

Mesenchymal stem cells (MSCs) have been demonstrated to be involved in the *in vivo* self-repair and self-regeneration processes of animal tissues. These cells can be isolated from different tissues such as bone marrow, adipose tissue, dental pulp, fetal appendages as well as umbilical cord blood, umbilical cord, and placenta. When cultured *in vitro*, they appear as spindle-shaped cells. MSCs express a specific marker profile; they are positive for CD29, CD51, CD73, CD90, and CD105 expression, yet negative for hematopoietic markers such as CD31 and CD45 (Wang et al., 2014). Interestingly, they do not express MHC class II and only express MHC class I at low levels. Moreover, they do not express Fas ligand and costimulatory molecules such as B7 and CD40, thus they have been suggested to be hypoimmunogenic cells (Atoui and Chiu, 2012). Other properties include their ability to self-renew, to create fibroblast colony forming units (CFU-F), and to differentiate into other cells such as bone, cartilage and fat. The aforementioned characteristics are part of criteria used to determine and identify MSCs (Dominici et al., 2006).

Although the term "mesenchymal" defines their origin and differentiation tendency, MSCs are flexible and can change *in vitro* to exhibit features of specific cells or progenitor cells belonging to the endoderm or ectoderm layers. From the culmination of many protocols focused on optimizing their culture, at present it is feasible to efficiently isolate and expand MSCs *in vitro* and *in vivo* (Ducret et al., 2016; Pham et al., 2014; Sensebe et al., 2011). Moreover, MSCs have been shown to play a role in the healing process in applications of MSC-based therapies for several diseases, such as heart dysfunction, neurodegenerative diseases, liver diseases, renal failure, and diabetes. Despite positive results of the therapy, it remains unclear as to the mechanisms exerted by MSCs. In this review, we will explore and discuss the role of MSCs in diabetic treatment.

ISSN: 2198-4093
www.bmrat.org

Table 1. Preclinical-trials of diabetes mellitus treatment by stem cell therapy

Species	Type of DM	Cell types/ transplant route	Immune suppression	Duration	Results
Mice (Wang et al., 2011)	1; NOD	UC-MSC-derived IPC/ retro-orbital vein	No	23 days	Improved body weights after 7 days; Lowered BG levels after 3 days; improved glucose tolerance at day 14; Prolonged survival of treated mice while NOD mice had died before end point; Human C-peptide and human cell nuclei appeared at the same sites within the lobules of mouse livers.
Mice (SCID) (Santamaria et al., 2011)	1; STZ	ESSC-derived IPC/ renal subcapsular transplant	No	35 days	BG levels were stablized in IPC-treated mice while ESSC-treated mice had peak increase in BG level at the early week 5 in the diabetic ones; Improved weight loss in IPC-grafted mice; Human insulin was only found in xenograft of IPCs and the serum of IPC-grafted mice with mean concentration 11.9 μIU/ml.
Mice NOD/ SCID (Phadnis et al., 2011)	1, pancreactomize and STZ	BM-MSC-derived IPC/ renal capsule	No	70 days	Transplanted IPCs could mature and secrete human c-peptide (insulin) in vivo; IPCs transplantation normalized BG levels and maintained for up to 8 weeks thereafter; Weight loss continued in the diabetic mice with 50% mortality by day 40 after the onset of diabetes whereas, IPC-grafted mice exhibited weight gain; After removal of grafted IPC, mouse BG increased to high levels within 3 days after and 93% mice died within 3 weeks; no detectable human C-peptide in plasma; and all mice had BG levels above 350 mg/dL at 2 h of glucose challenge.
Mice (Ngoc et al., 2011)	1, STZ	UCB-MSC-derived IPC/ portal vein	No	30 days	Improved weight loss: a slight decrease in unencapsulated IPCs-grafted mice versus significant increase in encapsulated IPCs-grafted mice compared with strong decrease in body weight in diabetic control; BG levels: obviously increased in unencapsulated IPC-grafted group whereas encapsulated IPC transplantation almost stabilized the BG levels; Encapsulated IPC alleviated the immune response at day 15 and 30 compared with the unencapsulated IPC-treated group.
Mice (Ho et al., 2012)	1; STZ	BM-MSC/ Tail vein infusion	No	6 months	Safe; Multiple Intravenous Transplantations were better than single injection; Reduced systemic oxidative stress levels from week 17; Markedly increased production of human insulin from week 11; Blood sugar level normalized at the end of week 15; At the end of 6 months, MSCs were proved to exist in liver tissues of the recipients and 51% of human cells in the recipient liver coexpressed human insulin.

ISSN: 2198-4093
www.bmrat.org

Mice (Kim et al., 2012)	1; STZ	hAMSC-derived IPCs/ Kidney	No	210 days	Safe; overcoming immune rejection for a long period; Improved survival rate: 120 days after transplantation: group control: 0/12 alive mice; group hAMSC-treated: 0/12 alive mice and group hAMSC-IPC: 9/16 alive mice; Restored body weight and maintained normoglycemia up to 210 days; Detected human insulin and C-peptide in the blood of normalized mice after 2 months; Human cell genes were detected in the transplanted kidneys.
Rat (Tsai et al., 2012)	1; STZ	UC-MSC-derived IPCs/ portal vein	No	8 weeks	Significantly decreased in BG levels at 4 weeks; Diabetic rats sustained hyperglycemia; Grafted cells were located in the liver.
Mice (Xiao et al., 2013)	1; STZ	UC-MSC and CB-MNC/ Tail vein infusion	Yes		Safe; Co-transplanted UC-MSCs and CB-MNCs at a ratio of 1:4 were effectively decreased the blood glucose levels in the first week and stabilized thereafter (7/10 mice); Detected Alu sequence indicated that the human cells had homed into the recipient's pancreas and kidney.
Mice (Kanafi et al., 2013)	1; STZ	SHED and DPSC-derived IPC/ subcutaneous transplantation	No	10 weeks	90% transplanted diabetic mice survived and normalized BG levels within 2 weeks and maintained 2 months or prolonged normoglycemia even after graft removal; also their body weight and glucose level in urine became normal; The diabetic mice remained hyperglycemia and reduced body weight and died within 10 – 13 days after STZ injection.
Mice (Dao et al., 2014)	1; STZ	PDPCs or PDPC-derived-IPCs/ Renal capsule	No	8 weeks	Decreased BG levels and improved glucose tolerance in cell-treated groups at 4 weeks and 8 weeks; Detected human insulin in the serum and kidney sections of IPC-grafted mice.
Rat (Boroujeni and Aleyasin, 2014)	1, Alloxan	UC-MSC-derived IPCs/ intraperitoneal transplant	No	17 days	The BG levels of MSC-treated rats were decreased at day 10 and increased thereafter; Transduced MSC-treated rats showed normalized BG within 3–4 days. Diabetic rats sustained hyperglycemia and died within 6 months.
Rat (Hu et al., 2014)	2, STZ with HFD	WJ-MSC/ intravenous infusion	No	10 weeks	Decreased significantly in BG levels at 2 weeks and modestly increased thereafter; Improved fasting C-peptide, glucagon and HbA1c levels after 10 weeks of transplantation; The number of beta cells in MSC-treated rats were more than two-fold compared with diabetic rats.
Rat (Tsai et al., 2014)	1; STZ	BM-MSC-derived IPC/ portal vein	No	8 weeks	Decreased in BG levels at 1 week, reached <250 mg/dl for 6 weeks; Decrease slowly in the body weights; Grafted cells were localized in the recipient's liver.
Mice (Zhang and Dou, 2014)	1, STZ	BM-MSC-derived IPC/ right-side testis	No	> 80 days	Normalized BG of diabetic mice for at least 80 days following xenograft. Blood glucose of grafted mice rose again after their graft removed. Human insulin existed in recipient mice.
Mice (Gabr et al., 2015)	1, STZ	BM-MSC-derived IPC/renal subcapsular space	No	12 weeks	The differentiated cells expressed low levels of pancreatic endocrine genes; Increased the percentage of IPCs among transplanted cells from under ≤3% to approximately 18% at 4 weeks; Normalized BG after 8±3 days of transplantation; Increased expression of insulin, glucagon, and somatostatin genes.

ISSN: 2198-4093
www.bmrat.org

Mice (Hu et al., 2015)	NOD	WJ-MSC/ intravenous infusion	No	18 weeks	Normalized FPG and fed blood glucose in 6-8 days and maintained for 6 weeks; Improved level of fasting C-peptide of these mice; Protected for 8-week delayed onset of diabetes; Increased the number of the $CD4^+CD25^+Foxp3^+$ Tregs in WJ-MSC-treated group; Decreased the levels of IL-2, IFN-c, and TNF-a; Depressed the degree of insulitis.
Mice (Tsai et al., 2015)	NOD	WJ-MSC/ retro-orbital vein	No	23 days	Increased survival rate; Significantly decreased BG three days after transplantation; at day 23, human C-peptide and serum insulin levels increased and glucose tolerance improved; Reduced T helper 1 cells; IL-17-producing T-cells and dendritic cells; Increased Tregs number and anti-inflammatory cytokine levels; The MSC could differentiate into IPC in vivo and performed the function in tissue repair.
Rat (Yu et al., 2015)	pancreatectomy	UC-MSC-derived IPCs/ portal vein	No	56 days	Decreased BG two weeks after transplantation (18.7 ± 2.5 mmol/L in treated group vs. 25.8 ± 1.25 mmol/L in diabetic rats); On day 56, glucose tolerance tests showed that after 45 min, BG levels were significantly lower (12.5 ± 4.7 mmol/L in treated group vs 42.2 ± 9.3 mmol/L in diabetic rats).
Rat (Zhou et al., 2015)	1, STZ	UC-MSC/Tail vein infusion	No	42 days	BG levels started to decrease at day 6 and modestly lowered thereafter; Restored islet structure: with the size at 63% and cell number at 42% or the normal rat; Human cell existed in the recipient's pancreas and activated the pancreatic PI3K pathway and its downstream anti-apoptotic signal; Induced the trophic effects on islets via the expression of β-cell growth factor genes and proteins.
Rat (Xie et al., 2016)	2, STZ with HFD	UC-MSC/ Tail vein infusion	No	7 days	Improved glucose homeostasis and promoted insulin sensitivity; Alleviated insulin resistance by producing IL-6 that induced M2 polarization; Obesity and insulin resistance were associated with increased pro-inflammatory adipose tissue macrophages infiltration.
Mice (Xin et al., 2016)	1, STZ	BM-MSC or IPC/ renal sub-capsular space	No	21 days	BG levels of the IPC-treated mice were normalized within 6 days and maintained throughout 21 days; whereas MSC-treated mice remained hyperglycemia; Transplanted IPCs expressed insulin, C-peptide, and PDX-1 without apparent apoptosis in vivo.

Mesenchymal stem cells and diabetes mellitus

It is known that MSCs are active participants in the healing process of damaged tissues. Although they are present and can be isolated from diabetic human and animal tissues, the repair process is limited due to inadequate and impaired functions of MSCs (Cianfarani et al., 2013; Tobita et al., 2015). Adult MSCs can proliferate and differentiate *in vitro* by the effects of several soluble and non-soluble factors in the culture medium. A diabetic or hyperglycemic environment reduces the proliferative potential of MSCs yet increases senescence and

ISSN: 2198-4093
www.bmrat.org

apoptosis of the cells (Cheng et al., 2016; Cianfarani et al., 2013; Cramer et al., 2010; Hankamolsiri et al., 2016; Kim et al., 2015; Wajid et al., 2015). Moreover, the differentiation and immunomodulation capacity of diabetic or high glucose treated MSCs are distinct from normal cells (Montanucci et al., 2016). A high glucose condition boosts adipogenic differentiation while decreasing osteogenic differentiation (Cramer et al., 2010; Hankamolsiri et al., 2016) and diminishing angiogenic capacity (Kim et al., 2015) of affected MSCs.

Interestingly, a small proportion (6%) of diabetic MSCs express proinsulin and C-peptide and these cells can differentiate rapidly into functional islet-like cells in the presence of a pseudo diabetic milieu (Phadnis et al., 2009). It has been suggested that the diabetic microenvironment may be a condition for MSC differentiation into insulin-producing cells *in vitro* and *in vivo*. Moreover, expression of stem cell specific surface markers on diabetic MSCs decreased (Cianfarani et al., 2013) while expression of pluripotent markers increased (Cheng et al., 2016). Additionally, hyperglycemia conditioned MSCs showed a reduction of cell migration (Cheng et al., 2016; Cianfarani et al., 2013) and of secretion of growth factors related to wound healing (Cianfarani et al., 2013). Therefore, the reduced expression of certain important features, such as the above, warrants more investigations in MSC applications for diabetes treatment.

Mesenchymal stem cell transplantation for diabetes mellitus

It is known that MSCs play a crucial role in healing damaged tissues. They can differentiate to replace the dead cells as well as secrete stimulant factors to activate surrounding cells in the microenvironment, enhancing the tissue repair process (Wang et al., 2014). Therefore, MSCs can be applied to treat tissues impaired by chronic hyperglycemia. For T1DM, MSC transplantation can theoretically increase beta cell mass via the following effects:

(1) beta cell replacement through *in vitro* or *in vivo* differentiation;

(2) local microenvironment modification by production of cytokines, chemokines and factors to stimulate endogenous regeneration;

(3) reduction or prevention of autoimmunity to beta cells (Ezquer, 2014). Although several MSC transplantation studies have clearly shown the outcome of controlled glucose metabolism, there have been observations of decreased insulin resistance as well as enhanced beta cell function effects. Moreover, the mechanisms of MSC treatment for T2DM still has not been well understood. Some studies have suggested that the immunomodulatory and inflammatory effects of MSCs are what contribute to the resulting reduction of insulin resistance (Liu et al., 2014; Xie et al., 2016).

ISSN: 2198-4093
www.bmrat.org

Safety of human mesenchymal stem cell transplantation

The first concern of any therapy is the risk of mortality. Similar to modern generation drugs, MSC-based therapies should be controlled and monitored for safety before their positive effects are determined. In diabetic animal models, graft rejection as well as acute adverse responses or sudden death were not noticed after xenograft MSC and/or differentiated MSC transplantations (Gabr et al., 2013; Ho et al., 2012; Hu et al., 2015; Lee et al., 2006; Xie et al., 2016). Interestingly, graft tolerance was observed when human cells were transplanted into diabetic mice without immunosuppressive treatment (Chao et al., 2008; Zhou et al., 2015). Moreover, MSC treated diabetic rodents survived and showed a prolonged life relative to non-treated diabetic animals (Kadam et al., 2010; Tsai et al., 2015). MSC transplantation was proven to be safe and well-tolerated in a small cohort of T2DM patients although approximately 22% of them had slight transient fevers (Kong et al., 2014).

Efficacy of human mesenchymal stem cell transplantation

Preclinical treatment

Many studies have shown that human MSCs (hMSCs) or/and hMSC-derived islet-like cells effectively safety transplanted into diabetic animals (Ho et al., 2012; Kim et al., 2012) (**Table 1**).

Human MSC transplantations lead to alleviated blood glucose levels in diabetic animals. It was confirmed that hMSC infusion could improve blood glucose homeostasis in both type 1 and type 2 diabetic animals. The glucose levels decreased markedly from a few days to 2 weeks after hMSC infusion and this decrease was maintained until 20 days to 10 weeks thereafter (Ammar et al., 2015; Ho et al., 2012; Hu et al., 2014; Hu et al., 2015; Kadam et al., 2010; Lee et al., 2006; Tsai et al., 2015; Xie et al., 2016; Yang et al., 2010; Zhou et al., 2015). Multiple transplantations of hMSCs could improve the effects of hyperglycemia (Ho et al., 2012; Lee et al., 2006). Glucose levels were notably normalized after seven administrations of hMSCs (Ho et al., 2012).

Other research studies have shown the efficiency of hMSC derived insulin-producing cells (IPCs) on diabetic treatments. These cells could be *in vitro* differentiated from many sources of hMSCs and exhibited many characteristics of actual beta cells, including C-peptide expression, insulin production, glucose response ability as well as pancreatic beta cell specific gene expression (Gabr et al., 2013; Kao et al., 2015; Kim et al., 2015; Seyedi et al., 2015; Seyedi et al., 2016; Thi-Tung Dang, 2015; Van Pham et al., 2014; Zhang and Dou, 2014). The IPCs were transplanted into liver (Chao et al., 2008), renal capsule (Hu et al., 2009; Kadam et al., 2010); blood glucose levels were reduced 3 days thereafter and normalized at more than 9 weeks out or until removal of graft (Chao et al., 2008; Kadam et al., 2010; Zhang et al., 2010). However, some results have shown that hIPC transplantations does not lower glucose levels (Hu et al., 2009; Ngoc et al., 2011).

ISSN: 2198-4093
www.bmrat.org

Clinical applications

Although many preclinical studies have shown evidence that hMSC therapy has beneficial effects in the treatment of diabetes mellitus, there are still not many clinical applications of MSC therapy for T1DM or T2DM in the world. The first problem is due to the safety issue of MSC transplantation (**Table 2**). However, this can be alleviated by understanding the special characteristics as well as *in vitro* and *in vivo* behavior of MSCs through experimental studies and evidence. MSCs themselves show unchanged morphology and phenotype as well as the normal karyotype. They also express tumor suppressors and oncogenes at normal levels even after they undergo long-term culture. There is no evidence that tumor formation is associated with MSC transplantation. Additionally, most of the clinical trials have proven that MSC transplantation for the treatment of diabetes is safe although there have been a few reported cases of fever. However, it is difficult to elucidate whether the fever was caused by the cell transplantation or from diabetic symptoms or certain infections.

The second problem or issue is whether MSC therapy is effective for the treatment of DM in humans. Although the mechanism has not been clearly demonstrated, MSC transplantation is capable of reducing blood glucose in various periods of follow-up time from a few months to several years. Moreover, it has been suggested that MSC transplantation can normalize or maintain the ameliorated blood glucose levels as well as improve serum insulin levels, C-peptide, HbA1C, and the daily insulin requirement for a long period. Beside evidence of these systemic effects, MSC transplantation has the potential to treat diabetic complications such as foot ulcers, thrombosis, heart failure, kidney failure, and blindness. Therefore, it is meaningful that MSC therapy can be applied to treat early and/or late stages of diabetes as well as relieve the pain of complications and delay or cease the need for amputations. Moreover, based on the successful clinical trials, many MSC therapies continue to be developed to improve the efficiencies of the following:

(1) prolonged time effect;

(2) reduced expenditure on treatment and increase in patients treated; and

(3) upgrade in cell products, from abundance and availability (commercial distribution) to development of diverse sources of MSCs and improvement of identifiable therapeutic characteristics of MSCs (e.g. immunomodulatory potential).

Moreover, MSCs have been suggested as universal therapeutic cells (Atoui and Chiu, 2012) based on their immune privilege and are being developed as ready-to-use products (e.g. Prochymal). Finally, the mechanisms related to MSC therapy, while still unclear, are gradually being discovered through more research findings. As of now, pertinent questions remain such as:

ISSN: 2198-4093
www.bmrat.org

Table 2. Clinical applications of stem cell transplantation for diabetes mellitus

Type of DM	Cases	Cell types	Methods	Immune suppress	Duration	Status	References
1	5	ASC and cultured BM	Autologou, intraportal	No	2.9 months	Safe 30% to 50% decreased insulin requirements 4- to 26-fold increased serum c-peptide levels	(Trivedi et al., 2008)
1	15	UCB	Autologou,Intravenous infusion	No	> 6 months	Safe Provides some slowing of the loss of endogenous insulin production increased Treg populations	(Haller et al., 2008)
	24	UCB	Autologou, Intravenous infusion	No	24 months	Completed clinic trial in 2013 Safe Increased regulatory T cells (Tregs) and naive Tregs 6 and 9 months after treatment; Fails to preserve C-peptide.	NCT00305344
1	11	hASC-IPC and cultured BM	Allogenic, omental vein infusion	N/A	mean follow-up of 23 months	Safe Decreased mean exogenous insulin requirement to 0.63 units/kgBW/day; Decreased Hb1Ac to 7.39%; Raised serum C-peptide levels to 0.38 ng/mL; Became free of diabetic ketoacidosis events.	(Vanikar et al., 2010)
1	63	PROCHYMAL® (human MSC)	Allogenic, Intravenous infusion	N/A	2 years	Complete Phase 2	NCT00690066
2	10	Placenta-derived MSC	Allogenic, 3 intravenous infusion	N/A	6 months	Safe Decreased insulin dose from 63.7±18.7 to 34.7±13.4 IU; Increased C-peptide level from 4.1±3.7 ng/mL to 5.6±3.8 ng/mL The renal function and cardiac function were improved after infusion.	(Jiang et al. 2011)
1	15	UCB-MSC (Stem Cell Educator therapy)	Allogenic, Intravenous infusion	N/A	40 weeks	Phase 1/ phase 2 study; Safe; Reduced HbA1C values; Decreased the median daily dose of insulin; Increased basal and glucose-stimulated C-peptide levels through 40 weeks; Increased expression of CD28 and ICOS; Increased the number of CD4+CD25+Foxp3+ Tregs, and restoration of Th1/Th2/Th3 cytokine balance	NCT01350219
1, 2	30	BM-MSC bone marrow enriched CD90+ cells	Autologou, Intramuscular injection	N/A	45 weeks	safe and feasible Improvements of microcirculation and complete wound healing; 18/22 patients showed wound healing after 45 weeks.	NCT01065337
2	118	BM-MNC	Autologous, Intra-pancreatic injection	N/A	36 months	Improved C-peptide levels.	(Hu et al. 2012)

ISSN: 2198-4093
www.bmrat.org

2	36	UCB-MSC (Stem Cell Educator therapy)	Allogenic, Intravenous infusion		12 months	Phase 1/ phase 2 study; Safe; HbA1C reduced from 8.61%+/-1.12 to 7.25% +/-0.58 at 12 weeks, and 7.33%+/-1.02 at one year post-treatment; Insulin sensitivity was improved post-treatment; Reversed immune dysfunctions through immune modulation on monocytes and balancing Th1/Th2/Th3 cytokine production.	NCT0141 5726
1	29	UC-MSC	Allogenic, Intravenous transfusion	N/A	21 months	Safe and effective; Fasting plasma glucose was not significant difference between treated and control group; HbA1c reached the lowest level at the sixth month (baseline 6.8 ± 0.57%, 6 months 5.5 ± 0.67%); C-peptide achieved the highest at the end of first year and slight decrease afterthat but still remained better than baseline.	(Hu et al. 2013)
2	21	BM-MSC	Autologous	N/A	12 months	The insulin requirement decreased by 66.7% in the intervention group and 32.1% in controls; 9/11 (91%) patients could maintain HbA1c <7% in the intervention group, whereas 6/10 (60%) in the control group.	(Bhansali et al. 2014)
1	20	BM-MSC	Autologou, Intravenous infusion	No	12 months	Phase 1 complete Safe; C-peptide peak values and C-peptide were preserved or even increased in the MSC-treated patients	NCT0106 8951
1	42	UC-MSC and BM-MNC	Allogenic UC-MSC and autologous BM-MNC, intra-arterial pancreatic infusion	N/A	12 months	Phase 1/ phase 2 Safe Improved metabolic measures: AUCC-Pep increased 105.7%, insulin area under the curve increased 49.3%, HbA1c decreased 12.6%, fasting glycemia decreased 24.4%. Daily insulin requirements decreased 29.2%	NCT0137 4854
2	18	UC-MSC	Intravenous transfusion	N/A	6 months	4/18 patients (22.2%) had slight transient fever. Safe and well tolerated; Alleviated blood glucose; and increased the generation of C-peptide levels and Tregs in a subgroup of T2DM patients.	NCT0141 3035

(i) how do MSCs survive,

(ii) how do MSCs behave in live bodies, and

(iii) what reactions and responses occur after cell transplantation over time. Therefore, studies of MSCs and their application for the treatment of diabetes are valuable to understand the underlying mechanisms.

What role do hMSCs play after they are administrated in diabetic bodies?

