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The Modulatory Role of Circulating Microvesicles in Endothelial Progenitor Cell
Function Is Altered in T2DM.

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

By

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2014
Wright State University

WRIGHT STATE UNIVERSITY

GRADUATE SCHOOL

April 28, 2014

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Hala Ammar ENTITLED The Modulatory Role of Circulating Microvesicles in Endothelial Progenitor Cell Function Is Altered in T2DM BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

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ABSTRACT

Ammar, Hala Mustafa. M.S., Department of Pharmacology and Toxicology, Wright State University, 2014. The Modulatory Role of Circulating Microvesicles in Endothelial Progenitor Cell Function Is Altered in T2DM.

Circulating microvesicles (cMVs) are the extracellular MVs released from the cells in the blood and on the vascular wall. Our previous study demonstrates that cMVs of diabetic mouse are detrimental to endothelial progenitor cells (EPCs), which are known to be very important for maintaining normal endothelial function and structure. In this study, we compared the levels of circulating EPCs and EPC-derived MVs (EPC-MVs) in diabetic and healthy subjects. Also, the migration ability, apoptosis rate and reactive oxygen species (ROS) production of EPCs cultured from diabetic and healthy subjects were determined. More importantly, we evaluated whether cMVs from healthy subjects (ch-MVs) improves the function of EPCs from diabetic patients (d-EPCs), and whether cMVs from diabetic patients (cd-MVs) impairs the function of EPCs from healthy subjects (h-EPCs). The d-EPCs or h-EPCs were incubated with ch-MVs or cd-MVs for 24 hours. The migration ability of EPCs was analyzed by an assay kit. The apoptotic rate and ROS production were analyzed by labeling with propidium iodide (PI) and dihydroethidium (DHE) respectively, followed with flow cytometric analysis. Our data showed that (1) there was a decrease in EPCs

and an elevation in EPC-MVs in diabetic patients when compared to healthy subjects; (2) The migration ability of d-EPCs were decreased, and the apoptosis rate and ROS production were increased in d-EPCs; (3) ch-MVs improve the function of d-EPC through improving its migration ability and decreasing the apoptosis and ROS production; (4) cd-MVs increase h-EPC apoptosis, and increase ROS production. We conclude that cMVs modulate EPC function and this role of cMVs is reversed in diabetes with the mechanism linked to ROS production.

Abbreviations

cMVs	Circulating Microvesicles.
ch-MVs	Circulating Microvesicles from Healthy Subjects.
cd-MVs	Circulating Microvesicles from Diabetic Patients .
EPCs	Endothelial Progenitor Cells.
h-EPCs	Endothelial Progenitor cells from Healthy Subject.
d-EPCs	Endothelial Progenitor cells from Diabetic Patients.
ROS	Reactive Oxygen Species.

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Acknowledgment

I would like to express the deepest appreciation to my advisor, Dr. Yanfang Chen, for his intellectual guidance, support and encouragement during the past two years. Without his guidance and persistent help this thesis would not have been possible.

I would like to thank my committee member Dr. David. R. cool for serving on my thesis committee and for his valuable comments and suggestions on my research work.

I would like to express very special appreciation to my committee member Dr. Ji. C. Bihl. This research was very challenging, and I could not have accomplished this thesis without the help and support of her. Thank you for your understanding, patients, encouragement, and guidance.

I would like to thank Dr. Thomas M. Koroscil and his nurses in the endocrinology department at Wright State Physicians Internal Medicine for offering the blood sample for this study.

A special thanks to my family. My husband thank you for supporting me for everything. I cannot thank you enough for encouraging me throughout this experience. My father and mother for all of the sacrifices that you have made on my behalf. Your prayer for me is what sustained me thus far.

To my beloved son and daughter Rashad and Renad for cheering me up always.

Finally, I thank my God, I have experienced your guidance day by day. Thank you for letting me through all the difficulties.

1. Introduction.

Diabetes mellitus (DM) is one of the major risk factors for vascular disease that is considered the leading cause of death in the United States. The incidence and mortality of cardiovascular disease are two- to eight fold higher in persons with diabetes than in those without diabetes (Howard et al., 2002). DM, is a chronic metabolic disease characterized by a glucotoxicity and lipotoxicity, considered as a principle cause of vascular endothelial damage, which ultimately leads to various cardiovascular complications. Endothelium dysfunction is a key initiator for vascular injury, and is characterized by a pro-inflammatory, pro-coagulative, and pro-vasoconstrictive endothelial constitution. (Burger et al., 2012). It results from increased oxidative stress in the vascular cells that is mostly attributable to NADPH oxidase (Nox) activation, and endothelial nitric oxide synthase (eNOS) uncoupling (Zhang et al., 2008). DM is associated with endothelial dysfunction which could be linked with functional impairment of endothelial progenitor cells (EPCs) (Thum et al., 2007).

EPCs are defined as bone marrow (BM)-derived circulating immature cells with the ability to differentiate into mature endothelial cells (ECs). EPCs are known to play important roles in maintaining normal endothelial function and structure by repairing or replacing dysfunctional or injured ECs. EPCs have been established to have therapeutic effects on cardiovascular disease through promoting

angiogenesis (De Biase et al., 2013). Our laboratory has demonstrated that EPCs also linked to ischemic stroke outcome (Chen et al, 2011). So the reduction in the number of EPCs or impairment in their functions may directly affect the endothelium balance and lead to endothelial dysfunction. DM has been reported to impair EPC proliferation, differentiation, adhesion, mobilization, and survival. (Avogaro et al., 2011; Petrelli et al., 2010 & Chen et al., 2011). As reported by Oikaw and Busik (Oikaw et al., 2010& Busik et al., 2009), one of the leading mechanisms, which impair EPC functions in DM, is BM alteration through oxidative stress; including BM neuropathy and microangiopathy. Likewise, studies also showed a negative association for EPC dysfunction with insulin, as well as with glucose level. Insulin has been demonstrated to have a detrimental effects to EPC in vitro and in vivo (Humper et al., 2005; 2008). On the other hand, Lev et al have explored that EPCs number is increase and its function is improved after intensive glycemic control program (Lev et al., 2013). Moreover, in vitro, hyperglycemia has been shown to impair EPC angiogenic feature (Krankel et al., 2005). Aicher et al, demonstrated NO as an essential factor for the mobilization of EPCs (Aicher et al., 2003). Since diabetes down-regulate the NO production, so it may subsequently affect EPC trafficking. Numerous studies have shown a significant reduction and dysfunction in EPCs associated with major diabetic micro and macro vascular complication. Fadini et al found that EPC is dysfunctional in type 2 diabetes and demonstrated a strong correlation between circulating EPC's depletion and diabetes peripheral vascular complication (Fadini et al., 2005; 2006). In diabetic rat model, Yoon et al found

that EPC's defect is one of the other features leading to myocardial VEGF's downregulation, which consider as an early event in diabetic cardiomyopathy (Yoon et al., 2005). Diabetic nephropathy is another diabetic complication related to EPC dysfunction. Diabetic nephropathy is associated with CD34+ cells decrease (Makino et al., 2009). Naruse et al have demonstrated an improvement in nerve vascular support and sciatic nerve conduction in diabetic rats after intramuscular injection of EPCs derived from human cord blood (Naruse et al., 2005). Also, neurotropic and angiogenic effect in diabetic mice neuropathy has established via BM derived EPCs (jeong et al., 2009).

Extracellular microvesicles (MVs) are submicrometric fragments, ranging from 0.1 to 1 μm , released from the cells in response to numerous conditions, including activation and apoptosis (Mause & Weber., 2010). Circulating microvesicles (cMVs) are the extracellular MVs released from the cells in the blood and on the vascular wall. cMVs are found in the blood of healthy donors and play important roles in intercellular communication. An elevation in cMV levels has been reported to correlate with several diseases such as, thrombotic diseases, diabetes, cardiovascular diseases, and renal failure (Burger et al., 2011; Nomura et al., 2009a & Chen et al., 2011). Furthermore, some studies showed that the level of cMVs could predict the severity of these diseases (Rautou et al., 2011). So, cMV levels could serve both as biomarkers and predictors.

All type of cells release MVs in highly controlled process for specific function. MVs express certain membrane proteins that reflect their parental cells. It also

carries their parent's cell components, such as membrane, cytoplasm, cytosolic proteins, and some nuclear component. More important, MVs carry RNAs, such as mRNA, microRNA, and non-coding (ncRNA) (Mause and Weber., 2010, Diehl et al., 2012).

The membrane proteins of MVs help to identify their origin by labeling the cell surface specific antigens. For examples, endothelial progenitor cells-derived microvesicles (EPC-MVs) can be recognized as CD34+ and vascular endothelial growth factor receptor-2 (VEGFR-2) + particles. Endothelial microvesicles (EMVs) can be characterized as CD144+ particles, and MVs from platelet (PMVs) can be labeled by CD42a or CD42b.

Accumulating evidence support the idea that MVs are able to transfer and deliver genetic information from their parent cells to the target cells. So, MVs represent a novel cell-cell communication avenue. MVs could also work as cytokines container and as mediators of immune response and inflammation (Lee et al., 2011). The function of MVs is complex and multifactorial, and might depend on their stimulator and origin. In previous study we establishes that preincubation of circulating MVs isolated from diabetic mice impairs the function of EPCs from control mice, whereas, MVs from healthy control does not have that effect on EPCs (Chen et al, 2011).

Several studies established a direct effect of MVs on ECs. Circulating MVs impair endothelial relaxation throughout; NO disturbing, inflammation and coagulation promotion, and apoptosis and angiogenesis alteration (Lovren & Verma., 2013).

On the opposite, Agouni et al established a restoration to impaired coronary relaxation in ischemic mouse model after MV administration (Aqouni et al., 2007). However, few evidence is found about the effect of MVs on EPCs.

Recently, MVs display as a therapeutic target. However, there are conflicted opinions regarding its positive or negative effect. EPC-MVs are found to be able to protect the kidney from ischemia-reperfusion injury (Cantaluppi et al., 2012). Also, EPC- MVs has been reported to have a positive effect on hypoxia/reoxygenation-induced endothelial dysfunction and apoptosis (Wang et al., 2012). The EPC-MVs also have been shown to protect cardiomyocyte from Ang II-induced hypertrophy and apoptosis (Gu et al., 2014). By contrast, EMVs released under high glucose treatment to ECs have been reported to cause vascular inflammation in atherosclerotic mouse (Jansen et al., 2013). Similarly, our laboratory has demonstrated a negative effect of diabetic MVs on EPC functions. As mentioned, preincubation of circulating MVs isolated from diabetic mice impairs the function of EPCs (Chen et al, 2011). In this study, we proposed to investigate both positive and negative effect of MVs depending on their origins in human study. We have explored whether circulating MVs isolated from the healthy subjects (ch-MVs), could improve the function of diabetic EPCs (d-EPCs). On the other hand, whether diabetic MVs isolated from diabetic patients (cd-MVs), could impair the function of healthy EPCs (h-EPCs) in vitro. Furthermore, we have compared the levels of EPCs and EPC-MV in diabetic patients and healthy subjects.

