folia medica

Folia Medica 63(6):875-83
DOI: 10.3897/folmed.63.e57636

Original Article

# The Role of Mesenchymal Stem Cells in Regulating PDGF and VEGF during Pancreatic Islet Cells Regeneration in Diabetic Animal Model

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**Received:** 15 Aug 2020 ♦ **Accepted:** 13 Jan 2021 ♦ **Published:** 31 Dec 2021

**Citation:** Putra A, Suwiryo ZH, Muhar AM, Widyatmoko A, Rahmi FL. The role of mesenchymal stem cells in regulating PDGF and VEGF during pancreatic islet cells regeneration in diabetic animal model. Folia Med (Plovdiv) 2021;63(6):875-83. doi: 10.3897/folmed.63.e57636.

## Abstract

**Introduction:** Diabetes is a heterogeneous group of metabolic diseases characterized by elevated blood glucose due to autoimmune disorder or a combination of insulin resistance and insulin deficiency. VEGF and PDGF are the main actors in the regeneration of damaged pancreatic tissue. However, the prolonged release of these molecules may induce fibrosis formation. Mesenchymal stem cells (MSCs) have a high potential to regenerate damaged pancreatic tissue by releasing PDGF and VEGF.

**Aim:** This study aimed to investigate the effect of MSCs on the levels of PDGF and VEGF on days 2 and 44 in diabetic mice and determine the number of pancreatic islet cells and blood glucose levels.

**Materials and methods:** This study used a post-control group design with animals divided into five groups: sham, control, and three treatment groups (P) which were given MSCs at doses of $1.5\times10^5$, $3\times10^5$, and $6\times10^5$ cells. The levels of PDGF, VEGF, and blood glucose were measured by enzyme-linked immunosorbent assay (ELISA), while the number of pancreatic islet cells was analyzed using H&E staining.

**Results:** This study showed a significant increase of VEGF and PDGF levels on day 2 and a significant increase in islet cell percentages on day 44 in line with the decreased blood glucose level. However, there was no difference between VEGF and PDGF levels on day 44.

**Conclusions:** MSCs regulate PDGF and VEGF levels in wound healing phases and remodel pancreatic islet β-cells regeneration to control blood glucose in diabetic model mice.

## Keywords

diabetes, MSCs, PDGF, VEGF

## INTRODUCTION

Type 1 diabetes (T1D) is a chronic metabolic disease characterized by a rise in blood glucose level as a consequence of pancreatic β-cell destruction in which the autoimmune response plays a crucial role in manifesting this phenomenon.[1] Previous studies reported that the number of diabetes cases in developing countries has significantly increased in the last 10 years, and more than 1.5 million people died from the consequences of the disease.[2] A major therapeutic approach to T1D is insulin replacement; however, this exogenous insulin cannot mimic natural insulin, and the long-term complications of using those insulins are insufficiently prevented.[3] On the other hand, the pancreatic β-cells transplantation is limited by donor issues and a potency rejection to host immune cells. In addition, a complete vascularization system is needed.[4] Therefore, developing new alternative treatments of T1D should be explored, including the use of mesenchymal stem cells (MSCs); these MSCs display profound immunomodulatory properties in ameliorating the autoimmune disorders and regeneration capacities to damaged tissues, including pancreatic tissue in T1D by releasing the platelet-derived growth factor (PDGF) and the vascular endothelial growth factor (VEGF).

MSCs are widely defined as plastic adherent stromal cells with a multipotent differentiation capacity. MSCs naturally express various surface markers such as CD73, CD90, CD105, and lack the expression of CD45, CD34, CD14 or CD11b, and CD79a or CD19.[5,6] Under standard *in vitro* differentiation conditions, MSCs differentiate into specific cells such as chondrocytes, osteocytes, and adipocytes, including pancreatic β-cells.[7,8] Although MSCs can be isolated from various mesenchymal tissues[9,10], the umbilical cord (UC)-derived MSCs have more stemness than the other sources[11,12]. MSCs have immunosuppressive properties to control autoimmune diseases[13-15], including T1D, by modulating regulatory T (Treg) cells.[16] Once the inflammation is under control, MSCs gradually initiate the regeneration process by producing VEGF and PDGF to promote pancreatic islets β-cells growth.[17,18] VEGF has a pivotal role in promoting neovascularization of pancreatic islet for maintaining a normal function of islet cells in insulin secretion.[19,20]