Survival

ISSN: 2198-4093
www.bmrat.org

Labeled MSCs have been transplanted into diabetic mice to determine their survival in body tissues. It was confirmed that the grafted cells presented and secreted functional human insulin and C-peptide (Xin et al., 2016; Zhang et al., 2010). Hess *et al.* found approximately 2.5% insulin-positive cells in diabetic mice whereas the cells were absent in the normal pancreas (Hess et al., 2003). Lack of certain surface antigens on MSCs may result in a lowered immune responses in recipients as described above (safety issue).

Differentiation potential and cell replacement

It has been shown that MSC transplantation can ameliorate blood glucose levels within the follow-up time from few weeks to several years. However, the underlying mechanism of this effect is still unclear. One proposed mechanism is the replacement potential of transplanted MSC-derived cells. MSCs are able to trans-differentiate into insulin-producing cells and have been suggested to compensate impaired beta cells in diabetic animals. It is necessary to carefully determine the surrogate capability of MSCs. Although MSCs can be induced to secrete insulin *in vitro* and/or *in vivo*, few MSCs become fully functional beta cells *in vivo* (only approximately 1.7 – 3% of transplanted cells). Moreover, the transient survival of transfused cells confirms that the replacement potential may not be the only mechanism of the therapeutic effect of MSCs. Sordi *et al.* have suggested the role of MSCs as helper cells when they observed normalized blood glucose levels and neovascular formation after co-transplantation of pancreatic MSCs and islet mass (Sordi et al., 2010).

Immune modulation - an important property of MSCs

Besides the differentiation potential, MSCs have the unique potential to modulate immune responses via several mechanisms. The *in vitro* immunosuppressive capability of MSCs was investigated since the late 1990s (Wang et al., 2014). When co-cultured with leukocytes, it is found that MSCs are able to alter the proliferation of several immune cells. They are able to inhibit B lymphocytes (Corcione et al., 2006), NK cells (Spaggiari et al., 2008), inhibit differentiation and function of monocyte-derived dendritic cells (Jiang et al., 2005), and suppress the activation and proliferation of T cells (Nauta and Fibbe, 2007). These results have been confirmed by others (Atoui and Chiu, 2012; Mattar and Bieback, 2015; Spaggiari et al., 2008; Wang et al., 2014; Yaochite et al., 2016; Zhao et al., 2010). In particular, MSCs are able to induce macrophages to convert from proinflammatory type 1 to anti-inflammatory phenotype, resulting in alleviation of insulin resistance in T2DM (Wang et al., 2014; Xie et al., 2016). Moreover, it is specifically described that MSCs can alter the ratio of regulatory T cells versus other subtypes of T cells while capable of inducing the generation of regulatory T cells (Tregs) and simultaneously inhibiting the proliferation of T helper 1 (Th1) and Th17 cells (Luz-Crawford et al., 2013). MSCs are capable of suppressing autoimmune responses in T1DM. Interestingly, these effects appear after direct interaction between MSCs and immune cells whereas the microenvironment components do not alter the immunomodulatory capability of MSCs *in vitro* (Wehner et al., 2013).

ISSN: 2198-4093
www.bmrat.org

It has been proposed that MSCs are not only multipotent adult stem cells but also universal donor cells due to their ability to avoid immune rejection (Atoui and Chiu, 2012). Moreover, systemic administration of MSCs derived from autologous or allogeneic or even xenogeneic sources have been reported which can create non-specific systemic immunosuppression (Nauta and Fibbe, 2007). The transplanted MSCs could survive and differentiate in allogeneic or xenogeneic recipients due to their immunotolerance capability. Atoui *et al.* have suggested that the underlying mechanism of immunotolerance capability of MSCs is due to their hypoimmunogenicity, T cell modulation and microenvironment immune suppression (Atoui and Chiu, 2012). Consequently, transplanted MSCs can be tolerated (in part) in the recipients and can induce local pancreatic stem cells to proliferate, leading to replacement of impaired cells in the diabetic animals.

It has been found that MSCs can produce many cytokines and factors which improve and modulate the surrounding microenvironment. These components include inflammatory cytokines, immunosuppressive molecules and growth factors responsible for the tissue repair process (Ma et al., 2014). The presence of inflammatory cytokines can initiate the immunomodulatory effects of MSCs via immunosuppressive factors such as nitric oxide (NO) in mice and indoleamine 2,3-dioxygenase (IDO) in humans, along with IL-10, TSG6, IL-6, LIF, PGE2, HO-1 and truncated CCL2 (Ma et al., 2014). The immunomodulatory ability of MSCs is supposedly plastic since it depends on the inflammation status and cytokine status (Ma et al., 2014; Wang et al., 2014). Both NO and IDO play a role as a switch; strong inflammation drives MSCs to induce immunosuppression while weak inflammation enhances immune responses induced by MSCs (Wang et al., 2014). Therefore, it is necessary to determine inflammation and cytokine levels as well as immunosuppressants to optimize the procedure of MSC transplantation.

Notably, it is perceived that MSCs derived from various tissues have different capabilities of immunomodulation (Mattar and Bieback, 2015). This difference may derive from their origins or different culture conditions (Menard et al., 2013).

Conclusion

Mesenchymal stem cells possess unique properties which make them suitable candidates for use in diabetes mellitus treatment strategies. Besides their potential to differentiate into various types of cells (e.g. beta cells), they also possess the ability to modulate immunity and angiogenesis via secreted paracrine factors. For Type 1 diabetes, the effects of MSCs on immune modulation are clearly evident; for Type 2 diabetes, some of the mechanisms exerted by MSCs are still unclear. However, for both T1DM and T2DM, MSC transplantation can dramatically improve the blood glucose levels, while reducing the insulin dose and side effects associated with DM. More importantly, both in preclinical trials and clinical trials, MSC transplantation has been

ISSN: 2198-4093
www.bmrat.org

demonstrated to be safe; there have been no observed adverse side effects or tumorigenesis. The data thus far have suggested that MSC transplantation is a promising therapy for DM. More controlled and randomized clinical trials are needed to further optimize the stem cell transplantation process.

Abbreviations

AMSC : Amniotic mesenchymal stem cell; BG: Blood glucose; BM: Bone Marrow; DPSC: Dental pulp stem cells from permanent teeth; DM: Diabetes mellitus; ESSC: Endometrial stromal stem cells; HFD: High fat diet; IPC: Insulin Producing Cell; MNC: Mononuclear Cell; MSC: Mesenchymal Stem Cell; NOD: Non-obese diabetic; PDPC: Periosteum-Derived Progenitor Cells; SCID: Severe Combined Immunodeficiency; SHED : Stem cells from pulps of human exfoliated deciduous teeth; STZ: Streptozotocin: T1DM: Type 1 Diabetes mellitus; T2DM: Type 2 Diabetes mellitus; UC: Umbilical Cord; UCB: Umbilical Cord Blood; WJMSC: Wharton's Jelly Mesenchymal Stem Cell.

Acknowledgement

This review was supported by the grant number DTDL.2012-G/23, Ministry of Science and Technology, Vietnam.

Author contribution

Ngoc Kim Phan has written the preclinical results showed in Table 1. Kiet Dinh Truong has written the clinical review. Loan Thi-Tung Dang has written the rest and completed and edited the review

ISSN: 2198-4093
www.bmrat.org

References

1. Ammar, H.I., Sequiera, G.L., Nashed, M.B., Ammar, R.I., Gabr, H.M., Elsayed, H.E., Sareen, N., Rub, E.A., Zickri, M.B., and Dhingra, S. (2015). Comparison of adipose tissue- and bone marrow- derived mesenchymal stem cells for alleviating doxorubicin-induced cardiac dysfunction in diabetic rats. *Stem cell research & therapy* 6, 148.
2. Atoui, R., and Chiu, R.C. (2012). Concise review: immunomodulatory properties of mesenchymal stem cells in cellular transplantation: update, controversies, and unknowns. *Stem cells translational medicine* 1, 200-205.
3. Bastaki, S. (2005). Diabetes mellitus and its treatment. *Int J Diabetes & Metabolism* 13, 111-134.
4. Chao, K.C., Chao, K.F., Fu, Y.S., and Liu, S.H. (2008). Islet-like clusters derived from mesenchymal stem cells in Wharton's Jelly of the human umbilical cord for transplantation to control type 1 diabetes. *PloS one* 3, e1451.
5. Cheng, N.C., Hsieh, T.Y., Lai, H.S., and Young, T.H. (2016). High glucose-induced reactive oxygen species generation promotes stemness in human adipose-derived stem cells. *Cytotherapy* 18, 371-383.
6. Cianfarani, F., Toietta, G., Di Rocco, G., Cesareo, E., Zambruno, G., and Odorisio, T. (2013). Diabetes impairs adipose tissue-derived stem cell function and efficiency in promoting wound healing. *Wound repair and regeneration : official publication of the Wound Healing Society [and] the European Tissue Repair Society* 21, 545-553.
7. Corcione, A., Benvenuto, F., Ferretti, E., Giunti, D., Cappiello, V., Cazzanti, F., Risso, M., Gualandi, F., Mancardi, G.L., Pistoia, V., *et al.* (2006). Human mesenchymal stem cells modulate B-cell functions. *Blood* 107, 367-372.
8. Cramer, C., Freisinger, E., Jones, R.K., Slakey, D.P., Dupin, C.L., Newsome, E.R., Alt, E.U., and Izadpanah, R. (2010). Persistent high glucose concentrations alter the regenerative potential of mesenchymal stem cells. *Stem cells and development* 19, 1875-1884.
9. Dave, S.D., Trivedi, H.L., Gopal, S.C., and Chandra, T. (2014). Combined therapy of insulin-producing cells and haematopoietic stem cells offers better diabetic control than only haematopoietic stem cells' infusion for patients with insulin-dependent diabetes. *BMJ case reports* 2014.
10. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315-317.
11. Dong, Q.Y., Chen, L., Gao, G.Q., Wang, L., Song, J., Chen, B., Xu, Y.X., and Sun, L. (2008). Allogeneic diabetic mesenchymal stem cells transplantation in streptozotocin-induced diabetic rat. *Clinical and investigative medicine Medecine clinique et experimentale* 31, E328-337.
12. Ducret, M., Fabre, H., Degoult, O., Atzeni, G., McGuckin, C., Forraz, N., Mallein-Gerrin, F., Perrier-Groult, E., and Fargues, J.C. (2016). A standardized procedure to obtain mesenchymal stem/stromal cells from minimally manipulated dental pulp and Wharton's jelly samples. *Bulletin du Groupement international pour la recherche scientifique en stomatologie & odontologie* 53, e37.

ISSN: 2198-4093
www.bmrat.org

13. Ezquer, M. (2014). Mesenchymal Stem Cell Therapy in Type 1 Diabetes Mellitus and Its Main Complications: From Experimental Findings to Clinical Practice. *Journal of Stem Cell Research & Therapy* 04.

14. Gabr, M.M., Zakaria, M.M., Refaie, A.F., Ismail, A.M., Abou-El-Mahasen, M.A., Ashamallah, S.A., Khater, S.M., El-Halawani, S.M., Ibrahim, R.Y., Uin, G.S., *et al.* (2013). Insulin-producing cells from adult human bone marrow mesenchymal stem cells control streptozotocin-induced diabetes in nude mice. *Cell transplantation* 22, 133-145.

15. Gao, X., Song, L., Shen, K., Wang, H., Qian, M., Niu, W., and Qin, X. (2014). Bone marrow mesenchymal stem cells promote the repair of islets from diabetic mice through paracrine actions. *Molecular and cellular endocrinology* 388, 41-50.

16. Halban, P.A. (2004). Cellular sources of new pancreatic beta cells and therapeutic implications for regenerative medicine. *Nature cell biology* 6, 1021-1025.

17. Hankamolsiri, W., Manochantr, S., Tantrawatpan, C., Tantikanlayaporn, D., Tapanadechopone, P., and Kheolamai, P. (2016). The Effects of High Glucose on Adipogenic and Osteogenic Differentiation of Gestational Tissue-Derived MSCs. *Stem cells international* 2016, 9674614.

18. Hess, D., Li, L., Martin, M., Sakano, S., Hill, D., Strutt, B., Thyssen, S., Gray, D.A., and Bhatia, M. (2003). Bone marrow-derived stem cells initiate pancreatic regeneration. *Nature biotechnology* 21, 763-770.

19. Ho, J.H., Tseng, T.C., Ma, W.H., Ong, W.K., Chen, Y.F., Chen, M.H., Lin, M.W., Hong, C.Y., and Lee, O.K. (2012). Multiple intravenous transplantations of mesenchymal stem cells effectively restore long-term blood glucose homeostasis by hepatic engraftment and beta-cell differentiation in streptozocin-induced diabetic mice. *Cell transplantation* 21, 997-1009.

20. Hu, J., Wang, F., Sun, R., Wang, Z., Yu, X., Wang, L., Gao, H., Zhao, W., Yan, S., and Wang, Y. (2014). Effect of combined therapy of human Wharton's jelly-derived mesenchymal stem cells from umbilical cord with sitagliptin in type 2 diabetic rats. *Endocrine* 45, 279-287.

21. Hu, J., Wang, Y., Wang, F., Wang, L., Yu, X., Sun, R., Wang, Z., Wang, L., Gao, H., Fu, Z., *et al.* (2015). Effect and mechanisms of human Wharton's jelly-derived mesenchymal stem cells on type 1 diabetes in NOD model. *Endocrine* 48, 124-134.

22. Hu, Y.H., Wu, D.Q., Gao, F., Li, G.D., Yao, L., and Zhang, X.C. (2009). A secretory function of human insulin-producing cells in vivo. *Hepatobiliary & pancreatic diseases international : HBPD INT* 8, 255-260.

23. IDF (2015). IDF Diabetes atlas Edition 7.

24. Jiang, X.X., Zhang, Y., Liu, B., Zhang, S.X., Wu, Y., Yu, X.D., and Mao, N. (2005). Human mesenchymal stem cells inhibit differentiation and function of monocyte-derived dendritic cells. *Blood* 105, 4120-4126.

25. Kadam, S.S., Sudhakar, M., Nair, P.D., and Bhonde, R.R. (2010). Reversal of experimental diabetes in mice by transplantation of neo-islets generated from human amnion-derived mesenchymal stromal cells using immuno-isolatory macrocapsules. *Cytotherapy* 12, 982-991.

26. Kao, S.Y., Shyu, J.F., Wang, H.S., Lin, C.H., Su, C.H., Chen, T.H., Weng, Z.C., and Tsai, P.J. (2015). Comparisons of Differentiation Potential in Human Mesenchymal Stem Cells from Wharton's Jelly, Bone Marrow, and Pancreatic Tissues. *Stem cells international* 2015, 306158.

ISSN: 2198-4093
www.bmrat.org

27. Kim, H., Han, J.W., Lee, J.Y., Choi, Y.J., Sohn, Y.D., Song, M., and Yoon, Y.S. (2015). Diabetic Mesenchymal Stem Cells Are Ineffective for Improving Limb Ischemia Due to Their Impaired Angiogenic Capability. *Cell transplantation* 24, 1571-1584.

28. Kim, J., Park, S., Kang, H.M., Ahn, C.W., Kwon, H.C., Song, J.H., Lee, Y.J., Lee, K.H., Yang, H., Baek, S.Y., *et al.* (2012). Human insulin secreted from insulinogenic xenograft restores normoglycemia in type 1 diabetic mice without immunosuppression. *Cell transplantation* 21, 2131-2147.

29. Kong, D., Zhuang, X., Wang, D., Qu, H., Jiang, Y., Li, X., Wu, W., Xiao, J., Liu, X., Liu, J., *et al.* (2014). Umbilical cord mesenchymal stem cell transfusion ameliorated hyperglycemia in patients with type 2 diabetes mellitus. *Clinical laboratory* 60, 1969-1976.

30. Le, P.T.-B., Van Pham, P., Vu, N.B., Dang, L.T.-T., and Phan, N.K. (2016). Expanded autologous adipose derived stem cell transplantation for type 2 diabetes mellitus. *Biomedical Research and Therapy* 3, 1034-1044.

31. Lee, R.H., Seo, M.J., Reger, R.L., Spees, J.L., Pulin, A.A., Olson, S.D., and Prockop, D.J. (2006). Multipotent stromal cells from human marrow home to and promote repair of pancreatic islets and renal glomeruli in diabetic NOD/scid mice. *Proceedings of the National Academy of Sciences of the United States of America* 103, 17438-17443.

32. Liu, X., Zheng, P., Wang, X., Dai, G., Cheng, H., Zhang, Z., Hua, R., Niu, X., Shi, J., and An, Y. (2014). A preliminary evaluation of efficacy and safety of Wharton's jelly mesenchymal stem cell transplantation in patients with type 2 diabetes mellitus. *Stem cell research & therapy* 5, 57.

33. Luz-Crawford, P., Kurte, M., Bravo-Alegria, J., Contreras, R., Nova-Lamperti, E., Tejedor, G., Noel, D., Jorgensen, C., Figueroa, F., Djouad, F., *et al.* (2013). Mesenchymal stem cells generate a CD4+CD25+Foxp3+ regulatory T cell population during the differentiation process of Th1 and Th17 cells. *Stem cell research & therapy* 4, 65.

34. Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., and Wang, Y. (2014). Immunobiology of mesenchymal stem cells. *Cell death and differentiation* 21, 216-225.

35. Mattar, P., and Bieback, K. (2015). Comparing the Immunomodulatory Properties of Bone Marrow, Adipose Tissue, and Birth-Associated Tissue Mesenchymal Stromal Cells. *Frontiers in immunology* 6, 560.

36. Menard, C., Pacelli, L., Bassi, G., Dulong, J., Bifari, F., Bezier, I., Zanoncello, J., Ricciardi, M., Latour, M., Bourin, P., *et al.* (2013). Clinical-grade mesenchymal stromal cells produced under various good manufacturing practice processes differ in their immunomodulatory properties: standardization of immune quality controls. *Stem cells and development* 22, 1789-1801.

37. Montanucci, P., Pescara, T., Pennoni, I., Alunno, A., Bistoni, O., Torlone, E., Luca, G., Gerli, R., Basta, G., and Calafiore, R. (2016). Functional Profiles of Human Umbilical Cord-Derived Adult Mesenchymal Stem Cells in Obese/Diabetic Versus Healthy Women. *Current diabetes reviews.*

38. Moorefield, E.C. (2012). Stem cell-based regenerative pharmacology for the treatment ò diabetes mellitus.

39. Nauta, A.J., and Fibbe, W.E. (2007). Immunomodulatory properties of mesenchymal stromal cells. *Blood* 110, 3499-3506.

ISSN: 2198-4093
www.bmrat.org

40. Ngoc, P.K., Phuc, P.V., Nhung, T.H., Thuy, D.T., and Nguyet, N.T. (2011). Improving the efficacy of type 1 diabetes therapy by transplantation of immunoisolated insulin-producing cells. *Human cell* 24, 86-95.

41. Phadnis, S.M., Ghaskadbi, S.M., Hardikar, A.A., and Bhonde, R.R. (2009). Mesenchymal stem cells derived from bone marrow of diabetic patients portrait unique markers influenced by the diabetic microenvironment. *The review of diabetic studies : RDS* 6, 260-270.

42. Pham, P.V., Vu, N.B., Pham, V.M., Truong, N.H., Pham, T.L., Dang, L.T., Nguyen, T.T., Bui, A.N., and Phan, N.K. (2014). Good manufacturing practice-compliant isolation and culture of human umbilical cord blood-derived mesenchymal stem cells. *Journal of translational medicine* 12, 56.

43. Ren, H., Sang, Y., Zhang, F., Liu, Z., Qi, N., and Chen, Y. (2016). Comparative Analysis of Human Mesenchymal Stem Cells from Umbilical Cord, Dental Pulp, and Menstrual Blood as Sources for Cell Therapy. *Stem cells international* 2016, 3516574.

44. Sensebe, L., Bourin, P., and Tarte, K. (2011). Good manufacturing practices production of mesenchymal stem/stromal cells. *Human gene therapy* 22, 19-26.

45. Seyedi, F., Farsinejad, A., Moshrefi, M., and Nematollahi-Mahani, S.N. (2015). In vitro evaluation of different protocols for the induction of mesenchymal stem cells to insulin-producing cells. *In vitro cellular & developmental biology Animal* 51, 866-878.

46. Seyedi, F., Farsinejad, A., Nematollahi-Mahani, S.A., Eslaminejad, T., and Nematollahi-Mahani, S.N. (2016). Suspension Culture Alters Insulin Secretion in Induced Human Umbilical Cord Matrix-Derived Mesenchymal Cells. *Cell journal* 18, 52-61.

47. Si, Y., Zhao, Y., Hao, H., Liu, J., Guo, Y., Mu, Y., Shen, J., Cheng, Y., Fu, X., and Han, W. (2012). Infusion of mesenchymal stem cells ameliorates hyperglycemia in type 2 diabetic rats: identification of a novel role in improving insulin sensitivity. *Diabetes* 61, 1616-1625.

48. Sordi, V., Melzi, R., Mercalli, A., Formicola, R., Doglioni, C., Tiboni, F., Ferrari, G., Nano, R., Chwalek, K., Lammert, E., *et al.* (2010). Mesenchymal cells appearing in pancreatic tissue culture are bone marrow-derived stem cells with the capacity to improve transplanted islet function. *Stem cells* 28, 140-151.

49. Spaggiari, G.M., Capobianco, A., Abdelrazik, H., Becchetti, F., Mingari, M.C., and Moretta, L. (2008). Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. *Blood* 111, 1327-1333.

50. Thi-Tung Dang, L., Nguyen-Tu Bui, A., Minh Pham, V., Kim Phan, N., Van Pham, P. (2015). Production of islet-like insulin-producing cell clusters in vitro from adipose derived stem cells. *Biomedical Research and Therapy* 2, 184-192.

51. Tobita, M., Tajima, S., and Mizuno, H. (2015). Adipose tissue-derived mesenchymal stem cells and platelet-rich plasma: stem cell transplantation methods that enhance stemness. *Stem cell research & therapy* 6, 215.

52. Tsai, P.J., Wang, H.S., Lin, G.J., Chou, S.C., Chu, T.H., Chuan, W.T., Lu, Y.J., Weng, Z.C., Su, C.H., Hsieh, P.S., *et al.* (2015). Undifferentiated Wharton's Jelly Mesenchymal Stem Cell Transplantation Induces Insulin-Producing Cell Differentiation and Suppression of T-Cell-Mediated Autoimmunity in Nonobese Diabetic Mice. *Cell transplantation* 24, 1555-1570.

ISSN: 2198-4093
www.bmrat.org

53. Van Pham, P., Thi-My Nguyen, P., Thai-Quynh Nguyen, A., Minh Pham, V., Nguyen-Tu Bui, A., Thi-Tung Dang, L., Gia Nguyen, K., and Kim Phan, N. (2014). Improved differentiation of umbilical cord blood-derived mesenchymal stem cells into insulin-producing cells by PDX-1 mRNA transfection. *Differentiation; research in biological diversity* 87, 200-208.

54. Van Pham, P., Vu, N.B., and Phan, N.K. (2016). Umbilical cord-derived stem cells (MODULATISTTM) show strong immunomodulation capacity compared to adipose tissue-derived or bone marrow-derived mesenchymal stem cells. *Biomedical Research and Therapy* 3, 687-696.

55. Voltarelli, J.C., Couri, C.E., Rodrigues, M.C., Moraes, D.A., Stracieri, A.B., Pieroni, F., Navarro, G., Leal, A.M., and Simoes, B.P. (2011). Stem cell therapies for type 1 diabetes mellitus. *Indian journal of experimental biology* 49, 395-400.

56. Wajid, N., Naseem, R., Anwar, S.S., Awan, S.J., Ali, M., Javed, S., and Ali, F. (2015). The effect of gestational diabetes on proliferation capacity and viability of human umbilical cord-derived stromal cells. *Cell and tissue banking* 16, 389-397.