2. HYPOTHESIS AND SPECIFIC AIMS

2.1 Hypothesis

The hypothesis is that Levels of circulating microvesicles modulate endothelial progenitor cell functions in T2DM by changing their migration ability, apoptosis rate, and ROS production. Circulating microvesicles from healthy subjects could improve the function of diabetic EPCs; conversely, circulating microvesicles from diabetic patients could have the ability to impair the function of EPCs from healthy subjects.

2.2. Specific aims

Three specific aims associated with this study.

1. Determine the levels of EPC and EPC-MV in diabetic patients and healthy subjects.
2. Compare the migration ability, apoptosis rate and ROS production in diabetic and healthy subjects.
3. Determine whether circulating MVs isolated from the blood sample of healthy subjects (ch-MVs) could improve the function of diabetic EPCs (d-EPCs) and conversely whether circulating MVs isolated from diabetic patients (cd-MVs) could impair the function of healthy EPCs (h-EPCs)

3. MATERIAL AND METHODS

3.1. Study Population

A total of sixteen subjects were included in the study between 25-55 yrs. seven with type 2 diabetes mellitus (T2DM), and nine were healthy control. Patients were recruited from the endocrinology department of Dr. Thomas Koroscil at the Wright State Physicians Internal Medicine building in Dayton, Ohio. The protocol was approved by the wright state university institutional review board (IRB). Each patient signed an informed consent after thorough explanation.

Exclusion criteria: pregnancy, patient with history of myocardial infarction, stroke, unstable angina, renal or heart failure, cancer, chronic alcohol abuse, hypertension requiring more than 3 antihypertensive medication, use of any multi-vitamin supplement, and patients with proliferative diabetic retinopathy or nephropathy or painful diabetic neuropathy requiring chronic narcotic analgesic therapy. The members of the Department of Pharmacology and Toxicology at Wright State University Boonshoft school of Medicine, Dayton, Ohio, performed the study.

3.2 Blood Sampling

After an 8 hour fast, blood samples were drawn. Approximately 60 ml of blood was obtained through a 20-gauge butterfly needle, which was inserted into a

forearm vein. Sample was collected in the presence of 3.13% citrate buffer (8ml) from all subject. The blood samples were directly transferred to the lab and processed within 1 hour. Samples were also tested for glucose, HbA1c, and lipid profile.

3.3. Study Design

EPCs and circulating MVs were isolated from each blood sample, then a cross study was achieved as described in figure 2. Circulating EPCs isolated from diabetic patients (d-EPCs) cultured for 10-17 days were incubated with fresh MVs isolated from healthy subjects (ch-MVs). On the other hand, the EPCs isolated from healthy subjects (h-EPCs) were incubated with diabetic MVs (cd-MVs). EPCs were cultured in 24-well plates and co-incubated with 100 µg/ ml of MVs. After 24-hour incubation, functional assay (migration), apoptosis and ROS production were performed. The levels of EPCs and circulating MVs were also measured using the flow cytometry at the day of MV isolation.

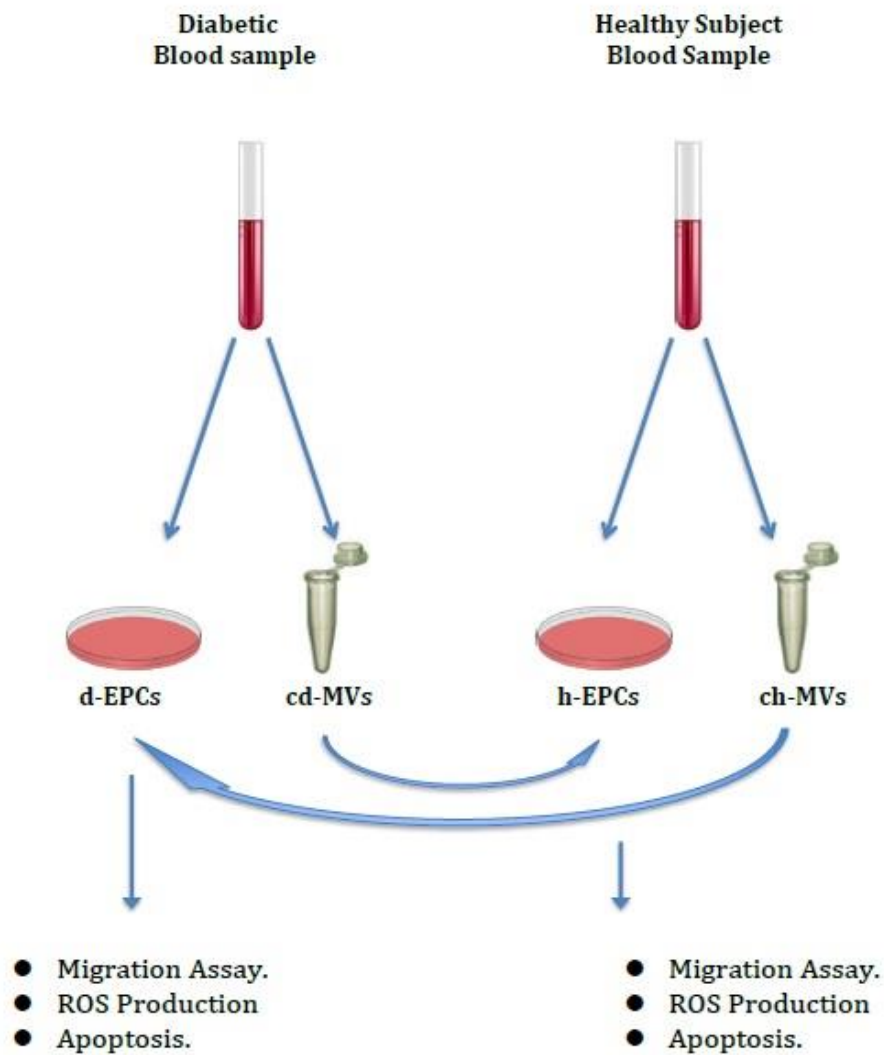


Figure 1. Diagram of the study design. A cross study was achieved. d-EPCs was incubated with ch-MVs, and h-EPCs was incubated with cd-MVs. Migration assay, ROS production, and apoptosis were measured in both groups.

3.4. Circulating EPC Isolation

Peripheral mononuclear cells (MNCs) were used for EPC culture. Briefly, from peripheral blood, using gradient density centrifugation at 400g for 35 minutes at 4°C, the MNCs in the interface layer were isolated and collected to two new tubes. The MNCs in the first tube, which was isolated from about 5 ml blood, was resuspended with 100 µl Phosphate-Buffered Saline (PBS) for the level of EPCs analysis using flow cytometry. The second was counted and cultured on 24 well plate at a density of 5×10^6 cells/well, and then grown in Endothelial cell Basal Medium-2 (EBM-2) supplemented with 10% FBS containing EPC growth cytokine cocktail (Lonza, Walkersville, MD). After 3 days, non-adherent cells were removed by washing with phosphate buffered saline (PBS). Thereafter, culture medium was changed every two days, and cells were cultured continuously for 10 to 17 days for MV co-incubation study. The upper layer of gradient density centrifugation (plasma) was collected for MV isolation.

3.5. Circulating MV Isolation

After gradient density centrifuge for isolation of MNCs, the upper layer, which is the platelet rich plasma, was collected and centrifuged (1500g, 15 min) at 10°C to get platelet-free plasma (PFP). PFP was then divided to numerous tubes 1 ml/tube, then centrifuged at 30,000g for 30 min, at 10°C to pellet circulating MVs. The MV pellet was resuspended in 100 µl PBS for each tube. Some (100 µg/ ml) were used to co-incubate with EPCs and some were used for flow cytometry analysis on the same day.

3.6. Flow cytometry analysis of the level of circulating EPCs and EPC-MVs.

EPCs isolated from 5 ml of blood, and MVs isolated from 1ml of PFP were both resuspended with 200 μ l PBS and divided to two tubes, respectively. One tube of each group was incubated with anti-human FITC-conjugated CD34 (eBioscience, San Diego, CA) and anti-human PI-conjugated VEGFR-2 (BD Biosciences, San Diego, CA). The second tube was incubated with FITC- labelled IgG1 (eBioscience, San Diego, CA) and PI- labelled IgG1 (BD Biosciences, San Diego, CA), as the isotopic control for color compensation. After incubation in the dark for 30 min, the percentages of EPCs and EPC-MVs were measured using flow cytometry (Accuri C6 Flow cytometry, San Jose, USA) as double stained with CD34 and VEGFR-2.

3.7. Dose- response Study of cd-MVs on h-EPCs Apoptosis Rate

To determine the effective dose of cd-MVs that increase h-EPCs apoptosis rate, h-EPCs were treated with different doses of cd-MVs (25, 50, 75, 100 μ g/ml). After 24h h-EPCs were trypsinized and the apoptosis rate analyzed using flow cytometer. Bradford assay (Bio-Rad, Hercules, CA) was used to quantify the protein concentration of cd-MVs.

3.8. Incubation of EPCs with Isolated Circulating MVs

EPCs were prepared, and circulating MVs were isolated as described above. MVs freshly isolated from 1 ml plasma were resuspended with 100 μ l EPC culture medium. Then EPCs (10-17 day old) were incubated with culture medium (Vehicle) or 100 μ g/ ml of MVs for 24 hours at 37°C before function assays. The

dose were determined by dose-response study. The co incubation time determined by previous animal study.

3.9. Migration Assay

EPC migration function was evaluated using the Boyden chamber system (Millipore, Temecula, CA). The Boyden Chamber system uses a porous membrane provides an interface between two chambers. After around 10 days of EPC culture, EPCs (2×10^4 cells) were detached with trypsin and placed into the upper compartment of the Boyden chamber, with the porous membrane at the bottom, and EPC serum free medium in the lower compartment.

Chemoattractants were already placed by the manufacture in the lower chamber, so the EPCs in the upper chamber should migrate toward the chemoattractant passing the porous membrane and moving to the other chamber. Circulating microvesicles solution 100 µg/ ml were added to the upper chamber of experimental ones. After 24 hour co-incubation, the EPCs that migrated across the membrane were stained and solubilized with extraction buffer, transferred to microplate, then read with Optical Density (OD) at a wavelength of 535 nm using Fusion Packard Reader, where scattered light passes through a cell suspension, greater scatter indicates more cells present.