57. Wang, Y., Chen, X., Cao, W., and Shi, Y. (2014). Plasticity of mesenchymal stem cells in immunomodulation: pathological and therapeutic implications. *Nature immunology* 15, 1009-1016.

58. Wehner, R., Taubert, C., Mende, T., Gaebler, C., de Andrade, A.V., Bornhauser, M., Werner, C., Tonn, T., Schakel, K., Bachmann, M., *et al.* (2013). Engineered extracellular matrix components do not alter the immunomodulatory properties of mesenchymal stromal cells in vitro. *Journal of tissue engineering and regenerative medicine* 7, 921-924.

59. Wu, C., Liu, F., Li, P., Zhao, G., Lan, S., Jiang, W., Meng, X., Tian, L., Li, G., Li, Y., *et al.* (2015). Engineered hair follicle mesenchymal stem cells overexpressing controlled-release insulin reverse hyperglycemia in mice with type L diabetes. *Cell transplantation* 24, 891-907.

60. Wu, X.H., Liu, C.P., Xu, K.F., Mao, X.D., Zhu, J., Jiang, J.J., Cui, D., Zhang, M., Xu, Y., and Liu, C. (2007). Reversal of hyperglycemia in diabetic rats by portal vein transplantation of islet-like cells generated from bone marrow mesenchymal stem cells. *World journal of gastroenterology* 13, 3342-3349.

61. Xiao, N., Zhao, X., Luo, P., Guo, J., Zhao, Q., Lu, G., and Cheng, L. (2013). Co-transplantation of mesenchymal stromal cells and cord blood cells in treatment of diabetes. *Cytotherapy* 15, 1374-1384.

62. Xie, Z., Hao, H., Tong, C., Cheng, Y., Liu, J., Pang, Y., Si, Y., Guo, Y., Zang, L., Mu, Y., *et al.* (2016). Human umbilical cord-derived mesenchymal stem cells elicit macrophages into an anti-inflammatory phenotype to alleviate insulin resistance in type 2 diabetic rats. *Stem cells* 34, 627-639.

63. Xin, Y., Jiang, X., Wang, Y., Su, X., Sun, M., Zhang, L., Tan, Y., Wintergerst, K.A., Li, Y., and Li, Y. (2016). Insulin-Producing Cells Differentiated from Human Bone Marrow Mesenchymal Stem Cells In Vitro Ameliorate Streptozotocin-Induced Diabetic Hyperglycemia. *PloS one* 11, e0145838.

64. Yang, Z., Li, K., Yan, X., Dong, F., and Zhao, C. (2010). Amelioration of diabetic retinopathy by engrafted human adipose-derived mesenchymal stem cells in streptozotocin diabetic rats. *Graefe's archive for clinical and experimental ophthalmology = Albrecht von Graefes Archiv fur klinische und experimentelle Ophthalmologie* 248, 1415-1422.

ISSN: 2198-4093
www.bmrat.org

65. Yaochite, J.N., de Lima, K.W., Caliari-Oliveira, C., Palma, P.V., Couri, C.E., Simoes, B.P., Covas, D.T., Voltarelli, J.C., Oliveira, M.C., Donadi, E.A., *et al.* (2016). Multipotent mesenchymal stromal cells from patients with newly diagnosed type 1 diabetes mellitus exhibit preserved in vitro and in vivo immunomodulatory properties. *Stem cell research & therapy* 7, 14.

66. Zhang, L., Gianani, R., Nakayama, M., Liu, E., Kobayashi, M., Baschal, E., Yu, L., Babu, S., Dawson, A., Johnson, K., *et al.* (2008). Type 1 diabetes: chronic progressive autoimmune disease. *Novartis Foundation symposium* 292, 85-94; discussion 94-88, 122-129, 202-123.

67. Zhang, Y., and Dou, Z. (2014). Under a nonadherent state, bone marrow mesenchymal stem cells can be efficiently induced into functional islet-like cell clusters to normalize hyperglycemia in mice: a control study. *Stem cell research & therapy* 5, 66.

68. Zhang, Y., Shen, W., Hua, J., Lei, A., Lv, C., Wang, H., Yang, C., Gao, Z., and Dou, Z. (2010). Pancreatic islet-like clusters from bone marrow mesenchymal stem cells of human first-trimester abortus can cure streptozocin-induced mouse diabetes. *Rejuvenation research* 13, 695-706.

69. Zhao, S., Wehner, R., Bornhauser, M., Wassmuth, R., Bachmann, M., and Schmitz, M. (2010). Immunomodulatory properties of mesenchymal stromal cells and their therapeutic consequences for immune-mediated disorders. *Stem cells and development* 19, 607-614.

70. Zhou, H., Tian, H.M., Long, Y., Zhang, X.X., Zhong, L., Deng, L., Chen, X.H., and Li, X.Q. (2009). Mesenchymal stem cells transplantation mildly ameliorates experimental diabetic nephropathy in rats. *Chinese medical journal* 122, 2573-2579.

71. Zhou, Y., Hu, Q., Chen, F., Zhang, J., Guo, J., Wang, H., Gu, J., Ma, L., and Ho, G. (2015). Human umbilical cord matrix-derived stem cells exert trophic effects on beta-cell survival in diabetic rats and isolated islets. *Disease models & mechanisms* 8, 1625-1633.

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Review

Improving stem cell engraftment to enhance functional efficacy in cardiovascular disease: where are we now?

Anh Thi Van Bui[1,*], Truc Le Buu Pham[1], Jitka Virag[2]

[1]Laboratory of Stem cell Research and Application, University of Science, Vietnam National University Ho Chi Minh City, Viet Nam
[2]Department of Physiology, Brody School of Medicine 600 Moye Blvd Greenville, NC 27834, United States

Abstract

Stem cell therapy is a promising therapy for repairing damaged tissue. A growing body of research shows that stem cells work effectively in several diseases such as cardiovascular disease, hepatic disease, and diabetes. It has been shown that stem cells not only differentiate into functional cells and replace dead cells, but also release growth factors and cytokines which can recruit autologous cells. The most significant barrier to achieve clinical relevance of this treatment mode is the poor survival rate of injected cells. To improve transplantation and enhance functional outcome, investigations of gene transfection (overexpression of anti-apoptotic and antioxidant proteins), growth factor supplementation, and scaffolding matrices are being conducted. In this review, we will focus on methods to increase cell survival in stem cell transplantation as a novel treatment for cardiovascular disease.

Keywords

Cardiovascular disease treatment; Cell transplantation improvement; Stem cell therapy

***For correspondence:**

vananhcsh@gmail.com

Competing interests: The authors declare that no competing interests exist.

Received: 05 January 2017
Accepted: 12 January 2017
Published: 18 January 2017

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Introduction

Cardiovascular disease is the leading cause of death globally. The World Health Organization (WHO) reported that about 17.5 million people died from this disease in 2012. Among many therapies and interventions that have been developed to treat the disease, stem cell therapy is one of the greatest potential candidates for treatment (Fuster, 2014). For instance, unlike heart replacement, various sources of progenitor cells/stem cells are available - umbilical cord derived stem cells, bone marrow derived mesenchymal stem cells, adipose derived stem cells, embryonic stem cells, cardiac stem cells, and induced pluripotent stem cells. Therefore, it is possible to have a suitable cell type for a specific patient. Moreover, the therapy does not require a surgery, so it is safer, more affordable and requires less recovery time and resources. Cells can be injected into the heart by catheter or needle and syringe. Patients do not even need to undergo anesthesia. This can reduce morbidity and mortality in an already high-risk population. As a consequence, there have been several clinical trials using cell therapy to treat both acute myocardial infarction and chronic ischemia in human (Fisher et al., 2014; Fisher et al., 2015).

The heart lacks self-regeneration ability. After infarction, hypoxia-sensitive cardiomyocytes die and an inflammatory response ensues, removing the debris. Fibroblasts then proliferate and occupy the space, creating a collagenous, non-contractile scar which continues to remodel, causing negative effectives on cardiac function. The hope in using stem cells resides in the idea that they not only become heart cells, but also recruit autologous stem/progenitor cells, mobilized to the infarcted area, to recapitulate the highly-vascularized environment to support the metabolic demands. Additionally, many cytokines and chemokines are secreted to induce paracrine effects.

The goals are that cell therapy can repair the damaged heart by mediating some combination of the following mechanisms:

Differentiate to cardiomyocytes

Many stem cell types have been showed that they can express markers of cardiomyocytes and cardio-progenitor cells (Boheler et al., 2002; Quevedo et al., 2009; Toma et al., 2002). For example, cardiomyocytes could be derived from embryonic stem cells when scientists cultivated embryoid body to day 7 and/or under the induction of several factors. When beating area appeared, they collected the cells from the population by enzyme (like collagenase) (Laflamme et al., 2007; Maltsev et al., 1993; Maltsev et al., 1994). Then, they adjusted the expression of cardio-specific proteins like myosin heavy chain (MHC), α-actin, desmin, and cardiac troponin T (Boheler et al., 2002). In addition to embryonic stem cells, messenchymal stem cells has also been studied the differentiation

ISSN: 2198-4093
www.bmrat.org

ability into cardiomyocytes both *in vitro* and *in vivo* (Toma et al., 2002; Xu et al., 2004). At the present, adipose tissue-derived stem cells (ADSC) and induced-pluripotent stem cells (iPSC) are the main focus. 5-azacytidine is used as an effective induced factor to start the differentiation process by activating transcription factor genes like GATA4, Nkx2.5 which then stimulate expression of cardiac-specific genes (Burridge and Zambidis, 2013; Carvalho et al., 2013; Gherghiceanu et al., 2011; Li et al., 2015). As a result of this potential, stem cell therapy has become a promising therapy to treat cardiovascular disease, especially diseases relevant to myocardial injury.

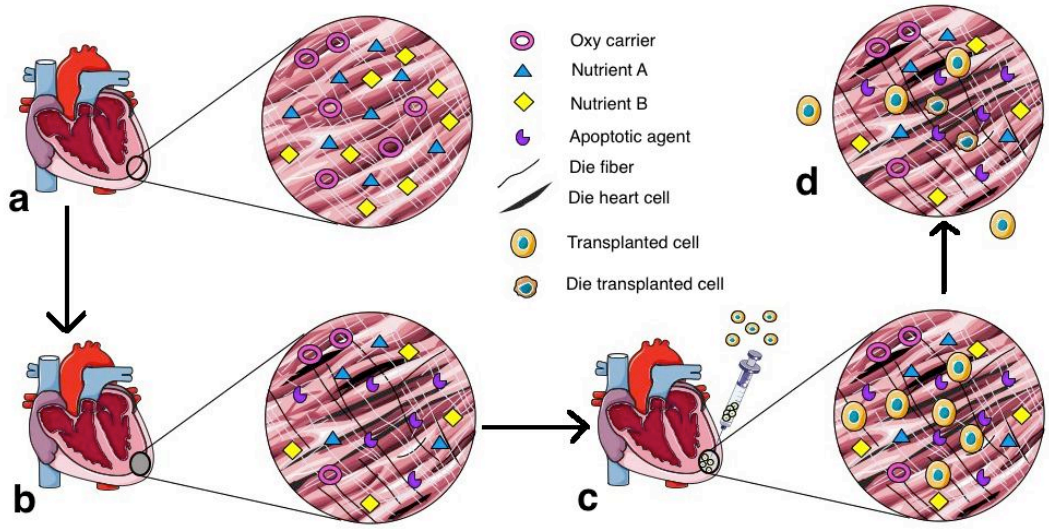


Figure 1. Reasons for cell loss after transplantation. (a) Normally, heart is supplied enough oxygen, nutrients as well as a good matrix for its life; (b) In ischemic condition, the infarcted area lacks of blood leading to the decreasing of these essential factors and the increasing of apoptotic factors; (c & d) When deliveried to the damaged heart area, the number of transplanted cells/stem cells could be lost by two ways: the cells die causing by hash environment in the infarcted zone and/or go out of the injuried tissue causing by weak adhesion to a poor extra cellular matrix.

Release of paracrine factors

In addition to differential ability, transplanted stem cells also release paracrine signaling and recruit endogenous cardiac stem cells (Gnecchi et al., 2008). A growing body of research has shown that the mechanism which stem cells can regenerate the damaged heart was the cells release soluble factors that stimulated the cardiac regeneration, neovascularization, and cardiac modeling after myocardial infarction (Gnecchi et al., 2008; Kinnaird et al., 2004; Zhang et

ISSN: 2198-4093
www.bmrat.org

al., 2007). These factors could activate pro-survival signaling pathway like PI3K/Akt or STAT3 which protect cardiomyocytes and enhance cardiac regeneration (Gnecchi et al., 2006). They contain factors such as bFGF, IGF-2, VEGF, IL-11. Additionally, these factors also recruit endogenous cardiac stem cells which, although the existence is controversial and if so, not present in significant numbers, have strong abilities to become heart cells, vascular smooth muscle cells, and endothelial cells *in vivo* (Leri et al., 2005). These factors like IGF-1 and HGF released by transplanted Mesenchymal stem cells-MSCs were shown that they could activate endogenous cardiac stem cells (Gnecchi et al., 2008). The broad spectrum of abilities of stem cells has been a significantly important issue that could explain the cardio-protection and remodeling of stem cell transplantation.

Cell fusion

Moreover, cell fusion between transplanted cells and domestic cells also has been used to explain the mechanism of damaged heart renewal by stem cell transplantation (Doppler et al., 2013). Injected cells were demonstrated to fuse with host cardiomyocytes and stimulate the regeneration of injured hearts. These stem cell types were cardiac progenitor cells (Oh et al., 2003), bone marrow stem cells (Nygren et al., 2004), and adipose tissue-derived stem cells (Metzele et al., 2011). This potential of stem cells has contributed to benefits of stem cell therapy for cardiovascular disease.

Although stem cell transplantation is a promising therapy and has been under development for a long time since the first study was published (Marelli et al., 1992), it also contains many challenges when cells are injected to hearts (**Fig. 1**). Firstly, poor cell survival caused by harsh environment in the infarcted area (Zhang et al., 2001). When cells are transplanted to injured heart, they are immediately embedded in the ischemic condition which lacks oxygen, nutrients, and contain many apoptotic signals, causing an abundance of cell death as soon as the first hour after transplantation (Haider and Ashraf, 2008; Muller-Ehmsen et al., 2002; Zhang et al., 2001). Secondly, cell engraftment has also been a major concern of stem cell therapy for heart disease. As a result of tenuous cell retention and cell survival, injected cells have difficulty integrating with the host tissue. The saline solution which is regularly used as a vehicle for delivery cannot keep the cells in the heart tissue because it does not contain a matrix for cell adherence of survival factors (Li et al., 2016). More studies are needed to optimize the transplant conditions.

To solve these two main problems in stem cell therapy for heart disease, scientists have suggested many solutions focusing on three approaches: gene transfection, growth factor introduction, and creation of hydrogel scaffolding (**Fig. 2**).

ISSN: 2198-4093
www.bmrat.org

Gene transfection

Akt

Overexpressing Akt helps stem cells (like MSCs) exhibit a significantly enhanced intramyocardial retention and engraftment. It is believed that Akt is necessary and sufficient for cell survival because it not only targets apoptotic family members, but also increases glucose metabolism and releases energy during hypoxia (Datta et al., 1999). In light of these advantages of Akt protein, Akt-modified MSCs genetically restore significantly greater myocardial volume than equal numbers of control cells (Mangi et al., 2003). In addition to Akt signal transduction, many investigators work with hypoxia inducible factor 1 alpha (HIF-1α) and Pim-1 kinase.

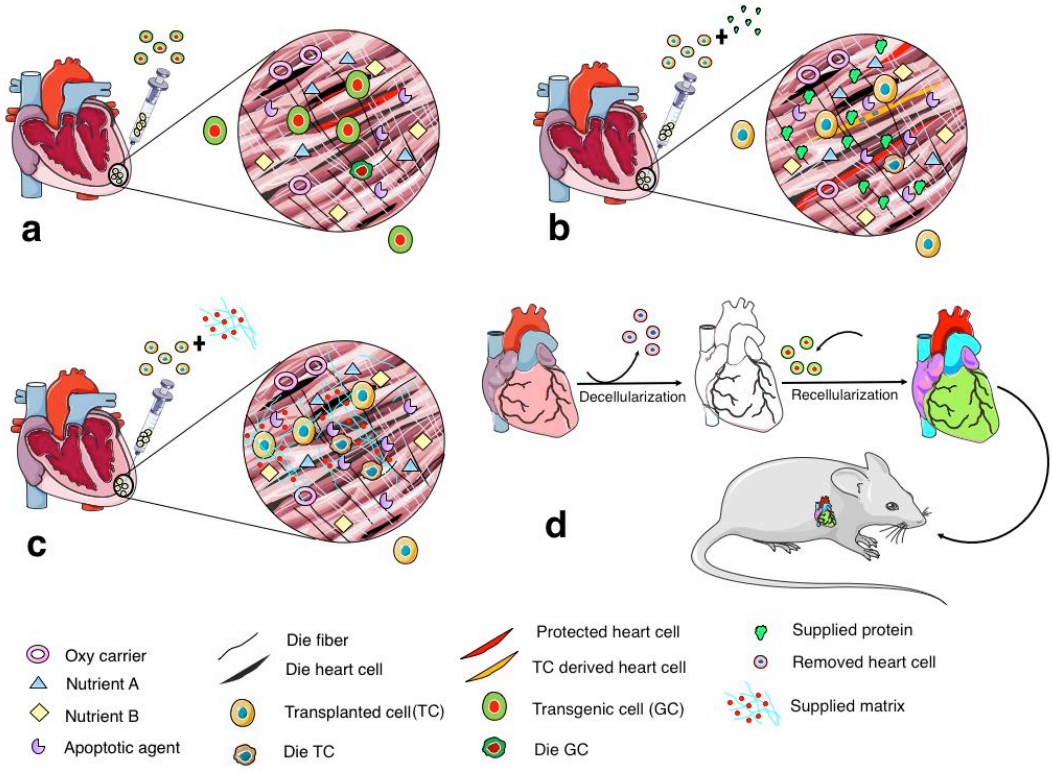


Figure 2. Stem cell engraftment improving methods. (a) The transgenic stem cells released helpful factors to protect recipient cardiomyocytes and themselves from a hash environment that contributed to increase the number of survival cells after injection; (b) Together with transplanted cells, supplied proteins promoted the differentiation of cells into myocardial cells and protected cells from ensuing lesions; (c) In order to enhance the adhesion of transplanted cells, the matrix was also added to the cell solution for implantation (c) Heart was removed all cells to form a natural heart scaffold which was then seeded with suitable cells to form a perfect heart for transplantation in vivo.

ISSN: 2198-4093
www.bmrat.org

HIF-1 alpha

HIF-1 alpha is a pro-angiogenic transcription factor which regulates the expression of many genes such as vascular endothelial growth factor (VEGF), angiopoietin 1 (ANGPT1) and ANGPT2, placental growth factor (PGF), and platelet-derived growth factor B (PDGFB) (Dai et al., 2007; Manalo et al., 2005). Under hypoxic conditions, hydroxyl reaction does not occur, causing HIF-1 protein to escape from inhibition and be activated, resulting in transcription of many genes and stimulates angiogenesis (Pugh and Ratcliffe, 2003). Basing on the important function of HIF 1 in hypoxic cells, it's expression was introduced in MSCs, which was expected that these MSCs could protect co-culture cardiomyocytes through HIF pathway (Dai et al., 2007).

Pim-1

Pim-1 is a proto-oncogenic serine-threonine kinase which was first described in hematopoietic cells. The kinase is responsible for cell survival, proliferation, and differentiation in the cell type (Wang et al., 2001). According to Aho et al, Pim-1 kinase inhibits pro-apototic Bad protein and increases Bcl-2 activity, promoting cell survival (Aho et al., 2004). Recently, it is also demonstrated that Pim-1 plays an important role in cardioprotection downstream of Akt signaling (Muraski et al., 2007). Fischer et al proves that Pim-1 overexpress – cardiac progenitor cells (Pim-1 – CPCs) survive and proliferate better than control CPCs (Fischer et al., 2009). In this study, Pim-1-CPCs improve cardiac repair and regeneration through increasing in vascular density, cell proliferation, and persistence (Fischer et al., 2009).

Protein

While genes must be introduced to cells before injection, growth factors can be supplied to the injected cells before or at the same time with transplantation. The following proteins have demonstrated promise as adjuncts to cellular therapy:

Ephrin

The Ephrin/Eph receptor tyrosine kinase (RTK) family is the largest of the RTK families. Unlike the other members of RTK family, Ephs specially can activated intracellular signals in both receptor exposing cells and ligand exposing cells that allows them to participate in many important biological processes such as development, proliferation, differentiation, and migration. There are two subclasses in Ephs family, including EphAs and EphBs that are segregated by their abilities to bind with the glycosylphosphatidylinositol-linked Ephrin-A ligands or the transmembrane-bound ephrin-B ligands. Typically, the receptors of specific groups respond to ligands of their corresponding groups , ie receptor

ISSN: 2198-4093
www.bmrat.org

EphA binds to ligand ephrinA. They are less or not bind to ligands of opposing groups , except ephrin B3 can attach and activate EphA4 and eprin A5 can bind and activate EphB2 (O'Neal et al., 2013). Ephrin as a target for cardiovascular treatment was first described by Mansson-Broberg et al. in 2008 (Mansson-Broberg et al., 2008). Since then, a few publications about the EphrinA/EphA-R signaling system in the heart have been produced (Frieden et al., 2010; Goichberg et al., 2011; O'Neal et al., 2013). Goichberg et al showed that motility and migration of human cardiac stem cells (hCSCs) were enhanced by the increasing of interactions between EphA2 on hCSCs and ephrinA1 which presented on cardiomyocytes (Goichberg et al., 2011). The Virag laboratory performed many studies to clarify the effects of this protein in cardio-protection after myocardial infarction (Dries et al., 2011; DuSablon et al., 2014; O'Neal et al., 2014). They demonstrated that mice lacking EphA2 receptor expression caused negative effects on survival after myocardial infarction and made the remodeling process stagnant (DuSablon et al., 2014). Furthermore, they demonstrated that EphrinA1 signaling could modulate inflammatory response and limit the infarct size (O'Neal et al., 2014). Unlike cancer treatment (Boyd et al., 2014), the approach using Ephrin signaling in heart disease treatment is still novel and more research is warranted to be assess clinical relevance.

Bone morphogenetic protein - BMP

BMP belongs to the transforming growth factor-beta (TGF-β) super family (Chen et al., 2004). It is a key protein in mesoderm development and cardiac differentiation (Chen et al., 2008). A growing body of research has shown that BMP is needed for pluripotent stem cells differentiating into cardiac cells. When supplied to the growth medium, BMP expands embryonic stem cells and has been demonstrated to promote cardiomyocyte differentiation (Pal and Khanna, 2007). Using BMP4 and Activin A to induce ESCs to differentiate into cardiomyocytes, Laflamme et al presented a cocktail of pro-survival factors to enhance post-transplantation cell survival. This approach significantly improved ESC engraftment in myocardial infarction treatment. For example, it prevented death pathways to protect the cells on the transplanted grafts and helped the grafts survive 1 to 4 weeks after implantation. Therefore, the grafts could cause positive effects on the cardiac function like remuscularization, wall thickening, ventricular dilation decreasing, global function improving, and wall motion enhancement (Behfar et al., 2002; Laflamme et al., 2007). With the advantages, BMP should be researched to apply in cell therapy for heart disease treatment.

Fibroblast growth factor – FGF

More than 20 years ago, FGF was shown to be cardioprotective, especially after ischemia-reperfusion injury by Kardami et al. (Kardami et al., 1993). FGF is a member of the family of heparin-binding growth factors and is involved in several important developmental processes (Thisse and Thisse, 2005). In the heart, FGF works as cardioprotective and angiogenic agent (Detillieux et al., 2003). It not only maintains cardiomyocyte survival, but also stimulates functional

ISSN: 2198-4093
www.bmrat.org

recovery after injury (Rosenblatt-Velin et al., 2005), leading to the notion that using FGFs is a feasible approach to improve stem cell transplantation effectiveness. Recently, to pre-treat stem cells with FGF before transplantation, scientists combined it in a cocktail of growth factors such as TGF β, BMP-2, Activin A, etc. (Behfar et al., 2010; Hahn et al., 2008). In the future, FGFs might be applied in both stem cell culture and global tissue restoration after heart damaged.

Insulin-like growth factor 1– IGF-1

IGF-1 has been shown to reduce the doubling time of mesenchymal stem cells (MSC), improve MSC proliferation, and enhance CXCR4 expression (Huang et al., 2012). IGF-1 also a demonstrated role in modulating cardiovascular function. It facilitates cardiac development, enhances cardiac contractility, and protects cardiac tissue after myocardial infarction (Ren et al., 1999). Therefore, using IGF-1 accompany with transplanted cells has become a promising strategy. Davis et al. has reported that, when the combination of cardiomyocytes and IGF-1 in a "biotin sandwich" transplanted into injured hearts resulted in recovery of heart function (Davis et al., 2006). This result suggested that IGF-1 could cause positive effects on both cardiac and injected cells and using IGF-1 in cell therapy needs further exploration.

Scaffold

To improve cell survival and cell engraftment in stem cell therapy for heart disease treatment, scaffolding to support cells is a valuable tool. Scaffolding helps us not only control the cell distribution, but also provide growth factors and structural support for transplanted cells.

Hydrogel

Although gene and growth factor strategies can improve cell survival, they do not contribute substantially to cell distribution and engraftment. To improve the stem cell transplantation effectiveness, a new approach called Matrix-Assisted Cell Transplantation (MACT) which creates a materials-based environment that enhances pro-survival paracrine signaling and then increase cell engraftment with the host tissues was born (Parisi-Amon et al., 2013; Prestwich, 2008). There are several materials that can be used in the strategy including natural and synthetic types like hydrogel, alginate, chitosan, collagen, etc. (Benoit et al., 2008; Kang et al., 2014). Subsequently, some scientists suggested that they will use hydrogel as a MACT to secrete growth factors and improve cell survival after transplantation (Jha et al., 2015). Hyaluronic acid –based hydrogel is a promising candidate which contains key advantages such as biocompatibility, tunable properties, and native biofunctionality (Highley et al., 2016; Travan et al., 2016). It is wide used for biomedical applications, especially in heart disease treatment

ISSN: 2198-4093
www.bmrat.org

therapy (Abdalla et al., 2013; Bonafe et al., 2014; Jha et al., 2015). More research is needed to validate its clinical potential as an adjunct to cellular engraftment therapies.

Natural heart scaffold

Decellularization of the heart is also an attractive and challenging approach. Scientists collect heart from deceased patients and used detergents like Triton-X-100 or SDS to decellularize the hearts. They pushed detergents through a tube to the heart's aorta to remove DNA, soluble protein, lipid, and other cellular material to perform a natural matrix. This matrix was then used to seed cells to create an engineered heart (Ott et al., 2008). Finally, they transplant the heart to animal model (Maher, 2013). The time and energy irequired to decellularize the tissue, seed the cells, grow the tissue to achieve functionality, and transplant it into an animal is an extremely labor- and time-intensive endeavor. However, "this train has left the station" and so there are several investigators that continue to focus on refining and expediting this strategy and the results are heartening (Lu et al., 2013; Tapias and Ott, 2014). Although it has tremendous challenges, it still holds remarkable advantages such as providing an excellent model to research cell development and differentiation in 3D, and improving our understanding of the complex, unique, and dynamic nature of the myocardial scaffolding. This strategy needs to be explored to optimize the decellulalrization process, which cell type(s) can be used, and the functional range of blood-pumping capacity.

Cell-derived matrices

Scaffold created from cell-derived matrices is also an attractive approach. Instead of using directly natural tissue (such as heart from the newly dead) to create scaffold, people used cells/stem cells to form matrices which mimicked natural extracellular matrices. Then, these cells would be removed. According to Fitzpatrick and McDevitt review, several approaches in using these scaffold for cardiovascular and other organs (like skeletal tissue or skin) regeneration have been conducted (Fitzpatrick and McDevitt, 2015). For example, to perform heart valve or blood vessel, scientists cultured fibroblast or smooth muscle cells in sheets, then these sheets were wrapped around a mandrel to have a tubular structure (Quint et al., 2012). Although there were several clinical trials using this strategy (McAllister et al., 2009; Quint et al., 2012; Wystrychowski et al., 2011), it needs to be explored more about engineered cells and decellularization techniques (Fitzpatrick and McDevitt, 2015) before applying on patients.

Conclusions and future directions

As stem cells provide a hopeful treatment for cardiovascular disease patients, despite controversial opinions about the potential of this therapy (Abbott, 2014),

ISSN: 2198-4093
www.bmrat.org

there are numerous pre-clinical trials and clinical trials using them (Kastrup et al., 2016; Krishna et al., 2011; Westerdahl et al., 2016). Our group has also reported that umbilical cord blood derived stem cells can differentiate into cardiac progenitor cells and that injection of induced cells into damaged heart reduced scar size, stabilized blood pressure, and maintained heart function (Pham et al., 2015). Van der Spoel et al. has a systematic review and meta-analysis about stem cell therapy for ischemic heart disease in large animals (van der Spoel et al., 2011). According to this review, stem cell therapy is safe and improves left ventricular ejection fraction (LVEF) after injury. Currently, Poulin and colleagues also have a similar review on 29 studies which showed that stem cell therapy could enhance LVEF, reduce infarcted size, and increase myocardial viability (Poulin et al., 2016). In the future, stem cell therapy with its safety, effective, and affordable price will be a promising therapy to save heart disease patients and improve the quality of life. Improving the outcome of the therapy by enhancing transplanted cell survival, distribution, and engraftment need further modifications and refinements to ensure optimal conditions before they can be routinely applied.

Abbreviations

ADSC: Adipose tissue-derived stem cell; Akt: Serine/threonine-specific protein kinase; ANGPT1, 2: Angiopoietin 1, 2; Bcl-2: B-cell lymphoma 2; bFGF: Basic fibroblast growth factor; BMP: Bone morphogenetic protein; CXCR4: Chemokine receptor type 4; Ephs: Ephrins protein; FGF: Fibroblast growth factor; HGF: Hepatocyte growth factor; HIF: Hypoxia inducible factor; IGF-2: Insulin-like growth factor 2; IL-11: Interleukin 11: iPSC: induced-Pluripotent stem cell; LVEF: left ventricular ejection fraction; MACT: Matrix-Assisted Cell Transplantation; MHC: Myosin heavy chain; MSC: Mesenchymal stem cell: PDGFB: Platelet-derived growth factor B; PGF: Placental growth factor; PI3K: Phosphoinositide 3-kinase: RTK: The Ephrin/Eph receptor tyrosine kinase; STAT3: Signal transducer and activator of transcription 3; TGF-beta: Transforming growth factor beta; VEGF: vascular endothelial growth factor.

Acknowledgement

I would like to thank Dr. Phuc Van Pham for his inspiration and guidance in stem cell research.

Author contribution

Anh Thi Van Bui created the outline and wrote the manuscript, Dr. Truc Le Buu Pham draw 2 figures and edited the manuscript, Assistant Professor Jitka Virag organized and edited the manuscript.

ISSN: 2198-4093
www.bmrat.org

References

Abbott, A. (2014). Doubts over heart stem-cell therapy. *Nature* 509, 15-16.

Abdalla, S., Makhoul, G., Duong, M., Chiu, R.C., and Cecere, R. (2013). Hyaluronic acid-based hydrogel induces neovascularization and improves cardiac function in a rat model of myocardial infarction. *Interactive cardiovascular and thoracic surgery* 17, 767-772.

Aho, T.L., Sandholm, J., Peltola, K.J., Mankonen, H.P., Lilly, M., and Koskinen, P.J. (2004). Pim-1 kinase promotes inactivation of the pro-apoptotic Bad protein by phosphorylating it on the Ser112 gatekeeper site. *FEBS letters* 571, 43-49.

Behfar, A., Yamada, S., Crespo-Diaz, R., Nesbitt, J.J., Rowe, L.A., Perez-Terzic, C., Gaussin, V., Homsy, C., Bartunek, J., and Terzic, A. (2010). Guided cardiopoiesis enhances therapeutic benefit of bone marrow human mesenchymal stem cells in chronic myocardial infarction. *Journal of the American College of Cardiology* 56, 721-734.

Behfar, A., Zingman, L.V., Hodgson, D.M., Rauzier, J.M., Kane, G.C., Terzic, A., and Puceat, M. (2002). Stem cell differentiation requires a paracrine pathway in the heart. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 16, 1558-1566.

Benoit, D.S., Schwartz, M.P., Durney, A.R., and Anseth, K.S. (2008). Small functional groups for controlled differentiation of hydrogel-encapsulated human mesenchymal stem cells. *Nature materials* 7, 816-823.

Boheler, K.R., Czyz, J., Tweedie, D., Yang, H.T., Anisimov, S.V., and Wobus, A.M. (2002). Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circulation research* 91, 189-201.

Bonafe, F., Govoni, M., Giordano, E., Caldarera, C.M., Guarnieri, C., and Muscari, C. (2014). Hyaluronan and cardiac regeneration. *Journal of biomedical science* 21, 100.

Boyd, A.W., Bartlett, P.F., and Lackmann, M. (2014). Therapeutic targeting of EPH receptors and their ligands. *Nat Rev Drug Discov* 13, 39-62.

Burridge, P.W., and Zambidis, E.T. (2013). Highly efficient directed differentiation of human induced pluripotent stem cells into cardiomyocytes. *Methods in molecular biology (Clifton, NJ)* 997, 149-161.

Carvalho, P.H., Daibert, A.P., Monteiro, B.S., Okano, B.S., Carvalho, J.L., Cunha, D.N., Favarato, L.S., Pereira, V.G., Augusto, L.E., and Del Carlo, R.J. (2013). Differentiation of adipose tissue-derived mesenchymal stem cells into cardiomyocytes. *Arquivos brasileiros de cardiologia* 100, 82-89.

Chen, D., Zhao, M., and Mundy, G.R. (2004). Bone morphogenetic proteins. *Growth factors (Chur, Switzerland)* 22, 233-241.

Chen, K., Wu, L., and Wang, Z.Z. (2008). Extrinsic regulation of cardiomyocyte differentiation of embryonic stem cells. *Journal of cellular biochemistry* 104, 119-128.

Dai, Y., Xu, M., Wang, Y., Pasha, Z., Li, T., and Ashraf, M. (2007). HIF-1alpha induced-VEGF overexpression in bone marrow stem cells protects cardiomyocytes against ischemia. *Journal of molecular and cellular cardiology* 42, 1036-1044.

Datta, S.R., Brunet, A., and Greenberg, M.E. (1999). Cellular survival: a play in three Akts. *Genes & development* 13, 2905-2927.

ISSN: 2198-4093
www.bmrat.org

Davis, M.E., Hsieh, P.C., Takahashi, T., Song, Q., Zhang, S., Kamm, R.D., Grodzinsky, A.J., Anversa, P., and Lee, R.T. (2006). Local myocardial insulin-like growth factor 1 (IGF-1) delivery with biotinylated peptide nanofibers improves cell therapy for myocardial infarction. *Proceedings of the National Academy of Sciences of the United States of America* 103, 8155-8160.

Detillieux, K.A., Sheikh, F., Kardami, E., and Cattini, P.A. (2003). Biological activities of fibroblast growth factor-2 in the adult myocardium. *Cardiovascular research* 57, 8-19.

Doppler, S.A., Deutsch, M.A., Lange, R., and Krane, M. (2013). Cardiac regeneration: current therapies-future concepts. *Journal of thoracic disease* 5, 683-697.

Dries, J.L., Kent, S.D., and Virag, J.A. (2011). Intramyocardial administration of chimeric ephrinA1-Fc promotes tissue salvage following myocardial infarction in mice. *The Journal of physiology* 589, 1725-1740.

DuSablon, A., Kent, S., Coburn, A., and Virag, J. (2014). EphA2-receptor deficiency exacerbates myocardial infarction and reduces survival in hyperglycemic mice. *Cardiovascular diabetology* 13, 114.

Fischer, K.M., Cottage, C.T., Wu, W., Din, S., Gude, N.A., Avitabile, D., Quijada, P., Collins, B.L., Fransioli, J., and Sussman, M.A. (2009). Enhancement of myocardial regeneration through genetic engineering of cardiac progenitor cells expressing Pim-1 kinase. *Circulation* 120, 2077-2087.

Fisher, S.A., Brunskill, S.J., Doree, C., Mathur, A., Taggart, D.P., and Martin-Rendon, E. (2014). Stem cell therapy for chronic ischaemic heart disease and congestive heart failure. *The Cochrane database of systematic reviews* 4, Cd007888.

Fisher, S.A., Zhang, H., Doree, C., Mathur, A., and Martin-Rendon, E. (2015). Stem cell treatment for acute myocardial infarction. *The Cochrane database of systematic reviews* 9, Cd006536.

Fitzpatrick, L.E., and McDevitt, T.C. (2015). Cell-derived matrices for tissue engineering and regenerative medicine applications. *Biomaterials science* 3, 12-24.

Frieden, L.A., Townsend, T.A., Vaught, D.B., Delaughter, D.M., Hwang, Y., Barnett, J.V., and Chen, J. (2010). Regulation of heart valve morphogenesis by Eph receptor ligand, ephrin-A1. *Developmental dynamics : an official publication of the American Association of Anatomists* 239, 3226-3234.

Fuster, V. (2014). Top 10 cardiovascular therapies and interventions for the next decade. *Nature reviews Cardiology* 11, 671-683.

Gherghiceanu, M., Barad, L., Novak, A., Reiter, I., Itskovitz-Eldor, J., Binah, O., and Popescu, L.M. (2011). Cardiomyocytes derived from human embryonic and induced pluripotent stem cells: comparative ultrastructure. *Journal of cellular and molecular medicine* 15, 2539-2551.

Gnecchi, M., He, H., Noiseux, N., Liang, O.D., Zhang, L., Morello, F., Mu, H., Melo, L.G., Pratt, R.E., Ingwall, J.S., *et al.* (2006). Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 20, 661-669.

Gnecchi, M., Zhang, Z., Ni, A., and Dzau, V.J. (2008). Paracrine mechanisms in adult stem cell signaling and therapy. *Circulation research* 103, 1204-1219.

ISSN: 2198-4093
www.bmrat.org

Goichberg, P., Bai, Y., D'Amario, D., Ferreira-Martins, J., Fiorini, C., Zheng, H., Signore, S., del Monte, F., Ottolenghi, S., D'Alessandro, D.A., *et al.* (2011). The ephrin A1-EphA2 system promotes cardiac stem cell migration after infarction. *Circulation research* 108, 1071-1083.

Hahn, J.Y., Cho, H.J., Kang, H.J., Kim, T.S., Kim, M.H., Chung, J.H., Bae, J.W., Oh, B.H., Park, Y.B., and Kim, H.S. (2008). Pre-treatment of mesenchymal stem cells with a combination of growth factors enhances gap junction formation, cytoprotective effect on cardiomyocytes, and therapeutic efficacy for myocardial infarction. *Journal of the American College of Cardiology* 51, 933-943.

Haider, H., and Ashraf, M. (2008). Strategies to promote donor cell survival: combining preconditioning approach with stem cell transplantation. *Journal of molecular and cellular cardiology* 45, 554-566.

Highley, C.B., Prestwich, G.D., and Burdick, J.A. (2016). Recent advances in hyaluronic acid hydrogels for biomedical applications. *Current opinion in biotechnology* 40, 35-40.

Huang, Y.-l., Qiu, R.-f., Mai, W.-y., Kuang, J., Cai, X.-y., Dong, Y.-g., Hu, Y.-z., Song, Y.-b., Cai, A.-p., and Jiang, Z.-g. (2012). Effects of insulin-like growth factor-1 on the properties of mesenchymal stem cells in vitro. *Journal of Zhejiang University Science B* 13, 20-28.

Jha, A.K., Tharp, K.M., Ye, J., Santiago-Ortiz, J.L., Jackson, W.M., Stahl, A., Schaffer, D.V., Yeghiazarians, Y., and Healy, K.E. (2015). Enhanced survival and engraftment of transplanted stem cells using growth factor sequestering hydrogels. *Biomaterials* 47, 1-12.

Kang, H., Wen, C., Hwang, Y., Shih, Y.R., Kar, M., Seo, S.W., and Varghese, S. (2014). Biomineralized matrix-assisted osteogenic differentiation of human embryonic stem cells. *Journal of materials chemistry B, Materials for biology and medicine* 2, 5676-5688.

Kardami, E., Padua, R.R., Pasumarthi, K.B.S., Liu, L., Doble, B.W., Davey, S.E., and Cattini, P.A. (1993). Basic fibroblast growth factor in cardiac myocytes: expression and effects. In Growth Factors and the Cardiovascular System, P. Cummins, ed. (Boston, MA: Springer US), pp. 55-75.

Kastrup, J., Mygind, N.D., Qayyum, A.A., Mathiasen, A.B., Haack-Sorensen, M., and Ekblond, A. (2016). Mesenchymal stromal cell therapy in ischemic heart disease. *Scandinavian cardiovascular journal : SCJ*, 1-20.

Kinnaird, T., Stabile, E., Burnett, M.S., Lee, C.W., Barr, S., Fuchs, S., and Epstein, S.E. (2004). Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote in vitro and in vivo arteriogenesis through paracrine mechanisms. *Circulation research* 94, 678-685.

Krishna, K.A., Krishna, K.S., Berrocal, R., Rao, K.S., and Sambasiva Rao, K.R.S. (2011). Myocardial infarction and stem cells. *Journal of Pharmacy and Bioallied Sciences* 3, 182-188.

Laflamme, M.A., Chen, K.Y., Naumova, A.V., Muskheli, V., Fugate, J.A., Dupras, S.K., Reinecke, H., Xu, C., Hassanipour, M., Police, S., *et al.* (2007). Cardiomyocytes derived from human embryonic stem cells in pro-survival factors enhance function of infarcted rat hearts. *Nature biotechnology* 25, 1015-1024.

Leri, A., Kajstura, J., and Anversa, P. (2005). Cardiac stem cells and mechanisms of myocardial regeneration. *Physiological reviews* 85, 1373-1416.

Li, Q., Guo, Z.K., Chang, Y.Q., Yu, X., Li, C.X., and Li, H. (2015). Gata4, Tbx5 and Baf60c induce differentiation of adipose tissue-derived mesenchymal stem cells into beating cardiomyocytes. *The international journal of biochemistry & cell biology* 66, 30-36.

ISSN: 2198-4093
www.bmrat.org

Li, X., Tamama, K., Xie, X., and Guan, J. (2016). Improving Cell Engraftment in Cardiac Stem Cell Therapy. *Stem cells international* 2016, 7168797.

Lu, T.Y., Lin, B., Kim, J., Sullivan, M., Tobita, K., Salama, G., and Yang, L. (2013). Repopulation of decellularized mouse heart with human induced pluripotent stem cell-derived cardiovascular progenitor cells. *Nature communications* 4, 2307.

Maher, B. (2013). Tissue engineering: How to build a heart. *Nature* 499, 20-22.

Maltsev, V.A., Rohwedel, J., Hescheler, J., and Wobus, A.M. (1993). Embryonic stem cells differentiate in vitro into cardiomyocytes representing sinusnodal, atrial and ventricular cell types. *Mechanisms of development* 44, 41-50.

Maltsev, V.A., Wobus, A.M., Rohwedel, J., Bader, M., and Hescheler, J. (1994). Cardiomyocytes differentiated in vitro from embryonic stem cells developmentally express cardiac-specific genes and ionic currents. *Circulation research* 75, 233-244.

Manalo, D.J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B.D., Ye, S.Q., Garcia, J.G., and Semenza, G.L. (2005). Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. *Blood* 105, 659-669.

Mangi, A.A., Noiseux, N., Kong, D., He, H., Rezvani, M., Ingwall, J.S., and Dzau, V.J. (2003). Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nature medicine* 9, 1195-1201.

Mansson-Broberg, A., Siddiqui, A.J., Genander, M., Grinnemo, K.H., Hao, X., Andersson, A.B., Wardell, E., Sylven, C., and Corbascio, M. (2008). Modulation of ephrinB2 leads to increased angiogenesis in ischemic myocardium and endothelial cell proliferation. *Biochemical and biophysical research communications* 373, 355-359.

Marelli, D., Desrosiers, C., el-Alfy, M., Kao, R.L., and Chiu, R.C. (1992). Cell transplantation for myocardial repair: an experimental approach. *Cell transplantation* 1, 383-390.

McAllister, T.N., Maruszewski, M., Garrido, S.A., Wystrychowski, W., Dusserre, N., Marini, A., Zagalski, K., Fiorillo, A., Avila, H., Manglano, X., *et al.* (2009). Effectiveness of haemodialysis access with an autologous tissue-engineered vascular graft: a multicentre cohort study. *Lancet (London, England)* 373, 1440-1446.

Metzele, R., Alt, C., Bai, X., Yan, Y., Zhang, Z., Pan, Z., Coleman, M., Vykoukal, J., Song, Y.H., and Alt, E. (2011). Human adipose tissue-derived stem cells exhibit proliferation potential and spontaneous rhythmic contraction after fusion with neonatal rat cardiomyocytes. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 25, 830-839.

Muller-Ehmsen, J., Whittaker, P., Kloner, R.A., Dow, J.S., Sakoda, T., Long, T.I., Laird, P.W., and Kedes, L. (2002). Survival and development of neonatal rat cardiomyocytes transplanted into adult myocardium. *Journal of molecular and cellular cardiology* 34, 107-116.

Muraski, J.A., Rota, M., Misao, Y., Fransioli, J., Cottage, C., Gude, N., Esposito, G., Delucchi, F., Arcarese, M., Alvarez, R., *et al.* (2007). Pim-1 regulates cardiomyocyte survival downstream of Akt. *Nature medicine* 13, 1467-1475.

Nygren, J.M., Jovinge, S., Breitbach, M., Sawen, P., Roll, W., Hescheler, J., Taneera, J., Fleischmann, B.K., and Jacobsen, S.E. (2004). Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nature medicine* 10, 494-501.

ISSN: 2198-4093
www.bmrat.org

O'Neal, W.T., Griffin, W.F., Dries-Devlin, J.L., Kent, S.D., Chen, J., Willis, M.S., and Virag, J.A. (2013). Ephrin-Eph signaling as a potential therapeutic target for the treatment of myocardial infarction. *Medical hypotheses* 80, 738-744.

O'Neal, W.T., Griffin, W.F., Kent, S.D., Faiz, F., Hodges, J., Vuncannon, J., and Virag, J.A. (2014). Deletion of the EphA2 receptor exacerbates myocardial injury and the progression of ischemic cardiomyopathy. *Frontiers in physiology* 5, 132.

Oh, H., Bradfute, S.B., Gallardo, T.D., Nakamura, T., Gaussin, V., Mishina, Y., Pocius, J., Michael, L.H., Behringer, R.R., Garry, D.J., *et al.* (2003). Cardiac progenitor cells from adult myocardium: homing, differentiation, and fusion after infarction. *Proceedings of the National Academy of Sciences of the United States of America* 100, 12313-12318.

Ott, H.C., Matthiesen, T.S., Goh, S.K., Black, L.D., Kren, S.M., Netoff, T.I., and Taylor, D.A. (2008). Perfusion-decellularized matrix: using nature's platform to engineer a bioartificial heart. *Nature medicine* 14, 213-221.

Pal, R., and Khanna, A. (2007). Similar pattern in cardiac differentiation of human embryonic stem cell lines, BG01V and ReliCellhES1, under low serum concentration supplemented with bone morphogenetic protein-2. *Differentiation; research in biological diversity* 75, 112-122.

Parisi-Amon, A., Mulyasasmita, W., Chung, C., and Heilshorn, S.C. (2013). Protein-engineered injectable hydrogel to improve retention of transplanted adipose-derived stem cells. *Advanced healthcare materials* 2, 428-432.