3.10. Flow Cytometry Analysis of Cell Apoptosis

The apoptotic rate of h-EPCs and d-EPCs after co-incubation was assessed using apoptosis assay kit (BD Biosciences, San Diego, CA). After co-incubation, the cell culture medium was removed and the cells were washed with PBS.

Cultured EPCs were detached by using 0.5% trypsin for 3-5 min at 37°C. Once the detachment happened, the complete media was added into the well to stop the reaction of trypsin and pipetted for several time to obtain single cell suspension. The cell suspension then centrifuged at 400g for 6 min to pellet the cells. After discarding the supernatant, pelleted cells were resuspended with 100 µl 1x annexin-binding buffer, and incubated with 5 µl FITC- conjugated Annexin V and 5 µl Propidium iodide(PI) for 30 min at room temperature in dark. The labelled cells then analyzed by flow cytometry. The EPCs stained with both annexin V and PI were considered to be late apoptosis EPCs (the upper right quadrant), and the cells stained only with annexin V were considered to be early apoptosis (the lower right quadrant). The apoptosis percentage counted as the total of both early and late apoptosis percentages. EPCs did not label with any antibody were used as control.

3.11. Intracellular ROS Production

Intracellular ROS production was determined by Dihydroethidium (DHE; Sigma-Aldrich, St. Louis, MO) staining. Briefly, cells were incubated with the DHE working solution (1µM) at 37° for 2 hours in dark. Then the cells were observed under an inverted microscope, red fluorescence was visualized by a fluorescence microscopy. After that the cells were washed with PBS twice, detached by using 0.5% trypsin for 3-5 min at 37°C. Once the detachment happened, the complete media was added into the well to stop the reaction of trypsin and pipetted for several time to obtain single cell suspension. The cell suspension then centrifuged at 400g for 6 min to pellet the cells. After discarding the supernatant,

pelleted cells were resuspended in 100ul PBS. Then the fluorescence intensity of cells was analyzed using a flow cytometer.

3.12. Statistical Analysis

All Data was expressed as mean \pm SD, and were analyzed using student's t-test. Statistica-Academic software Version 12 was used. For all tests, Values of $P < 0.05$ were considered statistically significant.

4. RESULTS

4.1. Study Participants

A total of sixteen participants were recruited (nine control, seven T2DM) as shown in table 1. The average of patient's age was 29 ± 4 yrs for control and 43 ± 8 yrs for diabetic patients. There was a significant difference on the age between two groups ($P < 0.05$). The HbA1c and glucose level was significantly higher in diabetic patients when compared to healthy control group (glucose: 85.4 ± 14.4 mg/dl and 155.5 ± 84.5 mg/dl, control vs. diabetes, $P < 0.05$; HbA1c: 5.4 ± 0.2 and 7.5 ± 1.7 % of total HGB, control vs. diabetes, $P < 0.05$). Also the level of triglycerides and very low density lipoprotein (VLDL) were significantly higher in diabetic patients (triglycerides: 99.77 ± 36.7 mg/dl and 157.28 ± 66.2 mg/dl, control vs. diabetes, $P < 0.05$; VLDL: 19.88 ± 7.3 mg/dl and 31.42 ± 13.4 mg/dl, control vs. diabetes, $P < 0.05$). However the level of total cholesterol has no significant difference between diabetic and control groups (176.88 ± 33.7 mg/dl and 160.7 ± 22.7 mg/dl, control vs. diabetes, $P > 0.05$). There were also no significant different between diabetic and control groups on the level of high density lipoprotein (HDL) and low density lipoprotein (LDL) (HDL: 50 ± 9.9 mg/dl and 40.7 ± 13.3 mg/dl, control vs. diabetes, $P > 0.05$; LDL: 107 ± 29.2 mg/dl and 88.5 ± 22.3 mg/dl, control vs. diabetes, $P > 0.05$).

Clinical Characteristics of the Studied Population

Characteristics	Healthy Subjects	Patients with T2DM
Total (n=16)	9	7
Average Age (years)	29±4	43±8*
Sex Ratio (male: female)	(7:2)	(6:1)
Fasting Blood Glucose (MG/DL)	85.4±14.4	155.5±84.5*
HbA1c (% Total HGB)	5.4±0.2	7.5±1*
Lipid Profile (MG/DL)		
Total Cholesterol	176.8±36.7	160.7±22.7
Triglycerides.	99.7±36.7	157.2±66.2*
HDL	50±9.9	40.7±13.3
LDL	107±29.1	88.5±22.2
VLDL	19.8±7.3	31.4±13.4*

Table 1. Clinical characteristic of studied population. Abbreviations: HbA1c, glycated hemoglobin; HDL, High Density Lipoproteins; LDL, Low Density Lipoproteins; VLDL, Very Low Density Lipoproteins. * Means significant changes $P<0.05$.

4.2. The Level of Circulating EPCs Decreased and EPC-MVs Increased in Diabetic Patients.

Using flow cytometry analysis, our finding showed that the percentages and the numbers of circulating EPCs (CD34⁺and VEGFR-2⁺) were significantly decreased in diabetic patient compared to control ($3.03 \pm 0.41\%$ and $0.76 \pm 1\%$, or 3119.8 ± 2414.6 and 421.5 ± 364.9 cells/ 1ml of blood. control vs. diabetes, $P < 0.01$; figure 2A, 2B and 2C.) By contrast, the percentages and the numbers of circulating EPC-MVs (CD34⁺and VEGFR-2⁺) were significantly increased in diabetic patient when compared to control ($1.06 \pm 0.58\%$ and $4.3 \pm 1.34\%$ or 2636.8 ± 1914.1 and 8309.2 ± 4122.05 MVs/1ml of plasma. control vs. diabetes, $P < 0.01$; figure 3A, 3B and 3C).

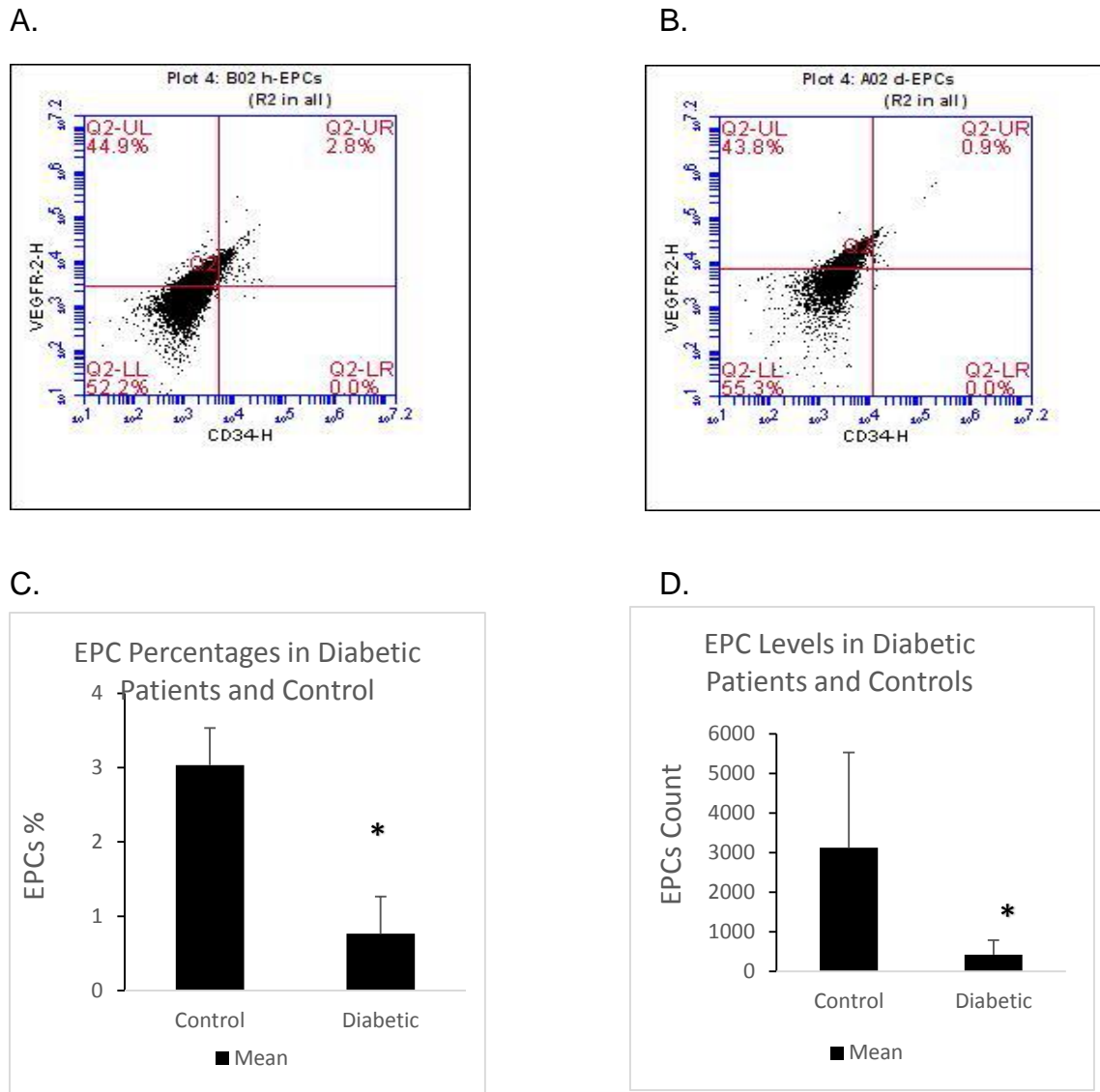


Figure 2. The level of circulating EPCs in diabetic patients and control. The level of circulating EPCs decreased in diabetic patients compared to control. A, B, Representative flow cytometry plots showing the level of circulating EPCs in control (A), and diabetic patient groups (B). C and D Bar charts summarizing the percentage and level of circulating EPCs respectively, in each group. EPCs were significantly reduced in diabetic patients compared to controls. *P < 0.05. Data are expressed as mean \pm SD, n=9 for control group, and 7 for diabetic patients.