Pham, T., Nguyen, T.T., Bui, A., Pham, T.H., Phan, K.N., Nguyen, M., and Pham, P. (2015). Preliminary evaluation of treatment efficacy of umbilical cord blood-derived mesenchymal stem cell-differentiated cardiac progenitor cells in a myocardial injury mouse model. *Biomedical Research and Therapy* 2, 1-11.

Poulin, M.F., Deka, A., Mohamedali, B., and Schaer, G.L. (2016). Clinical Benefits of Stem Cells for Chronic Symptomatic Systolic Heart Failure A Systematic Review of the Existing Data and Ongoing Trials. *Cell transplantation*.

Prestwich, G.D. (2008). Engineering a clinically-useful matrix for cell therapy. *Organogenesis* 4, 42-47.

Pugh, C.W., and Ratcliffe, P.J. (2003). Regulation of angiogenesis by hypoxia: role of the HIF system. *Nature medicine* 9, 677-684.

Quevedo, H.C., Hatzistergos, K.E., Oskouei, B.N., Feigenbaum, G.S., Rodriguez, J.E., Valdes, D., Pattany, P.M., Zambrano, J.P., Hu, Q., McNiece, I., *et al.* (2009). Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proceedings of the National Academy of Sciences of the United States of America* 106, 14022-14027.

Quint, C., Arief, M., Muto, A., Dardik, A., and Niklason, L.E. (2012). Allogeneic human tissue-engineered blood vessel. *Journal of vascular surgery* 55, 790-798.

Ren, J., Samson, W.K., and Sowers, J.R. (1999). Insulin-like growth factor I as a cardiac hormone: physiological and pathophysiological implications in heart disease. *Journal of molecular and cellular cardiology* 31, 2049-2061.

Rosenblatt-Velin, N., Lepore, M.G., Cartoni, C., Beermann, F., and Pedrazzini, T. (2005). FGF-2 controls the differentiation of resident cardiac precursors into functional cardiomyocytes. *Journal of Clinical Investigation* 115, 1724-1733.

Tapias, L.F., and Ott, H.C. (2014). Decellularized Scaffolds as a Platform for Bioengineered Organs. *Current opinion in organ transplantation* 19, 145-152.

ISSN: 2198-4093
www.bmrat.org

Thisse, B., and Thisse, C. (2005). Functions and regulations of fibroblast growth factor signaling during embryonic development. *Developmental Biology* 287, 390-402.

Toma, C., Pittenger, M.F., Cahill, K.S., Byrne, B.J., and Kessler, P.D. (2002). Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation* 105, 93-98.

Travan, A., Scognamiglio, F., Borgogna, M., Marsich, E., Donati, I., Tarusha, L., Grassi, M., and Paoletti, S. (2016). Hyaluronan delivery by polymer demixing in polysaccharide-based hydrogels and membranes for biomedical applications. *Carbohydrate polymers* 150, 408-418.

van der Spoel, T.I., Jansen of Lorkeers, S.J., Agostoni, P., van Belle, E., Gyongyosi, M., Sluijter, J.P., Cramer, M.J., Doevendans, P.A., and Chamuleau, S.A. (2011). Human relevance of pre-clinical studies in stem cell therapy: systematic review and meta-analysis of large animal models of ischaemic heart disease. *Cardiovascular research* 91, 649-658.

Wang, Z., Bhattacharya, N., Weaver, M., Petersen, K., Meyer, M., Gapter, L., and Magnuson, N.S. (2001). Pim-1: a serine/threonine kinase with a role in cell survival, proliferation, differentiation and tumorigenesis. *Journal of veterinary science* 2, 167-179.

Westerdahl, D.E., Chang, D.H., Hamilton, M.A., Nakamura, M., and Henry, T.D. (2016). Allogeneic mesenchymal precursor cells (MPCs): an innovative approach to treating advanced heart failure. *Expert opinion on biological therapy*, 1-7.

Wystrychowski, W., Cierpka, L., Zagalski, K., Garrido, S., Dusserre, N., Radochonski, S., McAllister, T.N., and L'Heureux, N. (2011). Case study: first implantation of a frozen, devitalized tissue-engineered vascular graft for urgent hemodialysis access. *The journal of vascular access* 12, 67-70.

Xu, W., Zhang, X., Qian, H., Zhu, W., Sun, X., Hu, J., Zhou, H., and Chen, Y. (2004). Mesenchymal stem cells from adult human bone marrow differentiate into a cardiomyocyte phenotype in vitro. *Experimental biology and medicine (Maywood, NJ)* 229, 623-631.

Zhang, M., Mal, N., Kiedrowski, M., Chacko, M., Askari, A.T., Popovic, Z.B., Koc, O.N., and Penn, M.S. (2007). SDF-1 expression by mesenchymal stem cells results in trophic support of cardiac myocytes after myocardial infarction. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology* 21, 3197-3207.

Zhang, M., Methot, D., Poppa, V., Fujio, Y., Walsh, K., and Murry, C.E. (2001). Cardiomyocyte grafting for cardiac repair: graft cell death and anti-death strategies. *Journal of molecular and cellular cardiology* 33, 907-921.

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Original Research

A preliminary investigation of *in vitro* anti-thrombotic and anti-platelet activity of *Pinus Gerardiana*

Atta-ur-Rehman*, Sara Naz, Muhammad Zaman, Syed Saeed-ul-Hassan, Javed Iqbal, Awais Ali Zaidi

Faculty of Pharmacy, University of Lahore, Lahore, Pakistan

Abstract

Introduction: Hemostasis is a process which preserves the stability of a closed and high-pressure circulatory system after any vascular injury. Circulating platelets are recruited to the site of injury, where they develop a major component of the developing thrombus, blood clotting, started by tissue factor, concludes in the generation of thrombin and fibrin. Thrombosis is a serious event in the arterial diseases and a major cause in the development of myocardial infarction, stroke and venous thrombo-embolism which justify prominent morbidity and mortality rate. The knowledge of molecular and cellular mechanism of the formation of thrombus has developed considerably in the recent studies by using different in-vitro and in-vivo models of diseases. *P. gerardiana* nut oil has been reported to possess anti-bacterial, anti-fungal, anti-viral, anti-septic, anti-neuralgic, diuretic, expectorant, hypertensive properties. However, hardly, any data is available regarding effects of nut oil on platelet function. In this study, fibrinolytic activity and effect on platelet aggregation were investigated. **Method**: *P. gerardiana* nut oil was extracted by using n-Hexane and then concentrated by rotary evaporator. Anti-thrombotic and fibrinolytic activities were evaluated on blood clot formation. Effects on platelet aggregation of the oil were determined based on collagen or epinephrine induced platelet aggregation. **Results**: *P. gerardiana* caused blood clot lysis in-vitro. *P. gerardiana* nut oil inhibited collagen dependent platelet aggregation while accelerated the epinephrine dependent platelet aggregation. In vitro whole blood coagulation was also reduced. In vivo *P. gerardiana* nut oil has no significant effect on blood cell indices. **Conclusion**: *P. gerardiana* nuts oil can be an effective therapy for the treatment of cardiovascular disorders and thromboembolism.

***For correspondence:**

rehman.atta.ur@gmail.com

Competing interests: The authors declare that no competing interests exist.

Received: 15 December 2016
Accepted: 06 January 2017
Published: 16 January 2017

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Keywords

Anticoagulant; Epinephrine; Haemostasis; *P. gerardiana*; Thrombosis

Introduction

Thrombosis is local coagulation or clotting of the blood in a part of the circulatory system. A more balanced clot is called a thrombus, must develop a seal which is long lasting on large damaged blood vessels. The clotting procedure is self-restricting and anti-thrombin prevent the clot from continuous enlargement, otherwise the developed clot block the blood flow in the vessel. Thrombosis (excessive blood clot formation) within healthy blood vessels can be harmful if not treated within time. Cardiovascular diseases like heart attack, stroke and pulmonary embolism and several others have been related to inappropriate blood clot formation (Furie and Furie, 2008). Synthetic drugs show many side effects and require closed monitoring e.g., tranexamic acid (anti-fibrinolytic drugs) and vitamin K has been used in the treatment of bleeding disorder (Sànchez-Lamar et al., 1999).

Platelets have important role in blood haemostasis as well as in pathogenesis of cardiovascular diseases (Campbell et al., 2008; Elliott et al., 1997). Platelet aggregation blood test (PABT) checks how well platelets, clump collectively and cause blood to become a clot (Elliott et al., 1997). Blood clot is the aggregation of platelets, red blood cells and fibrin formation through a specific pathway that helps maintain hemostasis. When injury of blood vessel wall occurs, clot formation is completed within 5 minutes in a healthy person (Sànchez-Lamar et al., 1999).

Chilgoza pine nuts (*Pinus gerardiana)* are the edible seed of the Chilgoza pine plant which belong to pinaceae family commonly found in Pakistan, India, China and Afghanistan (Destaillats et al., 2010). *P. gerardiana* nuts contain un-saturated fatty acids which are very beneficial for lowering high cholesterol level. *P. gerardiana* nuts are good source of energy (628 kcal) and nutrition consisting of protein (11.6g/100g), carbohydrates (19.3g/100g) and fatty acids (61g/100g) (Brufau et al., 2006). Regarding nutrition, *P. gerardiana* nuts contain vitamins, beta-carotene, thiamin (B1), riboflavon (B2), niacin (B3), pantothenic acid (B5), vitamin B6, folate (B9), vitamin C, vitamin E, vitamin k and minerals including calcium, iron, magnesium, manganese, phosphorus, zinc, potassium and phosphorus other constituents including water, so it can be used for both curative & nutritional purposes (Sagrero−Nieves, 1992; Savage, 2001).

P. gerardiana nut oil has anti-bacterial, anti-fungal, anti-viral, anti-septic, anti-neuralgic, choleretic, diuretic, expectorant, hypertensive properties (Amr and

ISSN: 2198-4093
www.bmrat.org

Abeer, 2011; Lawlees, 1992). Simillarly, hydro-alcoholic extract showed anti-inflammatory anti-bacterial and antioxidant properties (Sharma et al., 2016). Since oxidative stress contributes significantly in platelet activation (Bartimoccia et al., 2015) and also synthetic anti-inflammatory drugs like aspirin has antiplatelet activity (Roth and Majerus, 1975), the objectives of this study was to investigate the fibrinolytic activity and effect on platelet aggregation.

Materials-Methods

Preparation of *Pinus gerardiana* nuts oil concentrates (PGNC)

Pine nuts were purchased in March 2015 from a local market in Lahore-Pakistan. After removing impurities and broken kernels, pine nuts were crushed into powder. *P. gerardiana* nuts oil was extracted using soxholet apparatus (jisico Model No:GLHMP-F100) and N-hexane was used as extractor solvent. The extract was concentrated to a final volume of 350 ml using rotary evaporator at 50°C at reduced pressure. Oil concentrate was stored in amber green air-tight container and placed in dark at 20°C. The concentrate was used in all experiments without further dilution.

Preparation of platelet rich plasma and platelet aggregation

The research protocol was approved by the research ethics committee of Faculty of Pharmacy the University of Lahore. Blood was drawn into 15 ml falcon tube containing 1ml tri-sodium citrate (3.2%) solution from healthy volunteers after informed consents. Platelet rich plasma (PRP) was prepared as described in (Yavasoglu et al., 2010) with minor modification. Briefly, citratated supplemented blood was centrifuged for 15 minutes at 1200 rpm in centrifuge machine (Neuation Model no: I fuge D06) and stored at 37°C in water bath (jisico Model No: J-IWB-KOREA). Presence of platelets was confirmed by automated cell analyzer (Roche) according to standard laboratory procedure. 500µl PRP was taken in cylindrical cuvette provided with a magnetic stirrer at 700rpm. PRP was equilibrated for three min. 10 µl epinephrine (1mM) or 10 µl rat tail collagen (3mg/ml) were used as agonists to activate platelet aggregation. To evaluate the effect of extract PRP was pretreated with 2µl, 5µl or 10µl of PG nuts oil concentrate (PGNC). Aggregation time was compared with that of agonist epinephrine (adrenaline, Ameer Pharma Pakistan) or collagen (3mg/ml) alone. The process was performed in triplicate for at least three different blood samples.

Fibrinolytic activity

6ml blood was drawn from median cubital vein of healthy volunteers. 400µl blood was immediately taken in glass bottle (diameter 2 cm) with flat bottom. Blood was allowed to clot in the form of thin film at 37°C for half an hour. Blood

ISSN: 2198-4093
www.bmrat.org

clot film was washed twice with 1ml normal saline. The blood clot were treated with PGNC (30ul or 60ul) diluted in 1ml normal saline to observe the fibrinolytic effect. Control samples contained normal saline only. Samples were incubated at for 1 hour at 37°C. Glass bottles were removed and shake gently without disturbing the intact clot. 200µl blood suspension dilute with 1ml normal saline was transferred to 1 cm spectrophotometer cuvette and check the absorption (λ=550nm) by UV/Vis spectrophotometer (Model No: T 80) (Umesh et al., 2014). 100µl of blood suspension was again taken into eppendorf tubes that contained 1ml hydrochloric acid (0.1N). Acid hematin was quantified at λ=550nm spectrophotometrically.

Formation of clot

This test was carried out to conclude whole blood clotting time and the effect on the fibrin clot formation. PGNC were tested at different concentration 20 µl, 30 µl and 40 µl. 9ml blood was taken from healthy volunteers. Blood was prevented from clotting by adding 1ml tri-sodium citrate (3.2%) solution. 200µl blood was incubated for 1 hour at 37°C with 20 µl, 30 µl and 40 µl of PGNC. Normal saline used as control. Afterwards complete blood coagulation was observed by adding 50 µl of calcium chloride (100mM) solution. The time for the formation of clot was noted at 37°C in water bath (jisico Model No: J-IWB-Korea) (Al-Mamun et al., 2012).

In vivo experiment

Wistar rats (10 rats) with weight of 200-270g were purchased from the animal house of University of Lahore. All animal experiments were approved by animal research ethic committee of University of Lahore (fop-uol-2015-005). Animals were maintained in a controlled environment at a temperature of 22°C, humidity 40-60%.

Animals were grouped as follows: Group A: (Control Group): rats were provided with normal Saline. Group B: *Pinus gerardiana* nuts oil concentrate by using oral gavage at doses of 200 µl/kg/ per day. Animals were dosed daily for 15 days. At the 16th day animals were euthanized underanesthesis and blood was withdrawn by cardiac puncture and stored in EDTA tubes (ethylene-diamine-tetra-acetic acid) for the complete blood count (CBC) quantification on automated cell analyzer (Roche) according to standard laboratory procedure.

Statistical analysis

Data is presented as mean±SD of at least three independent experiments. Paired t-test or one-way ANOVA with Bonferoni post hoc test was applied to evaluate and compare the significant difference among means. A $p<0.05$ was considered significant.

ISSN: 2198-4093
www.bmrat.org

Results

P. gerardiana induces Fibrinolysis (release of RBC) from a blood clot

The release of RBCs from the blood clot (as a measure of fibrinolytic activity) was measured. Absorption was directly proportional to the number of cells in the suspension. **Figure 1** showed that *P. gerardiana* nuts oil increased clot lysis significantly in a dose dependent manner as compared with control (normal saline treated blood clots). The results were reassured by quantifying the haemoglobin (acid hematin) concentration of the suspension. The results showed that the acid haematin content also increased in the PGNC in a dose dependent way.

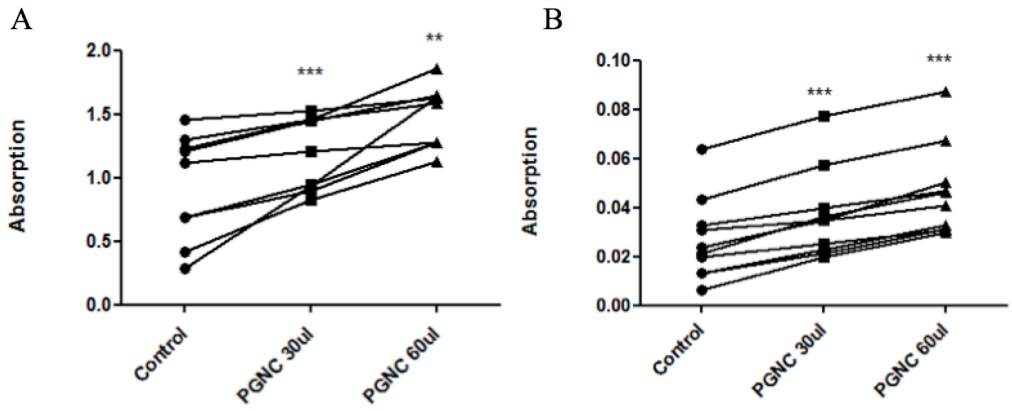


Figure 1. ***P. gerardiana*** **induces Fibrinolysis (release of RBC) from blood clot.** The effect of the PG nuts oil concentrates on fibrinolysis quantified by RBC release (**A**) and hemoglobin concentration; (**B**) Data is representation of 7 independent experiments. two tailed, paired t-test was applied to evaluate the difference in mean averages. **(p<0.002) and *** (p<0.001) significantly different (p<0.05) from control.

P. gerardiana has opposite effect on epinephrine and ollagen activated platelet aggregation

The effect of *P. gerardiana* nut oil (PGNC) on aggregation of platelet activated by collagen and epinephrine was tested. Platelets were incubated with different amounts of *P. gerardiana* and then activated by collagen or epinephrine. The control platelets were only treated with epinephrine (5μM) or 10μl rat tail collagen (3mg/ml). Treatment with PGNC showed opposite effects on the platelet aggregation activated by collagen or epinephrine (**Fig. 2**). PGNC significantly (p<0.001) and dose dependently increased the time for platelet

ISSN: 2198-4093
www.bmrat.org

aggregation when activated by collagen. This showed that the chemical constituents of PGNC interfere with the molecular mechanism of platelet aggregation induced or activated by collagen. The highest effect was observed when PRP was treated with 10µl PGNC. On the contrary, PGNC significantly (p<0.001) and dose dependently decreased the time for platelet aggregation when activated by epinephrine. These suggested that the chemical constituents mimic and facilitate platelet aggregation induction caused by epinephrine.

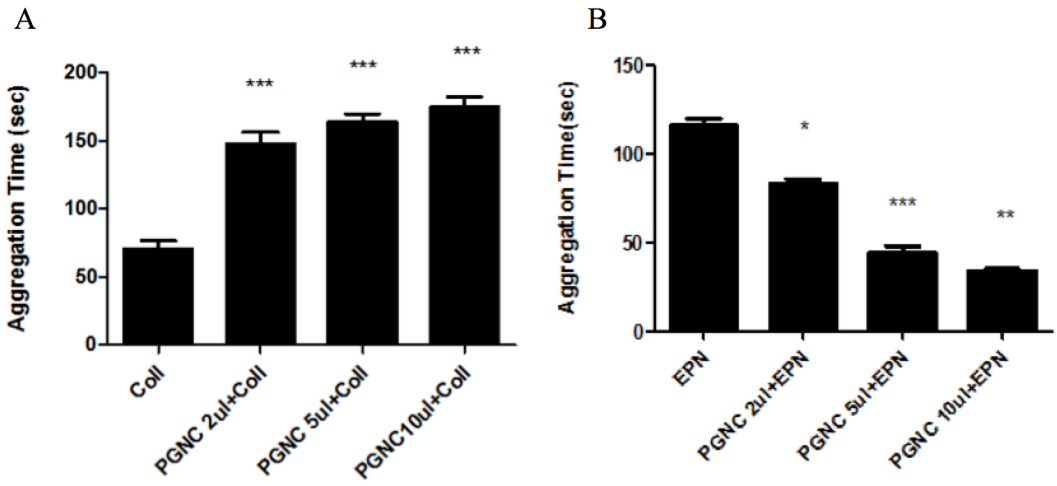


Figure 2. *P. gerardiana* has opposite effect on epinephrine and Collagen activated Platelet aggregation: platelet aggregation *in vitro*. (**A**) *Pinus gerardiana* nuts oil concentrate (PGNC) decrease Collagen activated platelet aggregation; (**B**) *Pinus gerardiana* nuts oil concentrate (PGNC) increased Collagen activated platelet aggregation. t-test with boneferonei post hoc test was applied to compare mean statistically. Data is representative mean±SD (n=8). * (p<0.05), ** (p<0.01), ***(p<0.001) vs epinephrine (EPN) 5uM or 10µl Collagen (Coll) (3mg/ml) treated controls.

PGNC delays *in-vitro* Ca^{+2} stimulated blood clot formation

Blood clotting is spontaneous phenomenon. Many medications like aspirin can delay blood clot formation. The effect of PGNC was evaluated on *in-vitro* blood clot formation. The samples of blood were collected and clotting time was measured as shown in **Figure 3**. The data showed that *P. gerardiana* nut oil concentrate (PGNC) significantly (p<0.01) and dose dependently (20µl, 30µl and 40µl) delayed the blood clotting time versus control. Normally, blood clotting was observed within 75 seconds after the addition of 50µl of $CaCl_2$ (100 mM) solution that acted as control. Pre-treatment with 20µl PGNC showed around 90% increase in blood clotting time and the effect was even stronger at higher amounts of PGNC. The maximum effect was observed at 40µl PGNC that was about 3 times more than the control group.

ISSN: 2198-4093
www.bmrat.org

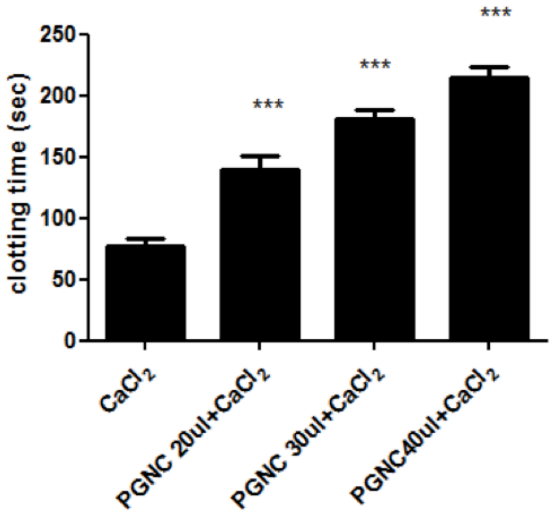


Figure 3. PGNC delays in-vitro Ca+2 Stimulated blood clot formation. *P.gerardiana* increase clotting time in a dose-dependent manner. Data is representative mean ± SD (n=8). Two talied, paired t-test was applied to compare the means. *** (p<0.001) vs. CaCl2 treated control.

PGNC does not reduce the platelet count

In order to check the effects of the PGNC on the blood cell indices, Wistar rats were treated with the concentrate 200mg/kg/day for 2 week. The blood was collected and analyzed for the complete blood count. No significant effect was observed on any blood indices parameter. Platelet, RBC, WBC counts were comparable to the control (**Fig. 4**).

Discussion

Platelets are blood cells that contribute in primary hemostasis. Primary hemostatic plug is formed by platelet-platelet interaction. Many studies have been carried out to develop anti-thrombotic agents with improved efficacy for preventing or treating arterial and venous thrombosis. The fibrinolytic enzyme prevents formation of fibrin clots in circulatory system. Some medicines like urokinase and streptokinase are widely used to inhibit homeostatic disorders, particularly thrombo emboli. The primary function of fibrinolytic activity is to disperse fibrin clot in a circulation. Alpha-2-anti-plasmin, plasminogen and tissue plasminogen activators all of which also play a vital role in clot lysis (Biggs, 1972; Li et al., 2010).

ISSN: 2198-4093
www.bmrat.org

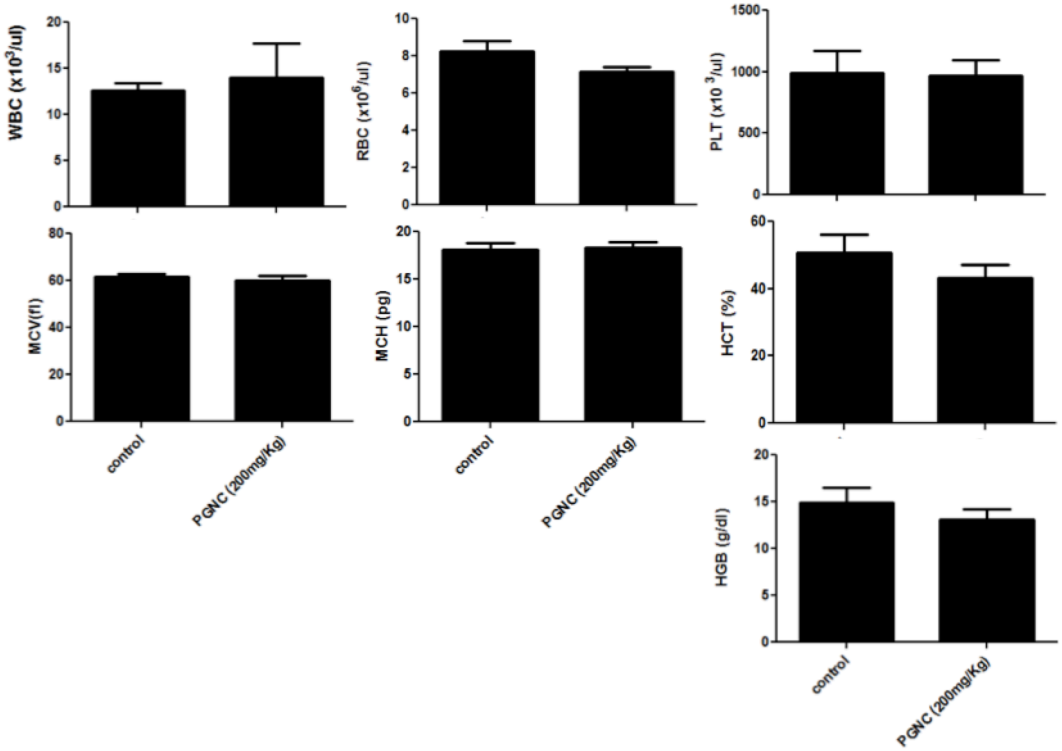


Figure 4. PGNC does not reduce the platelet count. Effect of *Pinus gerardiana* nuts oil PGNC on CBC parameters. Animal (n=5) were orally given 200mg/kg/day PGNC. Data is presented as the mean mean±SD t-test was applied to compare the means. $p<0.05$ was considered significant.

Current study describes the effect of *pinus gerardiana* nut concentrate (PGCN) on fibrinolytic activity and platelet aggregation *in-vitro* and *in-vivo. P.gerardiana* nut oil has increased the lysis of clot in comparison to control. Blood cells from fibrinolytic activity was observed by using a spectrophotometer (**Fig. 1A**) The heme released from haemoglobin of red blood cells when 0.1N hydrochloric acid (HCl) was added to the solution of the red blood cells and then acid haematin was formed (**Fig. 1B**). The release of RBCs and acid haematin were observed by spectrophotometry. In one of the study aqueous *Rue* extract was found effective as fibrinolytic effect by 12.53±5.67%. In our study, the fibrinolytic effect of *P. gerardiana* was found to be increased by 47% and 72%. A number of plant extracts and their products having fibrinolytic activity are recognized included Lumbricus rubellus, Pleurotus ostreatus, Spirodela polyrhiza, Flammulina velutipes, and Ganoderma lucidum, Ginger (*Zingiber officinale*), Garlic (*Allium sativum*) as well as from *Bacillus sp.* in Korean and Japanese fermented foods, chung kookjang, respectively (Michelson et al., 2006).

The interactions between platelets and a variety of adhesive proteins, such as collagen, and soluble agonists, such as ADP supply potential targets for developing anti-platelet agents. Different therapies are available to prevent

ISSN: 2198-4093
www.bmrat.org

irregular activation and aggregation of platelets. Epinephrine is an adrenergic agonist, bind with alpha 2-adrenergic receptors on platelets and also increases the effect of aggregation caused by other platelet agonists (Porth, 2005; Strukova, 2001). The capability of epinephrine induces the effect of aggregating agents on accumulation like fibrinogen binding, intracellular Ca2+ recruitment, granular relief and protein phosphorylation, and there are some factors correlate with platelet hyper-activity and resulting untimely coagulation of blood (Choi, 2002; Lanza et al., 1988). Similarly, collagen is the most thrombogenic component of the sub-endothelium (Baumgartner and Haudenschild, 1972). Following vascular damage, collagen is exposed to circulating platelets and both acts as a substrate for the adhesion of platelets (Cowan et al., 1981; Poole and Watson, 1995) and induces platelet activation (Poole and Watson, 1995).

The main evidence suggests that two receptors are involved in the platelet response to collagen, integrin α2β1 acts to adhere platelets to collagen, allowing platelets to interact with the lower affinity receptor glycoprotein VI, which is mainly responsible for platelet activation (Morton et al., 1989; Santoro et al., 1991).

At lower concentrations, many of the effects of collagen are enhanced by its production of thromboxane A2 (TXA) (Nakano et al., 1986; Pollock et al., 1986; Rittenhouse and Allen, 1982). The collagen-induced increase in [Ca2+] can be decreased by inhibiting the production of TXA via the pretreatment of platelets with cyclooxygenase inhibitors such as aspirin (Nakano et al., 1986).

P. gerardiana nuts oil concentrate by using its different concentrations was tested for platelet aggregation in the presence of both epinephrine and collagen showed opposite effect on platelet aggregation time (**Fig. 2A, B**). Previously unrefined methanolic extract of Nepeta juncea were examined for action against human platelet and have shown significant inhibitory effects on platelet aggregation (Hussain et al., 2009). Also it is investigated that extracts of garlic, onions, nettle and alfalfa are proved to be the most potent inhibitors of platelet aggregation in vitro (Pierre et al., 2005). Camomile, bramble are similarly powerful at repressing platelet accumulation *in-vitro* (Pierre et al., 2005). Toona microcarpa Harms leaf (TMHE) extract in-vitro significantly inhibited platelet aggregation induced by thrombin, but not by ADP or collagen (Choi, 2002). Parsley extract inhibits *in vitro* and *ex vivo* platelet aggregation and prolongs bleeding time in rats (Mekhfi et al., 2006). In this study, opposite effects of PGNC could be due to specific interference of phytochemicals with collagen or epinephrine dependent cell signaling in platelets.

Hemostasis is a process that is divided into two stages: platelet aggregation and coagulation. *P. gerardiana* nuts oil increased clotting time in comparison to control which was induced by calcium chloride (**Fig. 3**). This suggests that PGNC can be used cardiovascular diseases for anticoagulation. *In-vivo* effects of PGNC on blood indices were analysised. The result showed that *P. gerardiana* had no effect on CBC parameters. Result interpreted that there was no significant effect

ISSN: 2198-4093
www.bmrat.org

on RBCS, WBC, HGB, HCT and other parameter. *P. gerardiana* nuts oil has slightly increased the effect of MCHC but other parameters did not show any significant effect.

Conclusion

In summary, our data strongly suggest that *P. gerardiana* nut oil can be used in the treatment of thromboembolic disorders and this protective mechanism is associated with increased fibrinolysis and inhibition of collagen-stimulated platelet aggregation.

Abbreviations

CBC: complete blood count; Coll: collagen; EDTA: ethylene-diamine-tetra-acetic acid; EPN: epinephrine; HCT: hematocrit; HGB: hemoglobin; MCH: mean corpuscular hemoglobin; MCV: mean corpuscular volume; PGNC: *Pinus gerardiana* nuts oil concentrates; PLT: Platelets; PRP: platelet rich plasma; RBC: red blood cells; WBC: white blood cells.

Acknowledgement

We acknowledge technical help of central research lab, Faculty of Pharmacy,The university of Lahore and Armed forces institute of Pathology (AFIP), CMH Rawalpindi, for their technical support.

Author contribution

AUR give the concept, designed experiment, analyzed data and gave final approval of the manuscript and was the supervisor of the project. SN performed experiment, analyzed data and wrote draft of manuscript. MZ helped in data acquisition and experiment conditions optimization SSH and JA helped in editing and literature review for the paper AAZ did data acquisition, organization and data analysis and helped in write up of final draft.

ISSN: 2198-4093
www.bmrat.org

References

Al-Mamun, M.R., Amrin, N., Begum, J., and Mazid, M.A. (2012). Thrombolytic activity of some spices and plants available in Bangladesh. *The Thai Journal of Pharmaceutical Sciences* 36, 72-77.

Amr, A.R., and Abeer, E. (2011). Hypolipideimic and hypocholestermic effect of pine nuts in rats fed high fat, cholesterol-diet. *World Applied Sciences Journal* 15, 1667-1677.

Bartimoccia, S., Nocella, C., Pastori, D., Pignatelli, P., and Carnevale, R. (2015). Platelet Oxidative Stress and Antioxidant Nutrients. *Journal of Vascular Medicine & Surgery* 2014.

Baumgartner, H.R., and Haudenschild, C. (1972). Adhesion of platelets to subendothelium. *Annals of the New York Academy of Sciences* 201, 22-36.

Biggs, R. (1972). Human blood coagulation, haemostasis and thrombosis.

Brufau, G., Boatella, J., and Rafecas, M. (2006). Nuts: source of energy and macronutrients. *British Journal of Nutrition* 96, S24-S28.

Campbell, N.A., Reece, J.B., Urry, L.A., Cain, M.L., Wasserman, S.A., Minorsky, P.V., and Jackson, R.B. (2008). AP Edition Biology (Benjamin/Cummings).

Choi, J.W. (2002). Incidence of nonresponsiveness to epinephrine in platelets from healthy humans. *Acta haematologica* 108, 106-108.

Cowan, D.H., Robertson, A.L., Shook, P., and Giroski, P. (1981). Platelet adherence to collagen: role of plasma, ADP, and divalent cations. *British journal of haematology* 47, 257-267.

Destaillats, F.d.r., Cruz-Hernandez, C., Giuffrida, F., and Dionisi, F. (2010). Identification of the botanical origin of pine nuts found in food products by gas– liquid chromatography analysis of fatty acid profile. *Journal of agricultural and food chemistry* 58, 2082-2087.

Elliott, W.H., Elliott, D.C., Jefferson, J.R., and Wheldrake, J. (1997). Biochemistry and molecular biology (Oxford University Press Oxford).

Furie, B., and Furie, B.C. (2008). Mechanisms of thrombus formation. *New England Journal of Medicine* 359, 938-949.

Hussain, J., Jamila, N., Gilani, S.A., Abbas, G., and Ahmed, S. (2009). Platelet aggregation, antiglycation, cytotoxic, phytotoxic and antimicrobial activities of extracts of Nepeta juncea. *African Journal of Biotechnology* 8.

Lanza, F., Beretz, A., Stierle, A., Hanau, D., Kubina, M., and Cazenave, J.-P. (1988). Epinephrine potentiates human platelet activation but is not an aggregating agent. *American Journal of Physiology-Heart and Circulatory Physiology* 255, H1276-H1288.

Lawlees, J. (1992). The encyclopaedia of essential oils (Element Books Limited: Boston).

Li, H., Huang, W., Wen, Y., Gong, G., Zhao, Q., and Yu, G. (2010). Anti-thrombotic activity and chemical characterization of steroidal saponins from Dioscorea zingiberensis CH Wright. *Fitoterapia* 81, 1147-1156.

Mekhfi, H., ElHaouari, M., Bnouham, M., Aziz, M., Ziyyat, A., and Legssyer, A. (2006). Effects of extracts and tannins from Arbutus unedo leaves on rat platelet aggregation. *Phytotherapy research* 20, 135-139.

Michelson, A.D., Frelinger, A.L., and Furman, M.I. (2006). Current options in platelet function testing. *The American journal of cardiology* 98, S4-S10.

ISSN: 2198-4093
www.bmrat.org

Morton, L.F., Peachey, A.R., and Barnes, M.J. (1989). Platelet-reactive sites in collagens type I and type III. Evidence for separate adhesion and aggregatory sites. *Biochemical Journal* 258, 157-163.

Nakano, T., Terawaki, A., and Arita, H. (1986). Measurement of Thromboxane A2 Elevation of Ionized Calcium in Collagen-Stimulated Platelets with the Photoprotein, Aequorin. *Journal of biochemistry* 99, 1285-1288.

Pierre, S., Crosbie, L., and Duttaroy, A.K. (2005). Inhibitory effect of aqueous extracts of some herbs on human platelet aggregation in vitro. *Platelets* 16, 469-473.

Pollock, W.K., Rink, T.J., and Irvine, R.F. (1986). Liberation of [3H] arachidonic acid and changes in cytosolic free calcium in fura-2-loaded human platelets stimulated by ionomycin and collagen. *Biochemical Journal* 235, 869-877.

Poole, A.W., and Watson, S.P. (1995). Regulation of cytosolic calcium by collagen in single human platelets. *British journal of pharmacology* 115, 101-106.

Porth, C. (2005). Pathophysiology: Concepts of altered health states, Vol 1 (Lippincott Williams & Wilkins).

Rittenhouse, S., and Allen, C. (1982). Synergistic activation by collagen and 15-hydroxy-9 alpha, 11 alpha-peroxidoprosta-5, 13-dienoic acid (PGH2) of phosphatidylinositol metabolism and arachidonic acid release in human platelets. *Journal of Clinical Investigation* 70, 1216.

Roth, G.J., and Majerus, P.W. (1975). The mechanism of the effect of aspirin on human platelets. I. Acetylation of a particulate fraction protein. *Journal of Clinical Investigation* 56, 624-632.

Sagrero−Nieves, L. (1992). Fatty acid composition of Mexican pine nut (Pinus cembroides) oil from three seed coat phenotypes. *Journal of the Science of Food and Agriculture* 59, 413-414.

Sànchez-Lamar, A., Fiore, M., Cundari, E., Ricordy, R., Cozzi, R., and De Salvia, R. (1999). Phyllanthus orbicularis aqueous extract: cytotoxic, genotoxic, and antimutagenic effects in the CHO cell line. *Toxicology and applied pharmacology* 161, 231-239.

Santoro, S.A., Walsh, J., Staatz, W., and Baranski, K. (1991). Distinct determinants on collagen support alpha 2 beta 1 integrin-mediated platelet adhesion and platelet activation. *Cell regulation* 2, 905-913.

Savage, G.P. (2001). Chemical composition of walnuts (Juglans regia L.) grown in New Zealand. *Plant Foods for Human Nutrition* 56, 75-82.

Sharma, A., Goyal, R., and Sharma, L. (2016). Potential biological efficacy of Pinus plant species against oxidative, inflammatory and microbial disorders. *BMC Complementary and Alternative Medicine* 16, 35.

Strukova, S. (2001). Thrombin as a regulator of inflammation and reparative processes in tissues. *Biochemistry (Moscow)* 66, 8-18.

Umesh, M., Sanjeevkumar, C., Hanumantappa, B., and Ramesh, L. (2014). Evaluation of in vitro anti-thrombolytic activity and cytotoxicity potential of Typha angustifolia L leaves extracts. *Int J Pharm Pharm Sci* 6, 81-85.

Yavasoglu, I., Acar, B., Kadikoylu, G., and Bolaman, Z. (2010). Platelet aggregation tests are affected in pseudothrombocytopenia. *Laboratory Medicine* 41, 483-485.

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Original Research

Circulating apoptotic endothelial cell-derived microparticles are predicted metabolically unhealthy obesity

Alexander E. Berezin[1,2,*], Alexander A. Kremzer[3], Tatyana A Berezina[1], Yulia V. Martovitskaya[4], Tatyana A Samura[3]

[1]Private Medical Center "Vita-Center", Zaporozhye, Ukraine
[2]Internal Medicine Department, State Medical University, Zaporozhye, Ukraine
[3]Clinical Pharmacology Department, State Medical University, Zaporozhye, Ukraine
[4]Immunology Department, Clinical Laboratory "Dia-Service", Zaporozhye, Ukraine

Abstract

Introduction: Circulating apoptotic endothelial cell-derived micro particles (EMPs) are a marker of endothelial dysfunction and cardiovascular (CV) risk in type 2 diabetes mellitus patients. There is evidence regarding association between apoptotic EMP number and CV disease in obese individuals. The aim of the study to investigate whether increased number of circulating apoptotic EMPs may predict transformation of Met-HO into Met-UHO. **Methods**: The study was retrospectively evolved 89 patients with established abdominal obesity (47 patients with Met-UHO determined as MetS and 42 subjects with Met-HO) from the large cohort of abdominal obesity patients (n=268). Thirty five healthy volunteers matched for age and sex were involved in the study as a control cohort. Obesity-related biomarker (adiponectin, leptin, vistafin) and EMPs were measured at baseline. Flow cytometry was used to determine EMPs with immune phenotype CD31+/ annexin V+ and CD144+/annexin V+. **Results**: There was not found a significant difference between numbers of EMPs labeled $CD31^+$/ Annexin V^+ in Met-UHO and Met-HO patients, while Met-UHO patients had a significantly increased level of circulating $CD144^+$/ Annexin V^+ compared with Met-HO individuals. Multivariate logistic regression analysis has revealed the HOMA-IR, number of CV risk factors, serum leptin and hs-CRP independently predicted numbers of circulating $CD31^+$/ Annexin V^+ and $CD144^+$/ Annexin V^+ EMPs in Met-UHO. In Met-HO patients HOMA-IR remained an independent predictor of increased numbers of circulating $CD31^+$/ Annexin V^+ and $CD144^+$/ Annexin V^+ EMPs. **Conclusion**: in the investigation we found that the increased number of $CD31^+$/Annexin V^+ and $CD144^+$/ Annexin V^+ EMPs added to the based predictive model (HOMA-IR) may predict transformation of Met-HO into Met-UHO.

***For correspondence:**

dr_berezin@mail.ru

Competing interests: The authors declare that no competing interests exist.

Received: 16 January 2017
Accepted: 25 January 2017
Published: 29 January 2017

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Keywords

Endothelial cell-derived micro particles; Insulin resistance; Metabolically healthy obesity; Metabolically unhealthy obesity

Introduction

The prevalence of abdominal obesity and (T2DM) has been raised worldwide (Basterra-Gortari et al., 2017; Flegal et al., 2010). Obesity associates with substantially increased all cause and cardiovascular (CV) morbidity and mortality, as well as relates to highest risk of type 2 diabetes mellitus (T2DM) (Rey–López et al., 2014; Vanuzzo et al., 2008). Recent epidemiological investigations, clinical studies and some registers have shown higher prevalence of CV risk factors in obese individuals especially with morbid obesity (body mass index [BMI] more than 40 kg/m^2) (Antwi et al., 2012; Finucane et al., 2011; Sturm, 2007; Valdés et al., 2014). Because progressive increases in yearly pre-diabetes / T2DM prevalence has observed for all classes of BMI irrespective age and sex (Sterling et al., 2016), there is reason to expect that abdominal obesity mediates CV risk and risk of T2DM through underlying co-morbidities independently BMI (Grundy et al., 2005; Grundy et al., 2004).

In this context, obese individuals with similar BMI may be protected or opposite predisposed to obesity-related complications (i.e. T2DM, insulin resistance (IR), dyslipidaemia, hypertension) and CV disease (Cuthbertson et al., 2017). The heterogeneity of obesity leads to understanding of being of emerging metabolic phenotypes e.g. metabolically unhealthy obesity (Met-UHO) and metabolically healthy obesity (Met-HO) distinguished from each other for CV risk (Kim et al., 2015). Moreover, it is suggesting that Met-HO is a transient state in the pathway to cardiometabolic disease, i.e. Met-UHO and T2DM (Mongraw-Chaffin et al., 2016).

Based on the Adult Treatment Panel-III criteria subjects with established obesity and co-existing other metabolic abnormalities including dyslipidemia, insulin resistance (IR), increased fasting glucose and impaired glucose tolerance, are referred Met-UHO, whereas obese individuals without these abnormalities might be defined as Met-HO (Grundy et al., 2005; Ryden et al., 2013). The mechanisms underlying the change in phenotype from metabolically healthy to metabolically unhealthy obesity are still unclear (Ryden et al., 2013).

Micro particles (MPs) are defined a heterogeneous sub-population of extracellular vesicles with diameter average from 100 to1000 nm originated from plasma membranes of mother' cells (Berezin et al., 2015a). As a derivate of cellular membrane MPs are discussed powerful paracrine regulators of target cell structure and functions. MP released by apoptotic endothelial cells posse a wide spectrum of biological effects on intercellular communication by transferring

ISSN: 2198-4093
www.bmrat.org

different active molecules (proteins, peptides, hormones, growth factors, microRNAs) exhibiting coagulation activity, mediating cell growth and tissue differentiation (Alexandru et al., 2016). Additionally, apoptotic endothelial cell-derived MPs (EMPs) may directly worse endothelial integrity and vascular function playing a pivotal role in development of microvascular inflammation and IR (Alexandru et al., 2016; Berezin et al., 2016b).

Recent clinical studies have shown that the circulating levels of apoptotic EMPs were significantly increased in T2DM patients as compared with healthy volunteers (Berezin et al., 2016a) and they mediated CV risk in patients with established metabolic syndrome (MetS) and T2DM (Agouni et al., 2014; Berezin et al., 2015b; Berezin et al., 2016c). Whether apoptotic EMPs are involved in the transformation of Met-HO into Met-UHO determining the risk of T2DM and CV disease is not fully clear. The aim of the study: to investigate whether increased number of circulating apoptotic EMPs may predict transformation of Met-HO into Met-UHO.

Materials-Methods

The study was retrospectively evolved 89 patients with established abdominal obesity (47 patients with Met-UHO determined as MetS and 42 subjects with Met-HO) from the large cohort of abdominal obesity patients (n=268) who were examined between February 2012 and July 2016. We have enrolled obese subjects (body mass index was more 30 kg/m^2) without known CV disease including angina pectoris, previous myocardial infarction / stroke, heart failure, and asymptomatic atherosclerosis (defined by negative result of the contrast-enhanced multi-spiral tomography angiography). Thirty five healthy volunteers matched for age and sex were involved in the study as a control cohort. All patients have given their informed written consent for a participation in the study.

MetS was diagnosed based on the National Cholesterol Education Program Adult Treatment Panel III criteria (Williams, 2002). Patients were enrolled in the MetS cohort when at least three of the following components were defined: waist circumference ≥90 cm or ≥80 cm in men and women respectively; high density lipoprotein (HDL) cholesterol <1.03 mmol/L or <1.3 mmol/L in men and women respectively; triglycerides ≥1.7 mmol/L; blood pressure ≥130/85 mmHg or current exposure of antihypertensive drugs; fasting plasma glucose ≥5.6 mmol/L. Participants who had less than 2 criteria of MetS were classified as Met-HO patients. Those who had 2 or more criteria of MetS were classified as metabolically abnormal (Williams, 2002) and were not considered candidates for this study. Participants with abdominal obesity who had less than 3 criteria of MetS were classified those who had Met-HO. Therefore, individuals with non-alcoholic fatty liver disease, polycystic ovary syndrome, and those who had

ISSN: 2198-4093
www.bmrat.org

higher levels of HBV / HCV antibodies, were not enrolled in the study. The flow chart with inclusion / exclusion criteria is reported in **Fig. 1**.