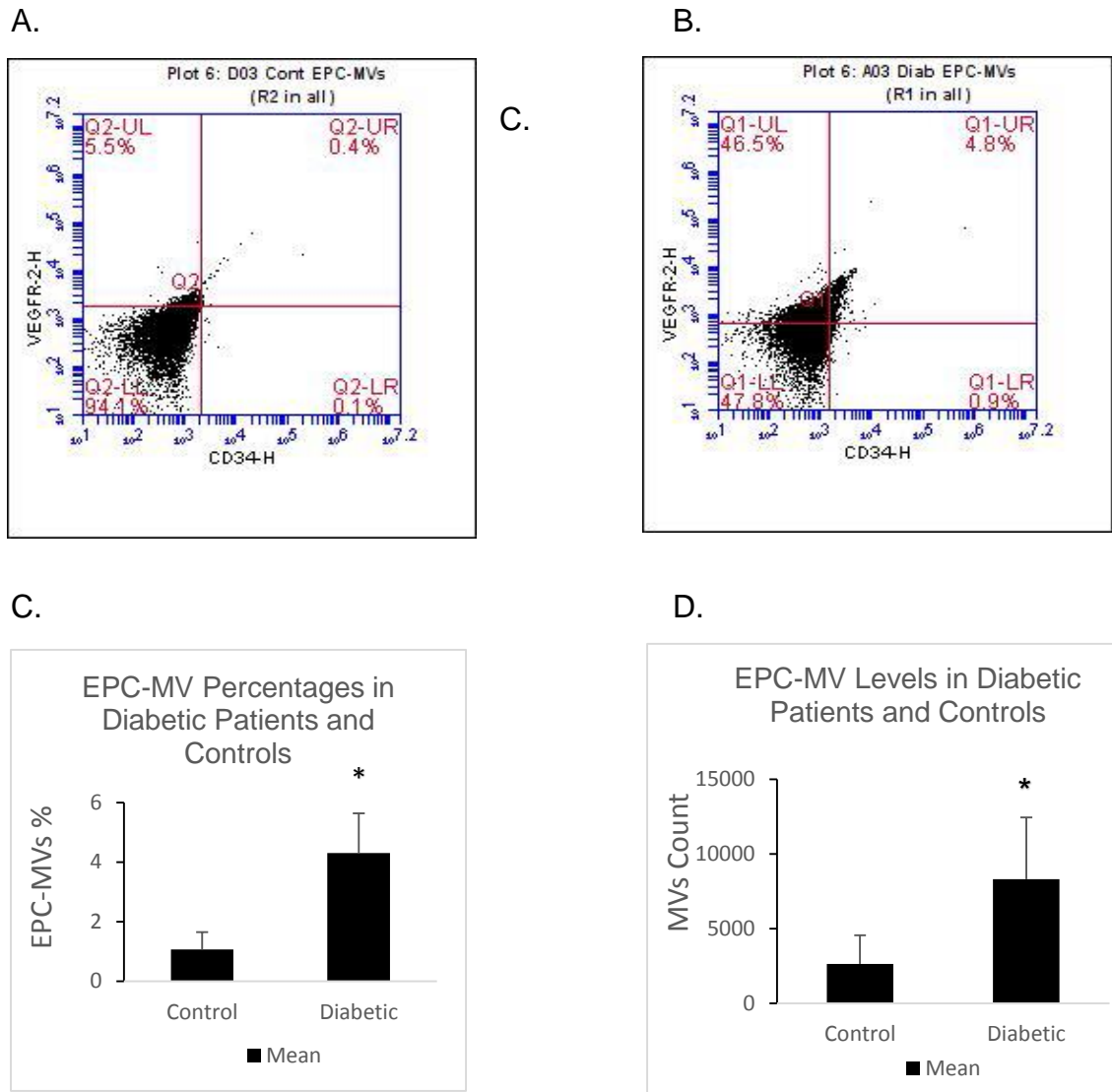


Figure 3. The level of circulating EPC-MVs in diabetic patients and controls. The level of circulating EPC-MVs elevated in diabetic patients compared to control. A, B, Representative flow cytometry plot showing the level of circulating EPC-MVs in control (A), and diabetic patient groups (B). C and D Bar charts summarizing the percentage and the count of circulating EPC-MVs respectively, in each group. EPC-MVs were significantly elevated in diabetic patients compared to controls. *P < 0.05. Data are expressed as mean \pm SD. n=9 for control group, and 7 for diabetic.

4.3. The Migration Ability of EPCs Decreased in Diabetic Patients.

To determine the difference of the migration ability in control group and diabetic group, Boyden Chamber Kit was used to detect the migration ability of EPCs in both groups. Results showed that the migration ability of EPCs from control group was significantly higher than the ones from diabetic patients. (0.229 ± 0.007 and 0.180 ± 0.025 , control vs. diabetes, $P < 0.05$; figure 3).

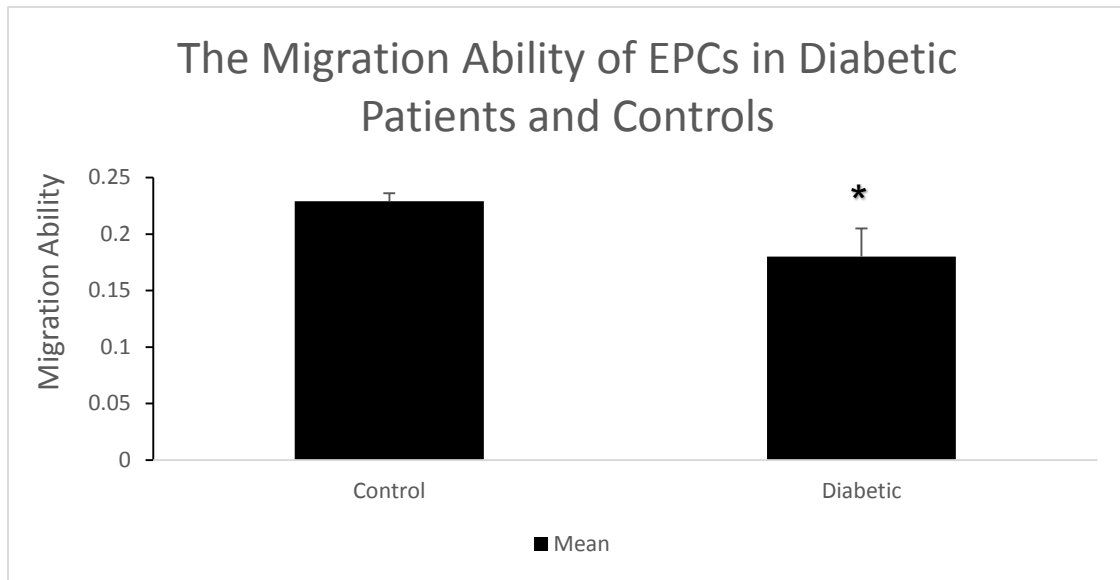


Figure 4. The migration ability of EPCs in diabetic patients and control. The migration ability of EPCs was significantly reduced in diabetic patients compared to control. * $P < 0.01$. Data are expressed as mean \pm SD, $n=3$.

4.4. The Apoptosis Rate of Cultured EPC Increased in Diabetic Patients.

To establish the difference in the apoptosis rate in control group and diabetic group, flow cytometry analysis was used. Results showed that apoptosis rate of d-EPCs was significantly higher than that of h-EPCs. (24.15 ± 10.15 and 70.80 ± 8.05 %, control vs. diabetic, $P < 0.01$. Figure 5).

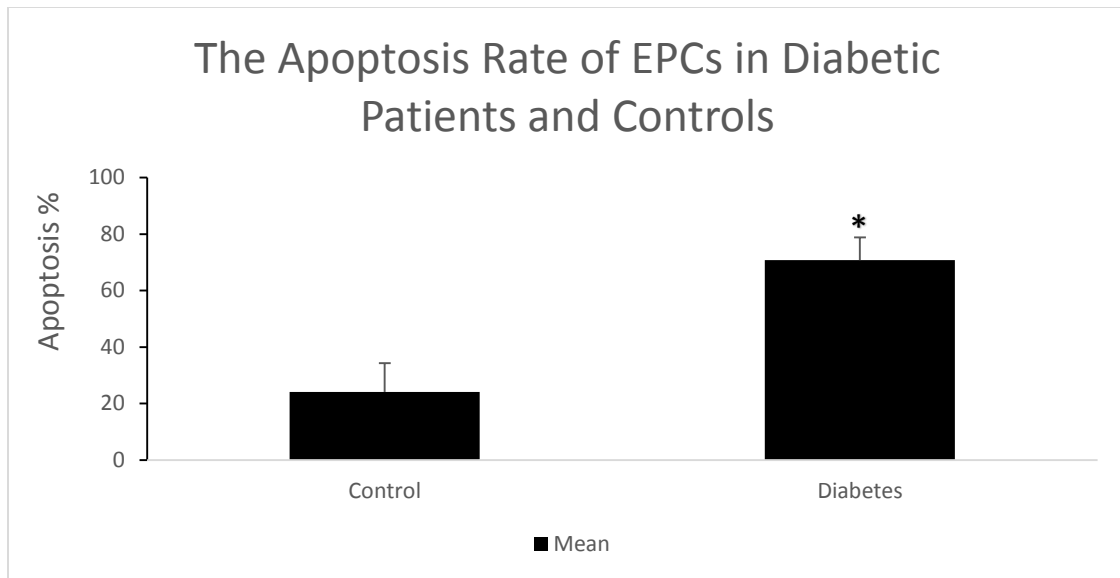


Figure 5. The apoptosis rate of EPCs in diabetic patients and controls. The apoptosis rate of EPCs was significantly increased in diabetic patients compared to control. * $P < 0.01$. Data are expressed as mean \pm SD, $n=3$.

4.5. The ROS Production of Cultured EPCs is elevated in Diabetic Patients

To evaluate the ROS production levels of EPCs in diabetic and healthy control, the DHE staining was used and the levels of ROS were checked under microscope and measured using a flow cytometer analysis. As shown in Figure 6 A, B, C, and D there were 2 populations, high ROS production population and low ROS production population. The percentage of ROS production in the diabetic patients was significantly higher in the high ROS production population compared to the control (46.05 ± 9.95 and 74.27 ± 5.32 , control vs. diabetic, $P < 0.05$). No significant change in the low ROS production population.