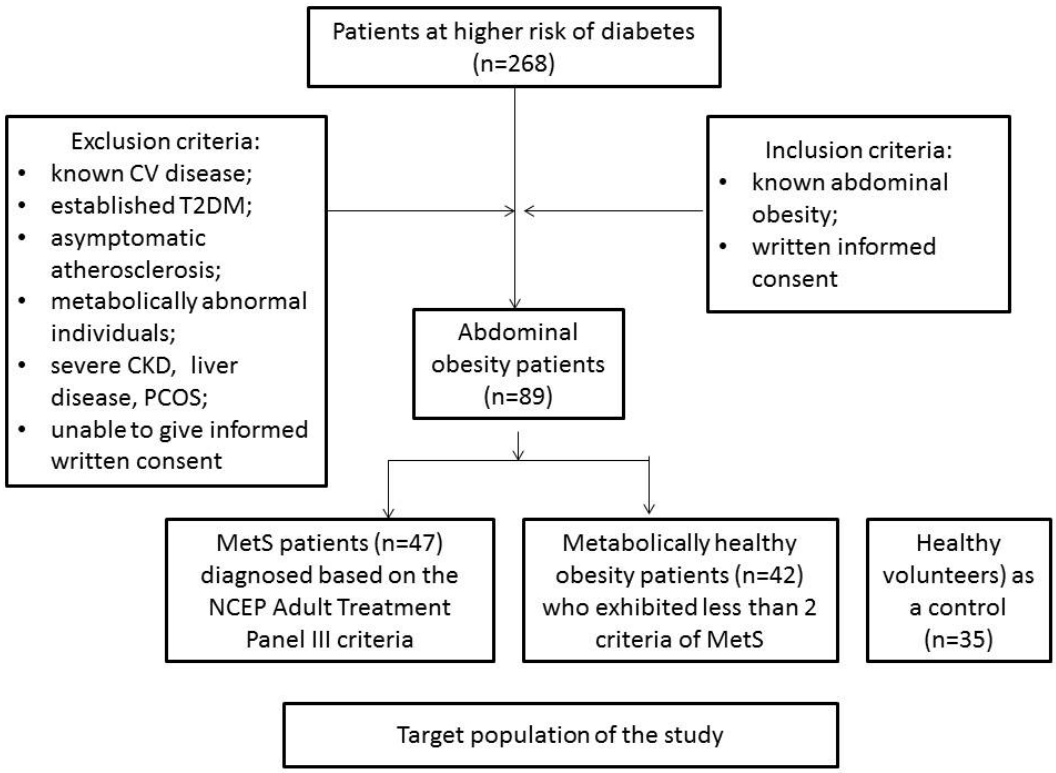


Figure 1. **The flow chart with inclusion / exclusion criteria**. Abbreviations: PCOS: polycystic ovary syndrome; Met-UHO: metabolically unhealthy obesity; Met-HO: metabolically healthy obesity.

Smoking status

Current smoking was defined as consumption of one cigarette daily for three months (Lindson-Hawley et al., 2013).

Anthropometric measurements

Anthropometric measurements (weight, height, body mass, body mass index, waist circumference, and waist-to-hip ratio) were made using standard procedures (Ashwell et al., 2012; Consultation, 2008). Height and weight were measured by professional health staff with the participants standing without shoes and heavy outer garments with a wall-mounted stadiometer (OMRON, Japan). Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). Waist circumference was measured at the level midway between the lower rib margin and the iliac crest with participants in a standing position

ISSN: 2198-4093
www.bmrat.org

without heavy outer garments and with emptied pockets, breathing out gently. Hip circumference was recorded as the maximum circumference over the buttocks.

Calculation of glomerular filtration rate

Glomerular filtration rate (GFR) was calculated with CKD-EPI formula (Levey et al., 2009).

Measurement of circulating biomarkers

To determine circulating biomarkers, blood samples were collected at baseline in the morning (at 7-8 a.m.) after at least 10 h fasting into cooled silicone test tubes wherein 2 mL of 5% Trilon B solution were added. Then they were centrifuged upon permanent cooling at 6,000 rpm for 3 minutes. Plasma was collected and refrigerated immediately to be stored at a temperature -70ºC.The levels of high-sensitive C-reactive protein (hs-CRP), adiponectin, leptin, vistafin were measured by commercially available standard kit (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). The intra-assay and inter-assay coefficients of variation were <5% for all cases.

Fasting insulin level was measured by a double-antibody sandwich immunoassay (Elecsys 1010 analyzer, F. Hoffmann-La Roche Diagnostics, Mannheim, Germany). The intra-assay and inter-assay coefficients of variation were <5%. The lower detection limit of insulin level was 1.39 pmol/L. Insulin resistance was assessed by the homeostasis model assessment for insulin resistance (HOMA-IR) (Matthews et al., 1985) using the following formula:

HOMA-IR (mmol/L × μU/mL) = fasting glucose (mmol/L) × fasting insulin (μU/mL) / 22.5

IR was arbitrarily defined as a homeostasis model assessment-IR index (HOMA-IR) value above the 75th percentile of normal glucose tolerance equal 2.45 mmol/L × μU/mL.

Hemoglobin A1c (HbA1c) were determined by high-pressure liquid chromatography method. Concentrations of total cholesterol (TC), cholesterol of high-density lipoproteins (HDL-C), triglycerides (TG), and low-density lipoproteins (LDL-C) were measured by direct enzymatic method (Roche P800 analyzer, Basel, Switzerland).

Quality control was assessed daily for all determinations.

Assay of circulating endothelial-derived microparticles

Circulating MPs were isolated from 5 ml of venous citrated blood drawn from the fistula-free arm. No hemolysis in the samples was found. All samples were not frozen before analysis. To prevent contamination of samples platelet-free plasma

ISSN: 2198-4093
www.bmrat.org

(PFP) was separated from whole blood. PFP was centrifugated at 70,476 × g for 70 min. MP pellets were washed with DMEM (supplemented with 10 µg/mL polymyxin B, 100 UI of streptomycin, and 100 U/ml penicillin) and centrifuged again (70,476 × g for 90 min) (Cvjetkovic et al., 2014). The obtained supernatant was extracted, and MP pellets were re-suspended into the remaining 200 µL of supernatant. PFP, MPs, and supernatant were diluted five-, 10-, and five-fold in PBS, respectively. Only 100 µL of supernatant was prepared for further analysis through incubation with different fluorochrome-labeled antibodies or their respective isotypic immunoglobulins (Beckman Coulter).

MPs were labeled and characterized by flow cytometry by phycoerythrin (PE)-conjugated monoclonal antibody against CD31 (platelet endothelial cell adhesion molecule [PECAM]-1), CD144 (vascular endothelial (VE)-cadherin), CD62E (E-selectin), and Annexin V (BD Biosciences, USA) followed by incubation with fluorescein isothiocyanate (FITC)-conjugated Annexin V (BD Biosciences, USA) per HD-FACS (High-Definition Fluorescence Activated Cell Sorter) methodology independently after supernatant diluted without freeze (Orozco and Lewis, 2010).

The samples were incubated in the dark for 15 min at room temperature according to the manufacturer's instructions. It was performed the analysis of area, height, and width forward scatter (FSC) and side scatter (SSC) parameters as well as side scatter width (SSC-W). Particle sizing by dynamic light scattering revealed a characteristic size of the MPs (Sigma, St Louis, MO, USA). A MPs' gate was established on the FACS Aria instrument by preliminary standardization experiments using a blend of size-calibrated fluorescent beads, with sizes ranging from 0.1 to 1.0 µm. Two size gates were defined based on forward angle light scattering from polystyrene microsphere (0.5-0.9 µm) accordingly standard protocol. The upper and the outer limit of the MP gate was established just above the size distribution of the 0.9-µm beads in a FSC-A and SSC-A setting (log scale) using the "auto-gate" function. Accordingly, MPs' gate was defined less than a 0.4 µm polystyrene microsphere extending down to the noise threshold level that is equivalent to cell-derived MPs < 1 µm diameter. The lower limit was the noise threshold of the instrument, and an absolute minimum threshold of 200 was set at the SSC-A parameter (instead of FSC-A) to avoid exclusion of the smallest events. In order to separate true events from background noise, we defined MPs as particles that were less than 1.0 µm in diameter, and expressed cell specific markers.

For each sample, 500 thousand events have been analyzed. Compensation tubes were used with similar reagents as were used in the sample tubes. Calculation of the number of MPs per liter plasma was based upon the particle count per unit time, the flow rate of the flow cytometer, and the net dilution during sample preparation of the analyzed MP suspension. MP-exposed antigen concentrations were calculated in each sample by multiplying the total concentration of positive MPs by the mean fluorescence intensity of the antigen

ISSN: 2198-4093
www.bmrat.org

exposure of the total positive MP population. The reproducibility of EPCs using standard protocol was 4.5%.

Determination of endothelial cell-derived MP populations

CD31 antigen was determined as common marker for endothelial cells, mononuclears and platelets. CD144+ antigen is essential for endothelial cells and used to identify a pure population of endothelial cells. To characterize entire population of MPs originated from endothelial cells we used both antigens' determination. CD31+/annexin V+ and CD144+/annexin V+ MPs were defined as apoptotic endothelial cell-derived MPs (Lacroix et al., 2013).

Statistical Analysis

Statistical analysis of the results obtained was performed in SPSS system for Windows, Version 22 (SPSS Inc, Chicago, IL, USA). The data were presented as mean (M) and standard deviation (±SD) or 95% confidence interval (CI); as well as median (Me) and 25%-75% interquartile range (IQR). To compare the main parameters of patient cohorts, two-tailed Student t-test or Shapiro–Wilk U-test were used. To compare categorical variables between groups, Chi2 test ($\chi 2$) and Fisher F exact test were used. Univariant and multivariant linear regression models were used to determine a relation between circulating number of microprticles and other biomarkers. C-statistics, integrated discrimination indices (IDI) and net-reclassification improvement (NRI) were utilized for prediction performance analyses. A two-tailed probability value of <0.05 was considered as significant.

Results

The demographic and anthropometric characteristics, the prevalence of CV risk factors are reported in **Table 1**. There was not a significant difference between healthy volunteers and entire cohort, as well as between subjects with metabolically unhealthy obesity (Met-UHO) and metabolically healthy obesity (Met-HO) in age, sex, adherence to smoke and haemodynamic performances. Abdominal obesity subjects exhibited higher BMI, WHR, HOMA-IR, as well as they had increased frequency of LVH, hypertension and IR presentation. Met-UHO patients had higher HOMA-IR, than those with Met-HO, while BMI, systolic and diastolic blood pressure, heart rate were similar in both cohorts. Additionally, hypertension, IR and dyslipidemia presentation were found frequently in Met-UHO patients compared to Met-HO individuals.

ISSN: 2198-4093
www.bmrat.org

Table 1. The characteristics of participants in the study

Variables	Healthy volunteers (n=35)	Entire patient cohort (n=89)	P value between healthy volunteers and entire patient cohort	Subjects metabolically unhealthy obesity (n=47)	Subjects metabolically healthy obesity (n=42)	P value between subgroups with obesity
Age, years	54.85±5.20	55.40±6.60	0.82	56.13±6.40	55.14±5.12	0.84
Male, n (%)	18 (51.4%)	47 (52.8%)	0.96	25 (53.2%)	22 (52.4%)	0.94
Hypertension, n (%)	-	54 (60.7%)	0.001	44 (93.6%)	10 (23.8%)	0.01
Dyslipidemia, n (%)	-	59 (66.3%)	0.001	47 (100.0%)	18 (42.9%)	0.046
IR, n (%)	-	55 (61.8%)	0.001	41 (87.2%)	14 (33.3%)	0.04
HOMA-IR, mmol/L × μU/mL	1.13 (0.98-1.24)	2.91 (1.20-3.80)	0.012	3.45 (3.22-3.78)	1.22 (1.02 -1.65)	0.001
Adherence to smoke, n (%)	9 (25.7%)	19 (21.3%)	0.76	12 (25.5%)	3 (7.1%)	0.82
BMI, kg/m2	22.3 (20.1 – 23.5)	27.9 (26.1 – 33.5)	0.001	27.5 (25.2 – 32.9)	27.2 (25.6 – 32.3)	0.89
WHR, units	0.85 (0.82 – 0.87)	1.02 (0.96 – 1.10)	0.001	1.02 (0.97 – 1.11)	1.01 (0.96 – 1.10)	0.96
Systolic BP, mm Hg	121±4	132±7	0.054	136±6	130±7	0.84
Diastolic BP, mm Hg	68±4	75±6	0.052	78±5	76±5	0.86
Heart rate, beat per min.	64.25±4.12	70.15±5.20	0.062	72.35±6.95	66.42±5.44	0.054

Notes: Data are expressed as mean (M) and standard deviation (±SD), median (Me) and interquartile range (IQR), numerous (n) and frequencies (%). Abbreviations: T2DM: Type two diabetes mellitus, LVEF: left ventricular ejection fraction; LVH: LV hypertrophy; HOMA-IR: homeostatic model assessment of insulin resistance index; IR: insulin resistance; WHR: weight to hip ratio.

Healthy volunteers and abdominal obesity individuals from entire cohort had similar level of GFR, hemoglobin, and creatinine. Consequently, abdominal obesity individuals had higher level of fasting glucose, HbA1c, total cholesterol, LDL cholesterol, triglycerides, hs-CRP, vistafin, leptin and lower adiponectin (Table 2). No difference was seen in GFR, hemoglobin, fasting glucose, creatinine, and lipids' level between Met-UHO and Met-HO patients. However, Met-UHO patients had higher HbA1c, vistafin, leptin and lower hs-CRP than those with Met-HO.

ISSN: 2198-4093
www.bmrat.org

Healthy volunteers had significantly decreased level of EMPs labeled CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ than those with abdominal obesity (Fig. 2). However, there was not found a significant difference between numbers of EMPs labeled CD31$^+$/ Annexin V$^+$ in Met-UHO and Met-HO patients. In contrast, Met-UHO patients had a significantly increased level of circulating CD144$^+$/ Annexin V$^+$ compared with Met-HO individuals.

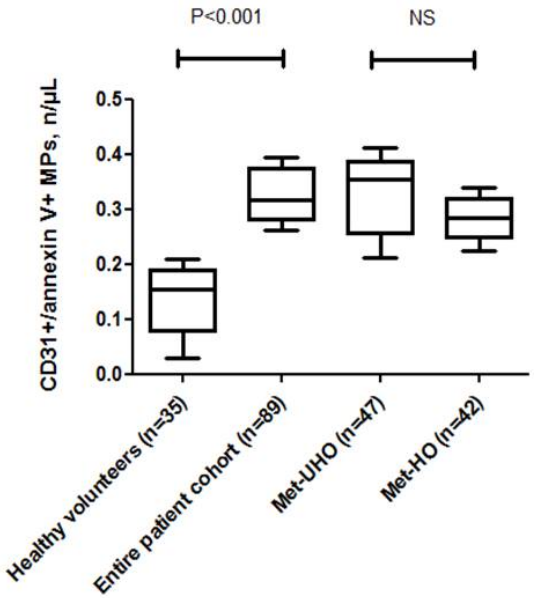


Figure 2a

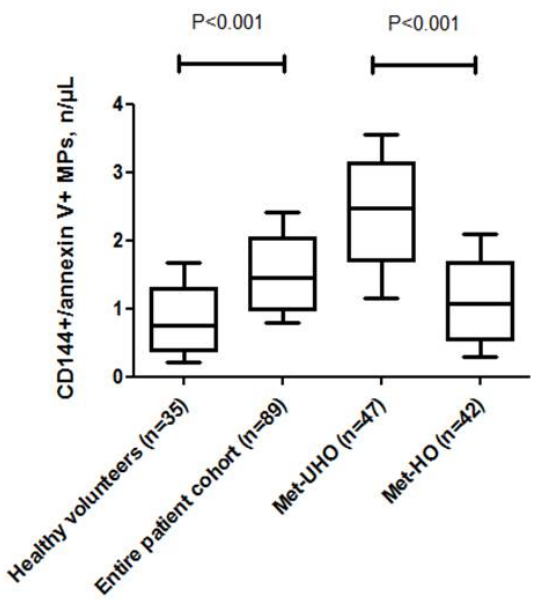


Figure 2b

Figure 2. The number of circulating apoptotic endothelial cell-derived micro particles in abdominal obesity patients and healthy volunteers. Abbreviations: Met-UHO, metabolically unhealthy obesity; Met-HO, metabolically healthy obesity.

The univariate linear regression analysis between numerous of EPMs with immune phenotypes determined CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$, CV risk factors, hemodynamic performances, and other biomarkers was performed. In Met-UHO patients the number of CD31$^+$/ Annexin V$^+$ EMPs received from peripheral blood positively related to HOMA-IR (r =0.35, P = 0.003), hs-CRP (r = 0.33, P = 0.001), number of CV risk factors (r = 0.32, P = 0.001), BMI (r = 0.31, P = 0.001), serum leptin (r = 0.31, P < 0.001), fasting glucose (r = 0.30, P < 0.001), serum vistafin (r = 0.29, P < 0.001), LDL cholesterol (r = 0.27, P = 0.003), but inversely associated with serum adiponectin (r = -0.31, P < 0.001). In contrast, in Met-HO individuals HOMA-IR (r =0.32, P = 0.001) and

ISSN: 2198-4093
www.bmrat.org

number of CV risk factors (r =0.31, P = 0.001) significantly related to number of CD31^{+}/ Annexin V^{+} EMPs. There were not sufficient relations between number of CD31^{+}/ Annexin V^{+} EMPs and biomarkers of obesity, i.e. leptin and vistafin, whereas between adiponectin and number of CD31^{+}/ Annexin V^{+} EMPs an inversely weak association was found (r = -0.25, P = 0.01).

Table 2. The biomarkers of the patients enrolled in the study

Variables	Healthy volunteers (n=35)	Entire cohort (n=89)	P1 value	Met-UHO (n=47)	Met-HO (n=42)	P2 value
GFR, mL/ min/1.73 m2	112.4 (102.2 – 123.4)	109.5 (101.2–117.5)	0.11	107.3 (98.7 – 114.1)	112.2 (100.1–118.3)	0.12
Hemoglobin, g/L	136.3 (129.8 – 147.2)	134.9 (126.7 – 143.9)	0.86	135.4 (128.5 – 142.1)	134.8 (127.3 – 144.8)	0.68
Fasting glucose, mmol/L	4.24 (3.60-4.91)	5.18 (4.51-5.92)	0.012	5.47 (4.43-5.92)	4.92 (4.1-5.80)	0.18
HbA1c, %	4.78 (4.21-5.15)	5.47 (4.82-5.90)	0.042	5.78 (5.42-6.16)	4.93 (4.54-5.48)	0.022
Creatinine, μmol/L	65.4 (58.2–81.2)	70.3 (60.7–83.56)	0.74	70.5 (58.7–85.7)	70.1 (60.2–84.5)	0.66
Total cholesterol, mmol/L	4.56 (3.25–4.88)	5.45 (4.46-6.15)	0.044	5.60 (4.71–6.52)	5.33 (4.32–6.17)	0.12
HDL Cholesterol, mmol/L	1.03 (0.98 – 1.08)	0.93 (0.90–1.12)	0.046	0.92 (0.88 – 1.13)	0.95 (0.9 – 1.15)	0.22
LDL Cholesterol, mmol/L	2.77 (2.33 – 3.10)	3.54 (3.34–3.66)	0.012	3.63 (3.21 – 3.70)	3.41 (3.30–3.65)	0.46
TG, mmol/L	1.67 (1.31 – 1.94)	2.27 (2.14 – 2.55)	0.014	2.31 (2.13 – 2.59)	2.24 (2.08 – 2.43)	0.52
hs-CRP, mg/L	3.27 (0 – 5.33)	5.15 (2.09-8.03)	0.001	7.10 (6.25-8.20)	3.04 (1.12-5.42)	0.044
Adiponectin, mg / L	13.65 (10.12-24.93)	10.12 (6.88-14.95)	0.001	8.36 (5.11-11.67)	11.25 (7.41-16.17)	0.026
Leptin, ng/ml	9.53 (5.12-14.22)	24.19 (15.55-33.17)	0.001	31.25 (19.67-43.22)	15.31 (11.32-21.67)	0.002
Vistafin, ng/mL	3.67 (1.85-4.50)	4.91 (2.55 – 7.13)	0.001	5.65 (3.22 – 8.64)	3.71 (1.92 – 5.03)	0.014

Note: The values correspond to medians and IQR of 25%–75%. Abbreviations: GFR: glomerular filtration rate; HbA1c: glycated hemoglobin, HDL: high-density lipoprotein; LDL: Low-density lipoprotein; P^1 : value of P between healthy volunteers and entire cohort patients with prediabetes; P^2 : value of P between Met-UHO and Met-HO patients; Met-UHO: metabolically unhealthy obesity; Met-HO: metabolically healthy obesity.

ISSN: 2198-4093
www.bmrat.org

Therefore, the number of CD144$^+$/ Annexin V$^+$ EMPs inversely related to a level of serum adiponectin (r = -0.28, P = 0.001) and positively associated with HOMA-IR (r =0.36, P = 0.001), hs-CRP (r = 0.31, P = 0.001), serum vistafin (r = 0.33, P = 0.001), number of CV risk factors (r = 0.32, P = 0.001), serum leptin (r = 0.32, P = 0.001), fasting glucose (r = 0.29, P = 0.012) in Met-UHO patients. In contrast, there was not a significant association between number of CD144$^+$/ Annexin V$^+$ EMPs and metabolic biomarkers of obesity, such as adiponectin, leptin and vistafin in Met-HO individuals, whereas they were related to HOMA-IR (r =0.36, P = 0.001), BMI (r = 0.32, P = 0.001) and hs-CRP (r = 0.30, P = 0.001).

Multivariate unadjusted linear regression analysis has shown that in Met-UHO patients the numbers of CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs related to HOMA-IR (r = 0.32, P = 0.001 and r =0.33, P = 0.001), hs-CRP (r = 0.30, P = 0.001 and r =0.31, P = 0.001), number of CV risk factors (r =0.29, P = 0.001 and r =0.30, P = 0.001), serum leptin (r = 0.30, P = 0.001 and r = 0.31, P = 0.001), serum vistafin (r = 0.28, P = 0.001 and r = 0.30, P = 0.001), serum adiponectin (r = -0.26, P = 0.001 and r = -0.29, P = 0.001) and LDL cholesterol (r = 0.27, P = 0.001 and r =0.28, P = 0.003) respectively. In Met-HO individuals the numbers of CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs related significantly to HOMA-IR (r =0.32, P = 0.001 and r =0.36; P<0.001), BMI (r = 0.27, P = 0.001 and r =0.31; P<0.001) and hs-CRP (r = 0.26, P = 0.001 and r = 0.30, P = 0.001) respectively.

After adjustment for BMI HOMA-IR remained the most profound factor related to numbers of CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs (r = 0.33, P = 0.001 and r =0.36, P = 0.001) in Met-UHO. We also determined a relation between numbers of CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs and number of CV risk factors (r =0.27, P = 0.001 and r =0.30, P = 0.001), serum leptin (r = 0.27, P = 0.001 and r = 0.29, P = 0.001), serum vistafin (r = 0.28, P = 0.001 and r = 0.30, P = 0.001). Therefore, mild association of CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs with HOMA-IR (r = 0.34, P = 0.001 and r =0.36, P = 0.001) was found in Met-HO patients. However, the multivariate adjusted for BMI linear regression analysis has shown that in Met-UHO numbers of CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs associated with serum hs-CRP (r = 0.27, P = 0.001 and r = 0.31, P = 0.001) respectively. In multivariate logistic regression analysis we found that HOMA-IR, number of CV risk factors, serum leptin and hs-CRP were independent predictors for increased numbers of circulating CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs in Met-UHO (**Table 3**). In Met-HO patients HOMA-IR remained an independent predictor of increased numbers of circulating CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs.

Statistics for model fit for the prediction of Met-UHO development is reported in **Table 4**. One can see increased number of CD31$^+$/ Annexin V$^+$ and CD144$^+$/ Annexin V$^+$ EMPs added to the based predictive model (Met-HO + HOMA-IR) may sufficiently improve prognostication of based model regarding development of Met-UHO.