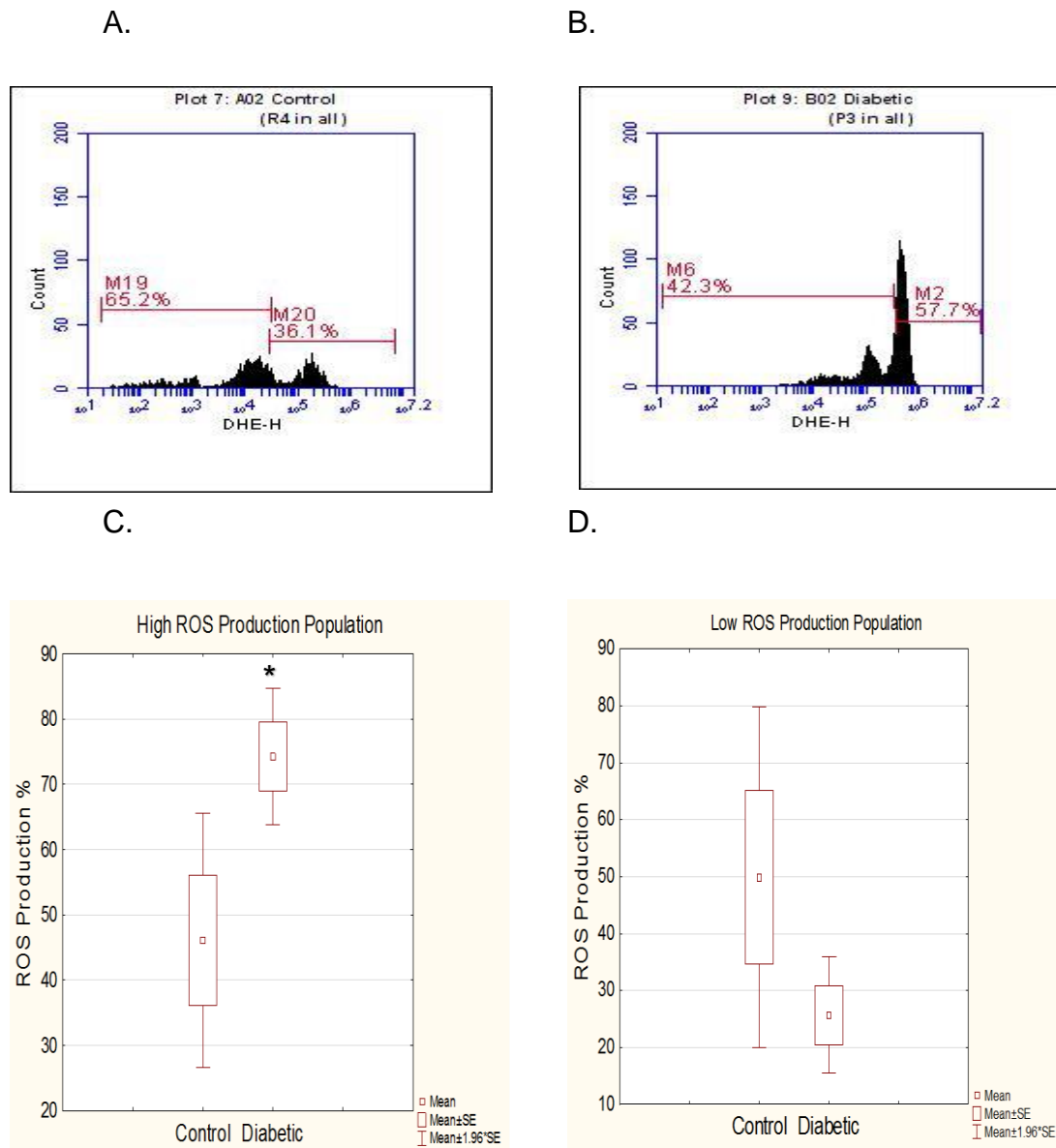


Figure 6. The ROS production of EPCs in diabetic patients and control. The ROS production of EPCs, in the high ROS production population was significantly increased in diabetic patients compared to control. (A). representative Flow cytometry plot for control group. B. representative Flow cytometry plot for diabetic group. C and D. Summarized Data. Data are expressed as mean \pm SE \pm SD, $n=4$ for diabetic and 2 for control * $P < 0.05$.

4.6. The Effect of cd-MV Different Doses on the Apoptosis Rate of h-EPCs.

To determine the effective dose of cd-MVs, h-EPCs were treated with different doses of cd-MVs solution (25, 50, 75, 100 µg/ml) for 24h, as seen in figure 7, cd-MVs dose dependently increase the cell apoptosis rate. Depend on these data, we select 100 µg/ ml for the following co- incubation experiments.

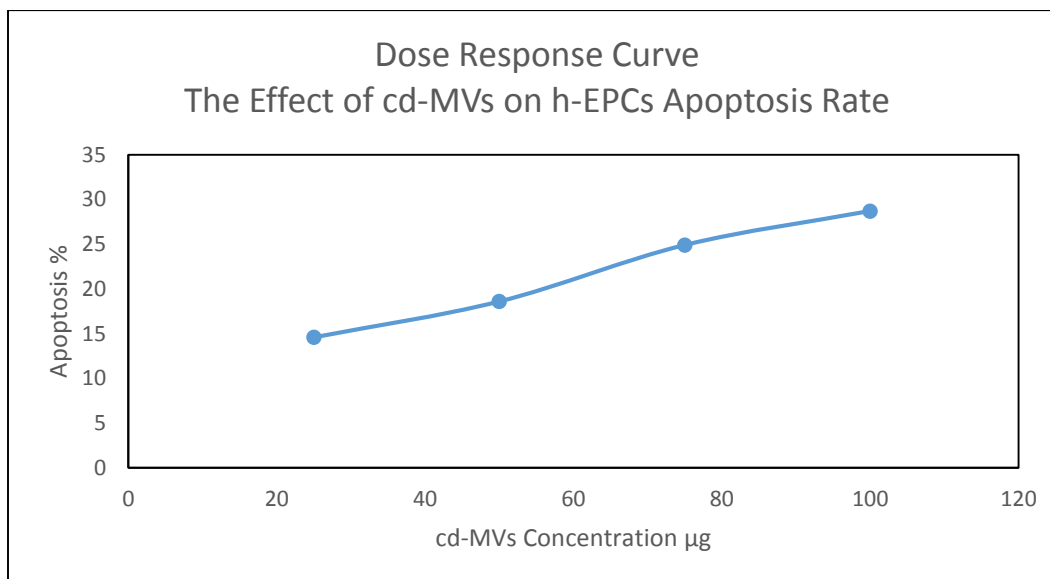


Figure.7.Dose- response study of cd-MVs on h-EPCs apoptosis rate.

Representative scatter plot showing dose dependent effect of cd-MVs on h-EPCs apoptosis rate.

4.7. The ch-MVs Enhanced the Migration Ability of d-EPCs

To establish the effect of ch-MVs on d-EPCs mobilization, d-EPCs were incubated with 36 μ l/ml of ch-MVs or with vehicle (culture media) in serum free EBM-2 medium for 24 hours. Boyden Chamber Kit was used to detect the migration ability of EPCs. Results showed that incubation with ch-MVs significantly improved the migration ability of d-EPCs, (0.18 ± 0.02 and 0.24 ± 0.3 , vehicle vs. ch-MVs, $P < 0.05$; figure 7).

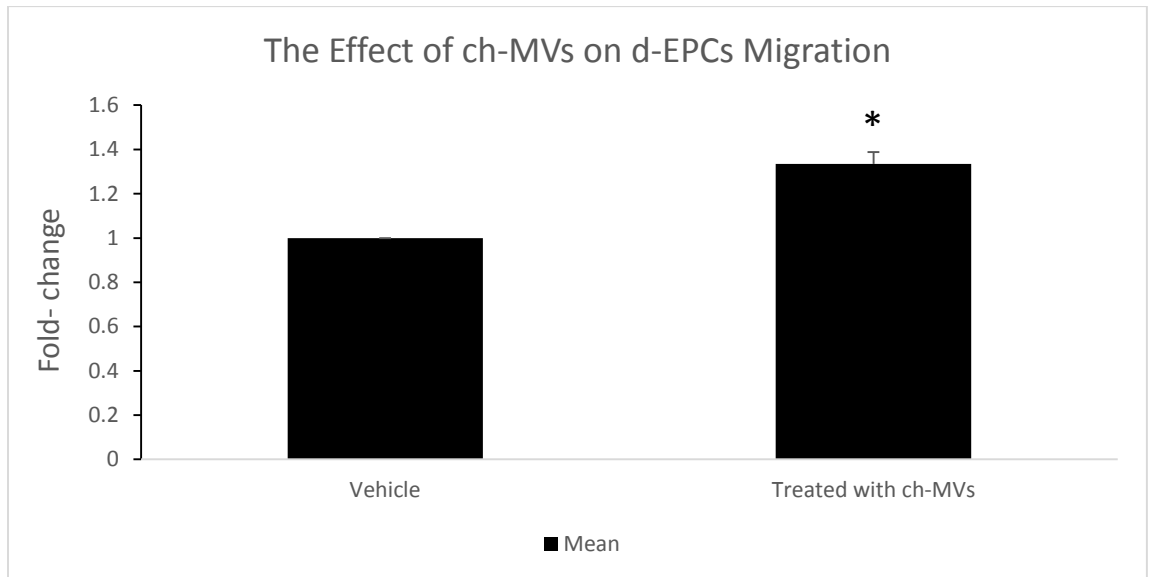
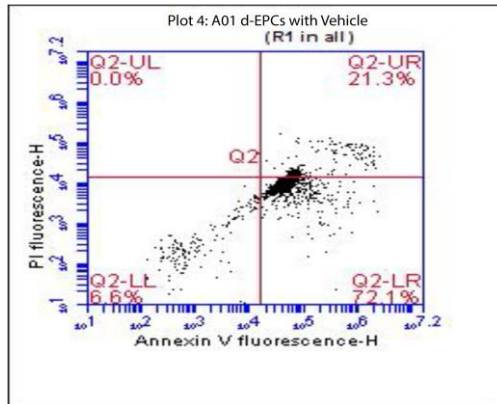


Figure.8.The effect of ch-MVs on d-EPCs migration ability. Preincubation of d-EPCs with ch-MVs (36ul/ml; 24 h) were significantly increased the migration ability of EPCs. Summarized data for the migration. n=3. * P <0.05. Data are expressed as mean \pm SD.

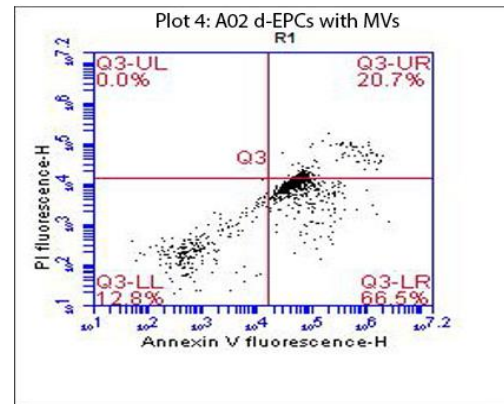
4.8. The ch-MVs Decreased the Apoptosis Rate of d-EPCs

According to flow cytometry analysis, 24 hours incubation of d-EPCs with 36 μ l/ml ch-MV significantly decreased the rate of apoptosis in d-EPCs when compared to vehicle, Figure 8A, 8B, and 8C. (72.1 ± 3.4 and $57.3 \pm 9.7\%$. vehicle vs. ch-MVs, $P < 0.05$).