ISSN: 2198-4093
www.bmrat.org

Table 3. The factors contributed in increased number of circulating apoptotic EMPs in abdominal obesity: The multivariate logistic BMI-adjusted regression

Factors	CD31+/ Annexin V+ EMPs		CD144+/ Annexin V+ EMPs	
	OR (95% CI)	P Value	OR (95% CI)	P Value
Met-UHO				
HOMA-IR, per 0.65 mmol/L × μU/mL	1.06 (1.03–1.11)	0.001	1.10 (1.05–1.17)	0.001
Number of CV risk factors, per 1 factor	1.02 (1.00–1.05)	<0.05	1.05 (1.01–1.10)	0.042
Serum leptin, per 5.5 ng/ml	1.02 (1.00–1.04)	<0.05	1.04 (1.01–1.09)	0.048
Serum vistafin, per 2.5 ng/mL	1.01 (0.97–1.06)	0.48	1.03 (1.00–1.07)	0.18
hs-CRP, per 4.50 mg/L	1.02 (1.00–1.05)	<0.05	1.03 (1.01–1.07)	<0.05
HOMA-IR, per 0.65 mmol/L × μU/mL	1.06 (1.03–1.11)	0.001	1.10 (1.05–1.17)	0.001
Number of CV risk factors, per 1 factor	1.02 (1.00–1.05)	<0.05	1.05 (1.01–1.10)	0.042
Met-HO				
HOMA-IR, per 0.65 mmol/L × μU/mL	1.05 (1.01–1.10)	0.001	1.09 (1.04–1.15)	0.001
Number of CV risk factors, per 1 factor	1.01 (0.97–1.04)	0.54	1.01 (1.00–1.03)	0.056
hs-CRP, per 4.50 mg/L	1.02 (0.98–1.06)	0.24	1.02 (1.00–1.05)	0.060

Abbreviations: CI: confidence interval; IQR: inter quartile range; BMI: Body mass index; hs-CRP: high sensitive C reactive protein; OR: odds ratio; HOMA-IR: homeostatic model assessment of insulin resistance index; IR: insulin resistance; Met-UHO: metabolically unhealthy obesity; Met-HO: metabolically healthy obesity.

Discussion

In this study we reported that increased number of CD31+/ Annexin V+ and CD144+/ Annexin V+ EMPs much more pretty accurate predict Met-UHO than based model (Met-HO). Taking into consideration that Met-UHO is considered a powerful risk factor of type 2 diabetes mellitus, we suggest that increased number of CD31+/ Annexin V+ and CD144+/ Annexin V+ EMPs related to HOMA-IR in both Met-UHO and Met-HO may predict probably obesity-related complications including higher risk of t2dm. Thus, early detection of abnormality in circulating levels of apoptotic EPCs may be a biomarker of IR and predictor of Met-UHO in patients with abdominal obesity when other metabolic disturbances are absent or evidence regarding them is fare limited.

ISSN: 2198-4093
www.bmrat.org

Recently it has been suggested that Met-HO is a stage of development of Met-UHO and T2DM (Mongraw-Chaffin et al., 2016). However, our results revealed that the most important factor that affects metabolic dysregulation in obesity is IR, which probably appears to be predominantly early stage of the Met-HO. There is evidence that an accumulation of visceral adiposity tissue (VAT) might associate with over-production of pro-inflammatory cytokines including hs-CRP, leptin and vistafin and induce IR (Brede et al., 2016). Therefore, infiltration of the sub-intima by LDL cholesterol may induce production of free radicals, oxidation of cytoskeleton and membrane vesiculation of endothelial cells. Finally, membrane vesiculation of endothelial cells is enhanced by inflammatory cytokines in conveying of VAT accumulation (Mause and Weber, 2010). Interestingly, the circulating number of apoptotic EMPs has well associated with conventionally obesity biomarkers (adiponectin, leptin, vistafin) in Met0UHO patients, but did not in Met-HO individuals. Indeed, in Met-HO patients we did not find severe metabolic abnormalities apart from leptin elevation compared with Met-UHO, however, IR was determined as common finding for both Met-UHO and Met-HO individuals without a difference in BMI.

Table 4. Statistics for model fit for the prediction of transformation of Met-HO to Met-UHO

Predictive Models	Depended variable: Met-UHO					
	AUC		NRI		IDI	
	M (95% CI)	P value	M (95% CI)	P value	M (95% CI)	P value
Based Model: Met-HO+ HOMA-IR	0.58 (0.52 – 0.63)	-	Reference	-	Reference	
Based Model + hs-CRP	0.59 (0.56 – 0.61)	0.66	0.21	0.42	0.015	0.80
Based Model + number of CV risk factors	0.64 (0.57 – 0.72)	0.14	0.26	0.12	0.022	0.12
Based Model + HOMA-IR + increased number of CD31+/ Annexin V+ and CD144+/ Annexin V+ EMPs	0.76 (0.66 – 0.87)	0.001	0.53	0.001	0.12	0.012

Abbreviations: AUC, area under the curve; 95% CI, 95% confidence interval; NRI, net reclassification index; IDI, integrated discrimination index; HOMA-IR, homeostatic model assessment of insulin resistance index; EMPs, endothelial micro particles.

The increased amount of VAT together with a chronic inflammation and IR predisposes to the development of endothelial dysfunction through attenuation synthesis and secretion of apoptotic EMPs. Indeed, pro-inflammatory cytokines, i.e. interleukin-6, tumor factor necrosis-alpha, leptin, and vistafin, may directly influence structure of endothelial cells and trigger a secretion of apoptotic EMPs (Berezin, 2016b; Rautou et al., 2011). The main biological function of this

ISSN: 2198-4093
www.bmrat.org

process is attenuation of endothelial cell repair and recovery of vascular function (Jansen et al., 2015). Unfortunately, co-existing IR affects endothelial progenitor cells and they are not able to differentiate into functionally mature endothelial cells even after stimulation by apoptotic EMPs (Martinez and Andriantsitohaina, 2011; Tetta et al., 2011). As a result, apoptotic EMP-induced endothelial dysfunction and IR may become an early predictor of shaping Met-UHO.

Recently we have reported that apoptotic EMPs may independent predict asymptomatic atherosclerosis and CV disease in T2DM patients (Berezin, 2016a; Berezin et al., 2016a), while their role in individuals with different phenotypes of obesity has remained controversial (Montoro-García et al., 2011). First, it is not clear whether increased number of apoptotic EMPs is adaptive mechanism of vascular repair or factor of endothelial injury. Indeed, circulating EPMs, which are enhanced in a large number of metabolic disorders including abdominal obesity, associated with IR and this has been linked to deleterious effects on endothelial cells (Martinez and Andriantsitohaina, 2011). At the same time, apoptotic EPMs are powerful factor contributing in endothelial progenitor cell mobbing and differentiation (Montoro-García et al., 2011). Secondary, it is not fully understand the innate molecular mechanisms, which correspond to triggers of secretion of these apoptotic MPs. Apoptotic MPs as cargo microvesicles consist of a variety of biomolecules including regulated proteins, DNA, mRNA, and non-coding RNA. The proportion of these components as well as an entire secretome is under a tight control of autocrine / paracrine mechanisms and inflammatory factors (i.e. tumor necrosis factor-alpha, interleukin-2, -6), which induces EMP formation in a time-dependent manner (Lee et al., 2014). Consequently, the final reply of the recipient cells, such as endothelial progenitor cells, is depends on epigenetic regulation of secretome secretion and primary trigger, which affects vesiculation (Berezin, 2016b; Berezin et al., 2015c). Obviously, an ability of apoptotic EMPs to modulate immune and inflammatory processes, coagulation and vascular function, angiogenesis and vascular injury may interact with other regulatory mechanisms the role of witch in the pathogenesis of abdominal obesity requires still being determined. It is no excluded that release of apoptotic EMPs might act as a direct endogenous survival signal for target cells (Lichtenauer et al., 2015).

The present results exhibited first the interrelationship between increased number of circulating apoptotic EMPs and IR in patient with Met-HO. Extrapolating these findings on entire obese population, we can suggest that a clinical diagnosis of abdominal obesity irrespective to its phenotypes (Met-UHO or Met-HO) is probably not sufficient to assess a risk of T2DM and CV disease (Berezin et al., 2015c). In this context, measurement of circulating apoptotic EMP number would be useful tool for stratification amongst obesity individuals at higher risk of T2DM and CV (Berezin, 2016a, b; Montoro-García et al., 2011), especially when conventional biomarkers of obesity are not detected in appropriate diagnostic level. Large investigations are required to understand the role of apoptotic EMPs in pathogenesis of different phenotypes of abdominal

ISSN: 2198-4093
www.bmrat.org

obesity, because they may be a target of the therapy as well as predictive biomarkers.

Study limitations

This study has some limitations. The first limitation is low number of the patient involved in the investigation. Another limitation is lack of standardization of MP measurements, while commercial flow cytometers are existed. It is necessary to note that a large pool of MPs might be produced after blood sampling due to destruction of platelets and blood cells. In this study we used platelet free plasma to prevent of contamination of samples with MPs originated from platelets. Therefore, preparation of MP isolates from samples is the most sophisticated step for further examination. The next limitation might relate to complicated assay and suffers from resolution of MP detection technique that is worth considering. Indeed, there were several technical-related difficulties in the measurement of MPs affected centrifugation of samples, labeling of MPs, using HD-FACS methodology and final assay of results obtained. Although HD-FACS methodology is widely used, theoretically overlap between two or more fluorochromes might reflect some obstacles for further interpretation of obtained results, especially including size gating in MP determination. Therefore, rotor type and centrifugation time theoretically may influence on purity of MPs. However, flow cytometery and HD-FACS methodology are commonly used procedure to determine and measure MPs.

Another limitation of the present study is that a specific role of MPs is also possible and has not been characterized. However, the authors suppose that these optionally technically restrictions might have no significant impact on the study data interpretation. Additionally, retrospective, relative small sample size may limit the significance of the present study.

Conclusion

In this investigation we first determined the increased apoptotic EMP number may predict transformation of Met-HO into Met-UHO. The evidence allow suggesting the apoptotic EMPs might serve as a biomarker higher risk of T2DM and CV disease. Future investigations are needed to confirm these suggestions and clear situation around discriminative value of apoptotic EMP number in abdominal obesity individuals.

Biomedical
Research & Therapy

ISSN: 2198-4093
www.bmrat.org

Abbreviations

AUC: area under the curve; BMI: body mass index; CI: confidence interval; CV: cardiovascular: GFR: glomerular filtration rate; EMPs: endothelial cell-derived mocroparticles; HDL-C: high-density lipoprotein cholesterol; hs-CRP: high sensitive C-reactive protein; IDI: integrated discrimination index; LDL-C: low-density lipoprotein cholesterol; Met-HO: metabolically healthy obesity; MetS: metabolic syndrome; Met-UHO: metabolically unhealthy obesity; MPs: microparticles; NCEP: National Cholesterol Education Program; NRI: net reclassification index; OR: odds ratio.

Acknowledgement

We thank all patients for their participation in the investigation, staff of the Regional Zaporozhye Hospital (Ukraine) and the doctors, nurses, and administrative staff in City hospital # 6 (Zaporozhye, Ukraine), City Hospital #10 (Zaporozhye, Ukraine), Regional Center of Cardiovascular Diseases (Zaporozhye, Ukraine) general practices, and site-managed organizations that assisted with the study.

Author contribution

AEB gave the concept, designed experiment, analyzed data and gave final approval of the manuscript and was the supervisor of the project. AAK performed experiment, analyzed data and wrote draft of manuscript. TAB helped in data acquisition and experiment conditions optimization. YVM and TAS helped in editing and literature review for the paper TAS did data acquisition, organization and data analysis and helped in write up of final draft.

ISSN: 2198-4093
www.bmrat.org

References

Agouni, A., Andriantsitohaina, R., and C Martinez, M. (2014). Microparticles as biomarkers of vascular dysfunction in metabolic syndrome and its individual components. *Current vascular pharmacology* 12, 483-492.

Alexandru, N., Badila, E., Weiss, E., Cochior, D., Stępień, E., and Georgescu, A. (2016). Vascular complications in diabetes: Microparticles and microparticle associated microRNAs as active players. *Biochemical and biophysical research communications* 472, 1-10.

Antwi, F., Fazylova, N., Garcon, M.-C., Lopez, L., Rubiano, R., and Slyer, J.T. (2012). The effectiveness of web–based programs on the reduction of childhood obesity in school–aged children: A systematic review. *JBI Database of Systematic Reviews and Implementation Reports* 10, 1-14.

Ashwell, M., Gunn, P., and Gibson, S. (2012). Waist–to–height ratio is a better screening tool than waist circumference and BMI for adult cardiometabolic risk factors: systematic review and meta–analysis. *Obesity reviews* 13, 275-286.

Basterra-Gortari, F., Bes-Rastrollo, M., Ruiz-Canela, M., Gea, A., and Martinez-Gonzalez, M. (2017). Trends in the prevalence of obesity and diabetes in Spanish adults 1987-2012. *Medicina clinica*.

Berezin, A., Zulli, A., Kerrigan, S., Petrovic, D., and Kruzliak, P. (2015a). Predictive role of circulating endothelial-derived microparticles in cardiovascular diseases. *Clinical biochemistry* 48, 562-568.

Berezin, A.E. (2016a). Biomarkers for cardiovascular risk in patients with diabetes (BMJ Publishing Group Ltd and British Cardiovascular Society).

Berezin, A.E. (2016b). Cardiac biomarkers in diabetes mellitus: New dawn for risk stratification? *Diabetes & Metabolic Syndrome: Clinical Research & Reviews*.

Berezin, A.E., Kremzer, A.A., Berezina, T.A., and Martovitskaya, Y.V. (2015b). Pattern of circulating microparticles in chronic heart failure patients with metabolic syndrome: Relevance to neurohumoral and inflammatory activation. *BBA clinical* 4, 69-75.

Berezin, A.E., Kremzer, A.A., Berezina, T.A., and Martovitskaya, Y.V. (2016a). The pattern of circulating microparticles in patients with diabetes mellitus with asymptomatic atherosclerosis. *Acta clinica Belgica* 71, 38-45.

Berezin, A.E., Kremzer, A.A., Cammarota, G., Zulli, A., Petrovic, D., Martell-Claros, N., Sabo, J., and Kruzliak, P. (2016b). Circulating endothelial-derived apoptotic microparticles and insulin resistance in non-diabetic patients with chronic heart failure. *Clinical Chemistry and Laboratory Medicine (CCLM)* 54, 1259-1267.

Berezin, A.E., Kremzer, A.A., Martovitskaya, Y.V., Berezina, T.A., and Gromenko, E.A. (2016c). Pattern of endothelial progenitor cells and apoptotic endothelial cell-derived microparticles in chronic heart failure patients with preserved and reduced left ventricular ejection fraction. *EBioMedicine* 4, 86-94.

Berezin, A.E., Kremzer, A.A., Samura, T.A., Berezina, T.A., and Kruzliak, P. (2015c). Immune phenotypes of endothelial-derived microparticles in dysmetabolic patients. *Journal of Proteomics & Bioinformatics* 8, 60.

Brede, S., Serfling, G., Klement, J., Schmid, S.M., and Lehnert, H. (2016). Clinical Scenario of the Metabolic Syndrome. *Visceral medicine* 32, 336-341.

ISSN: 2198-4093
www.bmrat.org

Consultation, W.E. (2008). Waist circumference and waist-hip ratio. Report of a WHO Expert Consultation Geneva: World Health Organization, 8-11.

Cuthbertson, D.J., Steele, T., Wilding, J.P., Halford, J.C., Harrold, J.A., Hamer, M., and Karpe, F. (2017). What have human experimental overfeeding studies taught us about adipose tissue expansion and susceptibility to obesity and metabolic complications.

Cvjetkovic, A., Lötvall, J., and Lässer, C. (2014). The influence of rotor type and centrifugation time on the yield and purity of extracellular vesicles. *Journal of extracellular vesicles* 3.

Finucane, M.M., Stevens, G.A., Cowan, M.J., Danaei, G., Lin, J.K., Paciorek, C.J., Singh, G.M., Gutierrez, H.R., Lu, Y., and Bahalim, A.N. (2011). National, regional, and global trends in body-mass index since 1980: systematic analysis of health examination surveys and epidemiological studies with 960 country-years and 9· 1 million participants. *The Lancet* 377, 557-567.

Flegal, K.M., Carroll, M.D., Ogden, C.L., and Curtin, L.R. (2010). Prevalence and trends in obesity among US adults, 1999-2008. *Jama* 303, 235-241.

Grundy, S.M., Cleeman, J.I., Daniels, S.R., Donato, K.A., Eckel, R.H., Franklin, B.A., Gordon, D.J., Krauss, R.M., Savage, P.J., and Smith, S.C. (2005). Diagnosis and management of the metabolic syndrome. *Circulation* 112, 2735-2752.

Grundy, S.M., Cleeman, J.I., Merz, C.N.B., Brewer, H.B., Clark, L.T., Hunninghake, D.B., Pasternak, R.C., Smith, S.C., and Stone, N.J. (2004). A summary of implications of recent clinical trials for the National Cholesterol Education Program Adult Treatment Panel III guidelines (Am Heart Assoc).

Jansen, F., Yang, X., Baumann, K., Przybilla, D., Schmitz, T., Flender, A., Paul, K., Alhusseiny, A., Nickenig, G., and Werner, N. (2015). Endothelial microparticles reduce ICAM−1 expression in a microRNA−222−dependent mechanism. *Journal of cellular and molecular medicine* 19, 2202-2214.

Kim, H.-N., Kim, S.-H., Eun, Y.-M., and Song, S.-W. (2015). Obesity with metabolic abnormality is associated with the presence of carotid atherosclerosis in Korean men: a cross-sectional study. *Diabetology & metabolic syndrome* 7, 68.

Lacroix, R., Judicone, C., Mooberry, M., Boucekine, M., Key, N.S., and Dignat−George, F. (2013). Standardization of pre−analytical variables in plasma microparticle determination: results of the International Society on Thrombosis and Haemostasis SSC Collaborative workshop. *Journal of Thrombosis and Haemostasis* 11, 1190-1193.

Lee, S.K., Yang, S.-H., Kwon, I., Lee, O., and Heo, J.H. (2014). Role of tumour necrosis factor receptor-1 and nuclear factor-B in production of TNF--induced pro-inflammatory microparticles in endothelial cells. *Thrombosis and haemostasis* 112, 580-588.

Levey, A.S., Stevens, L.A., Schmid, C.H., Zhang, Y.L., Castro, A.F., 3rd, Feldman, H.I., Kusek, J.W., Eggers, P., Van Lente, F., Greene, T., *et al.* (2009). A new equation to estimate glomerular filtration rate. *Annals of internal medicine* 150, 604-612.

Lichtenauer, M., Goebel, B., Fritzenwanger, M., Förster, M., Betge, S., Lauten, A., Figulla, H.-R., and Jung, C. (2015). Simulated temporary hypoxia triggers the release of CD31+/Annexin+ endothelial microparticles: A prospective pilot study in humans. *Clinical hemorheology and microcirculation* 61, 83-90.

Lindson-Hawley, N., Begh, R., McDermott, M.S., McEwen, A., and Lycett, D. (2013). The importance of practitioner smoking status: A survey of NHS Stop Smoking Service practitioners. *Patient education and counseling* 93, 139-145.

ISSN: 2198-4093
www.bmrat.org

Martinez, M.C., and Andriantsitohaina, R. (2011). Microparticles in angiogenesis. *Circulation Research* 109, 110-119.

Matthews, D., Hosker, J., Rudenski, A., Naylor, B., Treacher, D., and Turner, R. (1985). Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28, 412-419.

Mause, S.F., and Weber, C. (2010). Microparticles. *Circulation research* 107, 1047-1057.

Mongraw-Chaffin, M., Foster, M.C., Kalyani, R.R., Vaidya, D., Burke, G.L., Woodward, M., and Anderson, C.A. (2016). Obesity severity and duration are associated with incident metabolic syndrome: Evidence against metabolically healthy obesity from the Multi-Ethnic Study of Atherosclerosis. *The Journal of Clinical Endocrinology & Metabolism* 101, 4117-4124.

Montoro-García, S., Shantsila, E., Marín, F., Blann, A., and Lip, G.Y. (2011). Circulating microparticles: new insights into the biochemical basis of microparticle release and activity. *Basic research in cardiology* 106, 911-923.

Orozco, A.F., and Lewis, D.E. (2010). Flow cytometric analysis of circulating microparticles in plasma. *Cytometry Part A* 77, 502-514.

Rautou, P.-E., Vion, A.-C., Amabile, N., Chironi, G., Simon, A., Tedgui, A., and Boulanger, C.M. (2011). Microparticles, vascular function, and atherothrombosis. *Circulation research* 109, 593-606.

Rey–López, J., Rezende, L., Pastor–Valero, M., and Tess, B. (2014). The prevalence of metabolically healthy obesity: a systematic review and critical evaluation of the definitions used. *Obesity reviews* 15, 781-790.

Ryden, L., Grant, P.J., Anker, S.D., Berne, C., Cosentino, F., Danchin, N., Deaton, C., Escaned, J., Hammes, H.P., Huikuri, H., *et al.* (2013). ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD: the Task Force on diabetes, pre-diabetes, and cardiovascular diseases of the European Society of Cardiology (ESC) and developed in collaboration with the European Association for the Study of Diabetes (EASD). *European heart journal* 34, 3035-3087.

Sterling, S.A., Jones, A.E., and Cox, R.D. (2016). Longitudinal Trends in the Prevalence of Diabetes Mellitus in an Urban Emergency Department. *Southern medical journal* 109, 222-227.

Sturm, R. (2007). Increases in morbid obesity in the USA: 2000–2005. *Public health* 121, 492-496.

Tetta, C., Bruno, S., Fonsato, V., Deregibus, M.C., and Camussi, G. (2011). The role of microvesicles in tissue repair. *Organogenesis* 7, 105-115.

Valdés, S., García-Torres, F., Maldonado-Araque, C., Goday, A., Calle-Pascual, A., Soriguer, F., Castaño, L., Catalá, M., Gomis, R., and Rojo-Martínez, G. (2014). Prevalence of obesity, diabetes and other cardiovascular risk factors in Andalusia (southern Spain). Comparison with national prevalence data. The Diabetes study. *Revista Española de Cardiología (English Edition)* 67, 442-448.

Vanuzzo, D., Pilotto, L., Mirolo, R., and Pirelli, S. (2008). Cardiovascular risk and cardiometabolic risk: an epidemiological evaluation. *Giornale italiano di cardiologia (2006)* 9, 6S-17S.

Williams, L. (2002). Third report of the National Cholesterol Education Program (NCEP) expert panel on detection, evaluation, and treatment of high blood cholesterol in adults (Adult Treatment Panel III) final report. *Circulation* 106, 3143-3143.

Scope

Biomedical Research and Therapy (ISSN 2198-4093) is the major forum for basic and translational research into therapies. An international peer-reviewed journal, it publishes high quality open access research articles with a special emphasis on basic, translational and clinical research into molecular therapeutics and cellular therapies, including animal models and clinical trials. The journal also provides reviews, viewpoints, commentaries and reports. Biomedical Research and Therapy's Editorial Policies follow the recommendations of the International Committee of Medical Journal Editors (ICMJE), the World Association of Medical Editors (WAME), and the Committee on Publication Ethics (COPE) for guidance on policies and procedures related to publication ethics.

The journal is published monthly, **12 issues per year**.

Peer review policy

The decision to publish a manuscript is based on the opinion of the editor and at least two other reviewers. Articles containing statistical analysis will also receive a statistical review. Reviewers' names will not be revealed to the author, nor will authors' names be revealed to editors. Manuscripts are accepted for publication on the understanding that they have not been submitted simultaneously to another journal and that the work was not previously published. Prior publication of abstracts will not prejudice publishing of the complete study. The editors reserve the right to make editorial and grammatical corrections. The editors cannot be considered responsible for damage or loss of typescripts, illustrations or photographs. Statements and opinions expressed in the articles are those of the authors and the editors disclaim any responsibility or liability for this material.

Please read details at here: http://www.bmrat.org/index.php/BMRAT/peerreviewprocess

Manuscript preparation

Please read details at here: http://www.bmrat.org/index.php/BMRAT/guidelines

ISSN 2198-4093
9 772198 409309

BioMedPress
The Open Access Publisher