A.



B.



C.

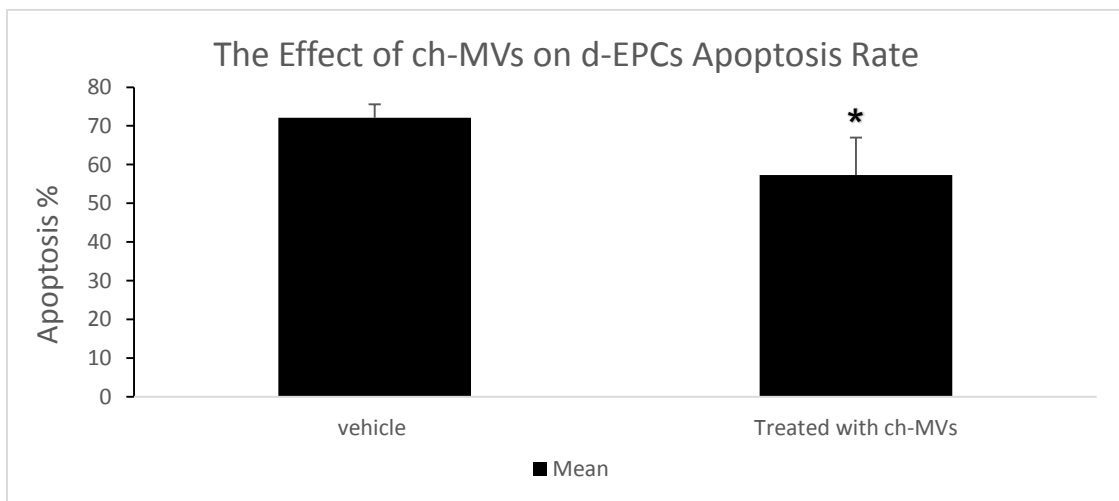


Figure 9. The effect of ch-MVs on d-EPCs apoptosis rate. Preincubation of d-EPCs with ch-MVs (36ul/ml; 24 h) were significantly decreased the apoptosis rate of d-EPCs compare to vehicle. (A). Representative flow cytometry plot showing the d-EPCs in vehicle group. (B). Representative flow cytometry plot showing the d-EPCs in MV co-incubation group. (C). Bar chart summarizing the apoptosis rate in d-EPCs in vehicle and MV treatment group, n=4. * P <0.05. Data are expressed as mean \pm SD.

4.9. The Effects of ch-MVs on the ROS production of d-EPCs

To evaluate the effect of ch-MVs on the levels of ROS production in d-EPCs, the d-EPCs were co-incubated with ch-MVs (100µg/ml) for 24 hrs. The levels of ROS were checked under microscope and analyzed using flow cytometry method. As shown in Figure 9A there were 2 populations, high ROS production population and low ROS production population. The percentage of ROS production in the co-incubation group was significantly decreased in the high ROS production population compared to the vehicle group (74.3 ± 10.6 and 61.5 ± 14.5 , vehicle vs. ch-MVs, $P < 0.05$). In the contrast, the percentage of ROS production was significantly increased in the low ROS production population when compared to the vehicle group (25.8 ± 10.4 and 37.2 ± 12.8 , vehicle vs. ch-MVs, $P < 0.05$). These data suggest that ch-MV could decrease the ROS production of db-EPCs by decrease the high ROS production population and increase the low ROS production population. (Figure 9B, $P < 0.05$ $n=4$). Figure 9C showing the significant change in high population, and 9D showing the significant change in low population.

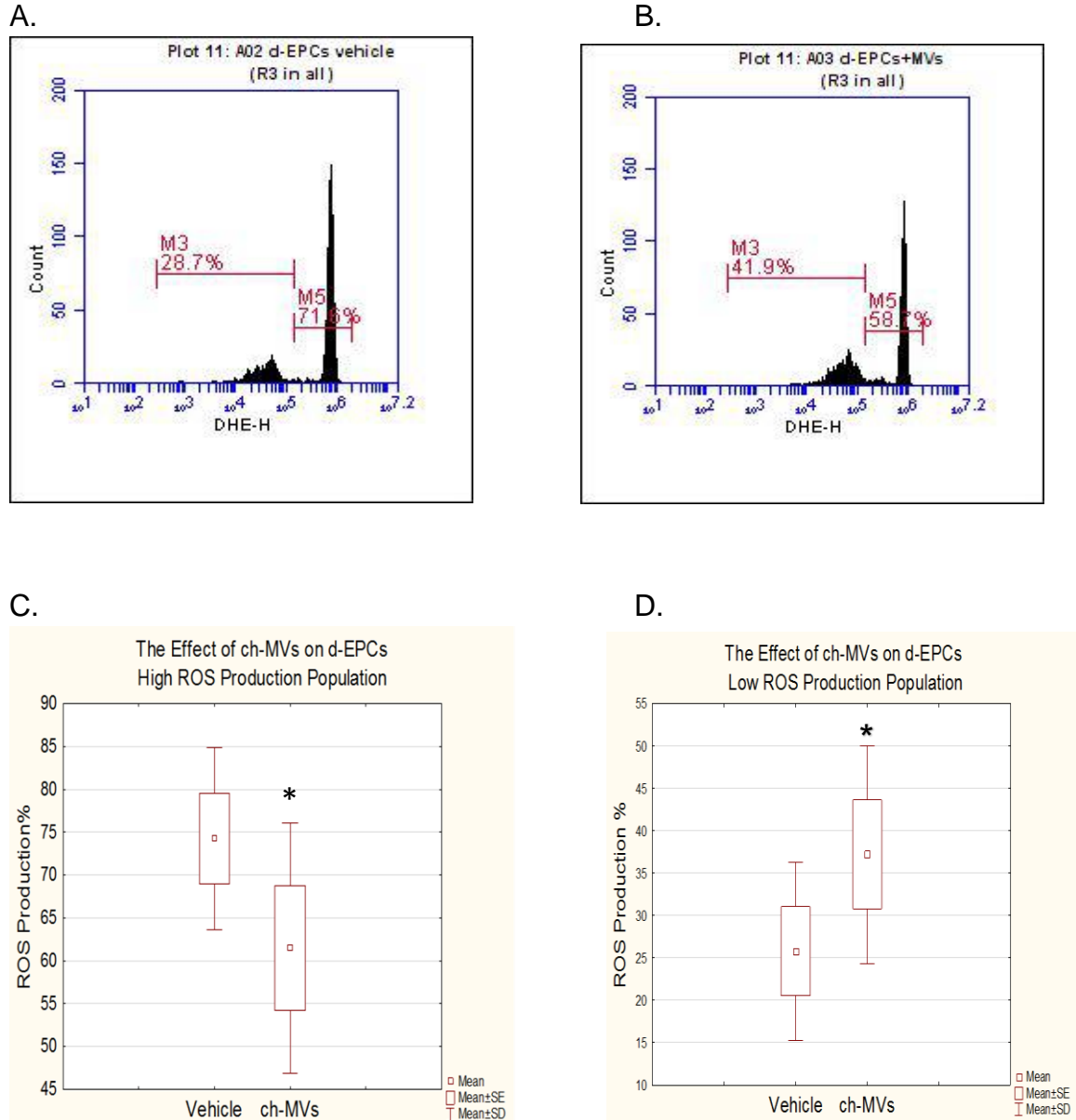


Figure 10. The effect of ch-MVs on d-EPCs ROS production. Preincubation of d-EPCs with ch-MVs (100ug/ml; 24 h) were significantly decreased the ROS production in the high ROS production population of d-EPCs, and increase it in the low ROS production population, compare to vehicle. (A). Representative flow cytometry plot for vehicle group. B. Representative flow cytometry plot for co-incubation group. C and D. Summarized Data, n=4. * P <0.05. Data are expressed as mean \pm SE \pm SD.

4.10. The effect of cd-MVs on the Migration Ability of h-EPCs.

As before, to establish the effect of cd-MVs on h-EPCs mobilization, h-EPCs were incubated with 36 μ l/ml of cd-MVs or with vehicle in serum free EBM-2 medium, for 24 hours using Boyden Chamber Kit. Results are shown in figure 10, after incubation with cd-MVs, the migration ability of h-EPCs has no significant change when compared to vehicle group. (0.22 ± 0.007 and $0.19 \pm 0.017\%$, vehicle vs. cd-MVs, $P > 0.05$).

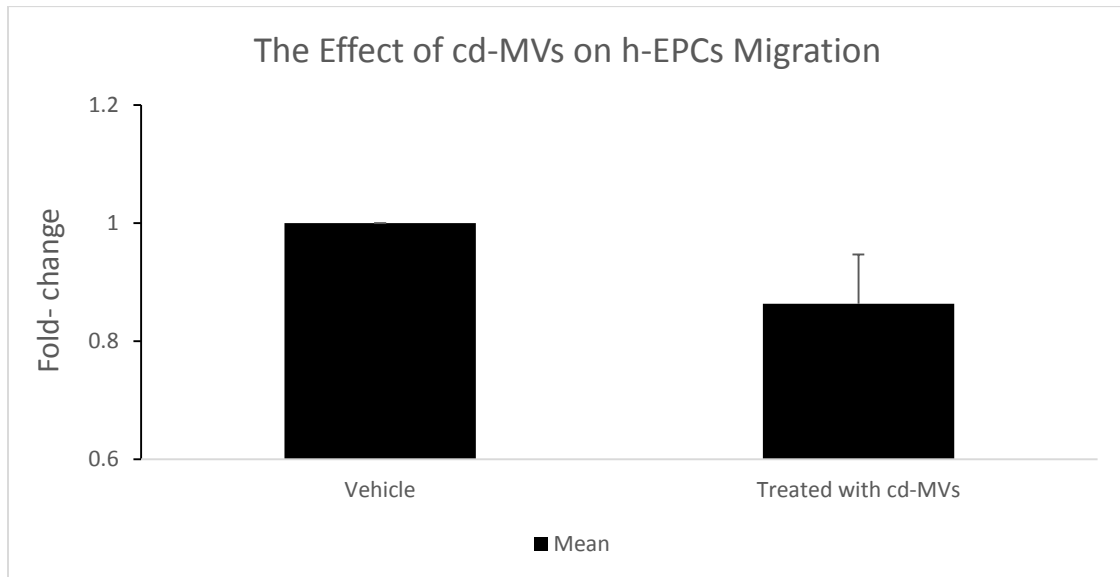


Figure.11.The effect of cd-MVs on h-EPCs migration. Preincubation of h-EPCs with cd-MVs (100ug/ml; 24 h) not significantly decrease the migration ability of h-EPCs. Summarized data for the migration. n=3. $P > 0.05$. Data are expressed as mean \pm SD. (fold of changes).

4.11. The cd-MVs Increased the Apoptosis Rate of h-EPCs.

According to flow cytometry analysis, 24 hours incubation of h-EPCs with 100 μ l/ml cd-MV significantly increased the apoptotic rate when compared to vehicle. (24.05 ± 9.9 and 42.7 ± 3.4 vehicle vs. with cd- MVs, $P < 0.05$; Figure 11 A, B and C).

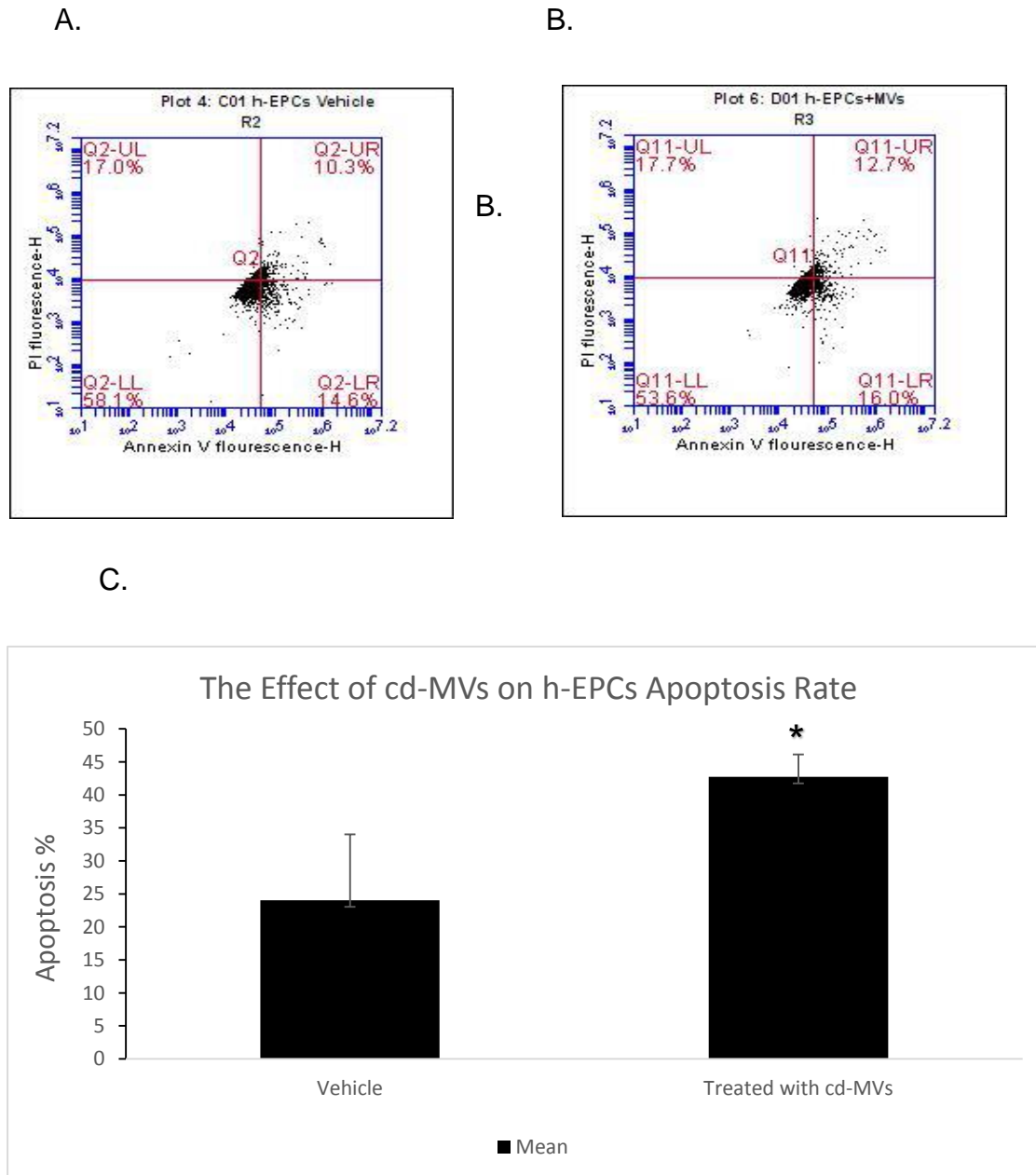


Figure 12. The effect of cd-MVs on h-EPCs apoptosis rate. Preincubation of h-EPCs with cd-MVs (36ul/ml; 24 h) significantly increases the apoptosis rate of h-EPCs compare to vehicle. (A). Representative flow cytometry plot showing the h-EPCs in vehicle group. (B). Representative flow cytometry plot showing h-EPCs treated with cd-MVs. (C). Bar chart summarizing the apoptotic rate in h-EPCs in vehicle group vs. in cd-MVs group, n=4. * P <0.05. Data are expressed as mean \pm SD.

4.12. The Effects of cd-MVs on the ROS production of h-EPCs

As previous, to evaluate the effect of cd-MVs on the levels of ROS production of h-EPCs, the h-EPCs were co-incubated with cd-MVs (100µg/ml) for 24 hrs. The levels of ROS were determined by DHE staining and were checked under microscope and analyzed using flow cytometry method. As shown in Figure 12, again there were 2 populations, high ROS production population and low ROS production population. The percentage of ROS production in the co-incubation group was significantly increased in the high ROS production population compared to the vehicle group (43.65 ± 18 and 68.4 ± 15 , vehicle vs. cd- MVs, $P < 0.05$). In the contrast, the percentage of ROS production was not significantly decreased in the low ROS production population when compared to vehicle group (51.2 ± 23.4 and 30.6 ± 14.4 , vehicle vs. with cd-MVs, $P > 0.05$; Figure 12, n=2)

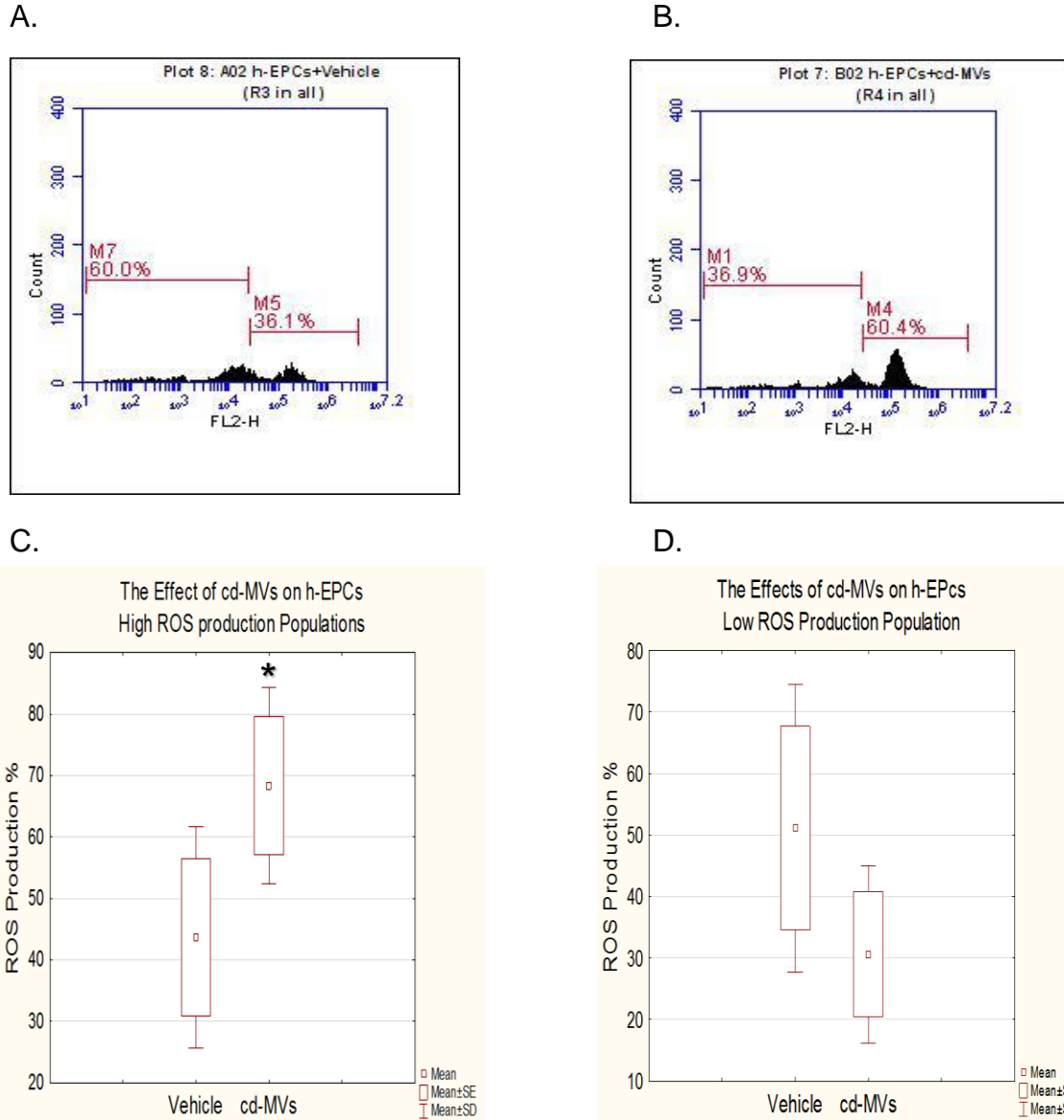


Figure 13. The effect of cd-MVs on h-EPCs ROS production. Preincubation of h-EPCs with cd-MVs (100ug/ml; 24 h) significantly increases the ROS production in the high ROS production population of h-EPCs. There was non-significant decrease in the low ROS production population, compare to vehicle. (A). Representative flow cytometry plot for vehicle group. B. Representative flow cytometry plot for co-incubation group. C and D. Summarized Data, n=2. * $P < 0.05$ for c only. Data are expressed as mean \pm SE \pm SD.

5. DISCUSSION.

There are four new findings for this study. First, the level of CD34⁺VEGFR⁺ MVs, which known as EPC-MVs, is elevated in diabetic patient compared to healthy subject and the level of EPCs is decreased. Second; the migration ability of d-EPCs were decreased, and the apoptosis rate and ROS production were increased in d-EPCs. Third; ch-MVs derived from healthy subject can improve the migration function of EPCs from diabetic patients and are able to decrease their apoptotic rate. In contract, cd-MVs derived from diabetic patients increase EPC apoptosis without affect the migration ability. Fourth; MV treatment can modulate ROS production of EPCs.

Diabetic environment including hyperglycemia, hyperlipidemia, and insulin resistance in type 2 diabetes mellitus patient, trigger and increase the oxidative stress, which targeting ECs and causing endothelial dysfunction leads to various cardiovascular complications (Tousoulis et al., 2012). Endothelium dysfunction is a key initiator for vascular injury, which is characterized by a pro-inflammatory, pro-coagulative, and pro-vasoconstrictive endothelial constitution. (Burger et al., 2012). EPCs play an important role in maintaining endothelium hemostasis, integrity and regenerate injured cells.

There are many studies have reported that the number and function of EPCs in diabetic patients and mice are decreased and impaired (Krankel et al., 2005; Fadini et al; 2006, Makino et al., 2009 & Tramontano et al., 2010). However until now the study determining the exact mechanism is lacking. Therefore, knowing EPC number and better understand of their function, as well as the mechanism by which diabetes deplete EPCs and impair their function, are very important for preventing and controlling cardiovascular complications in diabetes. Our previous study on mice find the decrease in circulating EPCs (Chen et al., 2011). To deepen previous finding, in the present study we evaluate the levels of circulating EPCs in diabetic patients. Our data showed that the level of circulating EPCs is reduced in diabetic patients compare to the healthy subjects. Moreover, we found that the migration ability of d-EPCs were decreased too, compare to an increase in the apoptosis rate and ROS production.

Improvement of EPC level and function could be very helpful for preventing and treating diabetic-related cardiovascular diseases. Previous studies are focusing on genetic transfection and chemokine stimulation to improve EPC function (Song et al., 2009; Kuliszewski et al., 2011 & Mohle et al., 1998). Our study group, in previous study, has demonstrated that over-expression of CXCR4 on EPCs could improve the EPC function which are impaired in db/db mice (Chen et al., 2012). Besides enhancing the EPC function, EPC transplantation is an effective therapeutic tool for the endothelial dysfunction, which is an imitation factor of cardiovascular diseases. The therapeutic effects of EPC transplantation include two beneficial facets. One is the physical replacement of injured ECs and

promotes the angiogenesis. The second is the secretion of supporting cytokines to promote repair and angiogenesis. More recently, some research reported that the mechanism might be partially ascribed to EPC released vesicles.

MVs are submicrometric fragments derived from different types of cells and tissues. Cell types shed MVs in response to activation, apoptosis and stress. MVs express certain membrane proteins, which reflect their cell origin and stimulator. Accumulating evidence demonstrated that the level of MVs could be used as biomarkers for disease process and outcome. Our previous study find that the decrease in EPCs and the increase in EPC-MVs (Chen et al., 2011).

In this human study, likewise we goes beyond the circulating level of EPCs, and measured the level of EPC-MVs. Circulating EPC-MVs could be the indicator of impairment or incompetence EPC (Pirro et al., 2008). Considering CD34⁺VEGFR⁺ MVs as EPC-MV, and using flow cytometry, which is a well-known method, to evaluate MV level, we revealed that the level of EPC-MVs is increased in diabetic patients compared to healthy subjects. These finding raises the concept of using MV levels as a biomarker to some diseases. A possible interpretation of EPC levels decrease and EPC-MV levels increase is a higher rate of EPCs breakdown into EPC- MV in diabetes.

Most important, MVs present a novel cell- cell communication avenue. They carry and transfer the parent information to the received cell, which could modulate the target cell function. Moreover it can be used as a therapeutic approach. However, MVs have both beneficial and detrimental biologic effects.

Some studies have shown positive effects for MVs, while others showed negative effects, which depends on the origins and the stimulus (Lovren & Verma, 2013). For example, EMVs generated by using starvation as ECs stimulator, protect ECs from apoptosis induced by camptothecin, by way of p38 MAPK activation inhibition (Jansen et al., 2012). By contrast, EMVs released by ECs treatment with high glucose have deleterious effects recognized as, adhesion proteins expression increase and enhanced macrophage infiltration in an atherosclerotic mouse model (Jansen et al., 2013). Constantly, our previous study showed that circulating MVs derived from diabetic mice could impair EPC functions such as migration and tube formation in vitro and in vivo, whereas, the MVs from healthy control mice do not have those detrimental effects (Chen et al., 2011). Similarly, platelet microvesicles (PMVs) isolated from healthy subjects promotes post-ischemic angiogenesis and revascularization in a myocardial ischemic rat (Brill et al., 2005), while PMVs, isolated from blood stored for transfusion, may cause thrombosis in recipients during the transfer process (Simak & Gelderman., 2006). EPC-MVs released under serum deprivation stress had protective effects on hypoxia/reoxygenation-induced brain microvascular EC dysfunction and apoptosis; whereas, EPC-MVs produced under apoptotic stimulation induced by tumor necrosis factor- α had opposite effects (Wang et al., 2013).

It is known that the level of many circulating MVs is elevated in diabetes such as endothelial MVs, platelet MVs, and monocytes MVs (Nomura et al., 2009b).

However, it is still not completely understood whether diabetic MVs have a

positive or negative effects on EPC Function. Establishing a positive effect of MVs on EPCs might be the miracle to endothelial dysfunction restoration. Also, knowing that diabetic MVs have a depraved effect on healthy EPCs could promote a big concern in the prevention prospective. Moreover, the research conducted on human samples is still blank. For address our hypothesis that MVs can improve or impair EPC function depending on its origin, we create a cross study where d- EPCs incubated with ch- MVs, and h- EPCs incubated with cd- MVs. The function (migration), ROS production and apoptosis of EPCs of two groups were evaluated after co-incubation. Our present study for the first time demonstrated that human circulating MVs derived from healthy subject have positive effect on human diabetic EPCs; while, the human diabetic MVs have negative effect on human EPCs isolated from healthy subjects. This agrees with and provides with further information of our previous study demonstrates an impairment of EPC function by co-incubating with circulating MVs from diabetic mice (Chen et al., 2011).

As known EPCs migrate from BM toward ischemic tissue or injured ECs, to be involved in EC regeneration (Urbich and Dimmeler., 2004). EPC migration impairment and endothelial dysfunction, both have established in diabetic patients (Menegazzo et al., 2012). Our data showed that 24 hours preincubation of diabetic EPCs with ch-MVs improves the migration ability of diabetic EPCs. Since improving the migration process would enhance EPC ability to restore endothelium, and avoid vascular complication in diabetes, this is a very important finding for further research to use it as endothelial dysfunction's restoration

avenue. These results suggest that the circulating MVs derived from different origins might carry different contents which exert opposite effects on EPCs. Our future research will focus on finding the different contents, such as proteins, mRNAs and miRNAs in these two types of MVs by using Mass spectroscopy, RNA sequencing.

For the mechanisms responsible for these effects, we observed the ROS production and apoptosis of EPCs after different MV treatment. It has become widely accepted that enhanced oxidative stress is responsible for EPC number and function reduction in diabetes and cardiovascular diseases (Lin et al., 2013; Rossig et al., 2004 & He et al., 2004). Also accumulating evidence showed MV elevation in diabetic and cardiovascular diseases (Lovern and Verma., 2013). MVs could be the missing link in this process; they might be involved regulating of ROS production. Agouni et al have found that activated T- cells derived MVs showing an ability to decrease ROS and increase NO production (Agouni et al., 2007). Conversely, Brodsky et al have found that MVs decrease NO production, and increase superoxide anion O_2^- (Brodsky et al., 2004). Also monocyte-MVs mediate ROS production in ECs through eNOS uncoupling, NADPH oxidase, xanthine oxidase, and cyclo-oxygenase (Essayagh et al., 2007). Burger et al concluded that MVs have the ability to modulate ROS production, through different mechanisms. The stimulator and the origin for MV release both play a crucial role in this process (Burger et al., 2012). These provide the rationale for our findings. Our data showing an ability of MV to alter ROS production depends on its origin and stimulator. Different MVs incubation changed the ratio of ROS

production. ch-MVs down-regulate ROS production. In contrast, cd-MVs increased the ratio of ROS production. Taken together, we might conclude that in normal situation MVs have positive effects on diabetic EPCs. They protect EPC against oxidative stress and enhance its function by decreasing ROS production. As we know, EPCs impairment and oxidative stress lead to endothelial dysfunction and the destructive sequence. Having that in mind, MVs could be considered the revolutionary treatment for ROS-mediated EPC dysfunction in diabetes and diabetic cardiovascular complications.

In addition, apoptosis known as the downstream mechanism of MV-mediated function. MVs isolated from hypertensive patients induce EC apoptosis (Huang et al, 2010). Also Jorg et al establish that MVs derived from activated ECs induce apoptosis in circulating angiogenic cells (Distler et al., 2011). Caspase 3 has been reported to be the related mechanism. For examples, MVs are able to deliver Caspase 3 to the target cells and induce apoptosis (Digant-George & Boulanger., 2011, Hussein et al., 2005; 2007). In contrast, Gatti et al established a protective effect against apoptosis of mesenchymal cell derived MVs (Gatti et al., 2011). In our study, apoptosis level was another mechanism to evaluate the MV function after co-incubation. As we expected, the ch-MVs have the ability to attenuate apoptosis in d-EPCs. By contrast, cd-MVs increase the apoptosis levels. These finding in agreement with our previous study that circulating MVs from diabetic mice impairs the function of EPCs. We believe that ROS production might be one of the responsible mechanisms for apoptosis level changes. From

these, we may conclude that the origin of MVs and the environment involved in their release consider the most critical factors that determine the function of MVs.

This study is the first study to evaluate the effect of human cMV on human EPCs, however it has several limitations. First, we used circulating MVs, so we cannot identify which type of MVs is responsible for the effects. Second, we did not test the effect of circulating diabetic-MVs on d-EPCs, in comparison the effect of circulating healthy-MVs on h-EPCs. For the future study, we will expand the number of participants and explore the contents of cMV and their mechanism of that action.

6. CONCLUSION

Taken all together, we conclude that the level of EPCs is decreased, and the level of EPC-MVs is elevated in diabetic patients. The MVs from healthy subject could improve the function of diabetic EPCs, in contrast, MVs from diabetic patients may impair the EPC function from healthy control. The mechanism for these effects might via the ROS production and apoptosis.

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