A previous study reported that the growth of functional islet cells depends on vascularization in the first-two weeks post-grafting in which delayed and incomplete vascularization is a major problem in improving the pancreatic β-cell function.[17] On the other hand, PDGF is the most important factor involved in stimulating proliferation, differentiation, and cellular migration, including endothelial migration.[21] PDGF and VEGF are also pivotal migration signals to recruit the most cells, including endogenous MSCs and endothelial cells, to the damaged area to initiate proliferation of pancreatic cells, particularly β-cell and endothelial cells.[18-21] However, releasing VEGF and PDGF continuously from an initial inflammation to the remodelling phase in wound healing processes can cause a deleterious effect on an islet cell function instead of regeneration. Prolonged activation of PDGF and VEGF are associated with necroinflammation extension of damaged areas leading to fibrosis development.[22,23] Therefore, although the VEGF and PDGF play a major role in pancreatic β-cells regeneration, the prolonged excessive VEGF and PDGF levels also have the opposite effects. On the other hand, MSCs can control VEGF and PDGF release in the initial phase of inflammation to initiate the proliferation phase in accelerating wound healing.[24] However, the ability of MSCs to decrease VEGF and PDGF levels to normal metabolic levels in the remodelling phase, a late phase of regeneration, remains unclear.

## AIM

Hence, in this study, we investigated the role of MSCs in decreasing PDGF and VEGF levels to a normal metabolic level on day 44 in the remodelling phase associated with the increase of pancreatic islet β-cells and normal plasma glucose levels. We also examined the ability of MSCs to increase the PDGF and VEGF levels on day 2.

## MATERIALS AND METHODS

### MSC isolation and characterization

The umbilical cords (UCs) were collected from 19-day pregnant female BALB/c mice under general anesthesia. After blood vessels were removed, the tissue parts of the UC under aseptic conditions were cut into smaller pieces and transferred to a T25 culture flask containing Dubelco Modified Eagle Medium (DMEM) (Sigma-Aldrich, Louis St, MO) supported with 10% fetal bovine serum (FBS) (Gibco™ Invitrogen, NY, USA) and 1% penicillin (100 U/mL)/streptomycin (100 μg/mL) (Gibco™ Invitrogen, NY, USA) and 0.25% amphotericin B (Gibco™ Invitrogen, NY, USA). These flasks were then incubated at 37°C in a humid atmosphere consisting of 5% $CO_2$, the medium being renewed every three days. After reaching 80% confluency, the cells were passaged, and at the fifth passage, the MSCs-like were used for the following experiments. All procedures were under the provisions of the experimental Ethics Commission of the Medical Faculty of UNISSULA.

To confirm the MSCs-like surface antigens, plastic adherent stromal cells at the fourth passage were characterized by flow cytometry assays. After trypsinized and pelleted, the cells were subsequently incubated using fluorescein allophycocyanin (APC)-, isothiocyanate (FITC)-, and peridinin-chlorophyll-protein (perCP)-Cy5.5.1- conjugated anti-mouse CD73, CD90, and CD105 antibodies (BD Bioscience, San Jose, CA, USA) for 30 minutes at room temperature in the dark. On the other hand, an isotype-specific conjugated anti-IgG BD Bioscience, San Jose,

CA, USA) was used as negative controls. The cells were then washed twice using PBS. The analyses were performed using a BD Accuri C6 Plus flow cytometer (BD Bioscience, San Jose, CA, USA). The post-acquisition analysis was performed using the BD Accuri C6 Plus software (BD Bioscience, San Jose, CA, USA).

## In-vitro differentiation

We further performed the osteogenic differentiation assay in the fifth passage. The MSC-like cells were cultured in a standard medium containing DMEM with 10% FBS, 1% penicillin (100 U/mL)/streptomycin (100 µg/mL) and 0.25% amphotericin B at 37°C and 5% $CO_2$ until reaching 95% confluency. Then the standard medium was replaced using an osteogenic differentiation medium containing Mouse MesenCult™ Osteogenic Differentiation Basal Medium (Stem Cell Technologies, Singapore) with 20% Mouse MesenCult™ Osteogenic Differentiation 5X Supplement (Stem Cell Technologies, Singapore), 1% L-glutamine (Gibco™ Invitrogen, NY, USA), 1% penicillin (100 U/mL)/streptomycin (100 µg/mL), and 0.25% amphotericin B. The osteogenic differentiation medium was replaced every 3 days. Calcium deposition was shown by Alizarin Red staining (Sigma-Aldrich, Louis St, MO), followed by 21 days of induction.

## Induction of diabetic mouse model and intraperitoneal MSCs administration

Thirty male BALB/c mice (6-8 weeks old) weighing 50 g were caged at 24±2°C and 60% relative humidity, with 12:12-hour light-dark cycle (laboratory standard). The control and treatment groups of mice were fasted for 8 hours and then were intraperitoneally injected using single-dose streptozotocin (STZ: Sigma, St. Louis, MO) (60 mg/kg body weight) to induce a diabetic animal model. For the sham mice, sterile PBS injections were used. Seven days after the STZ injection, the blood glucose levels were examined from lateral saphenous vein blood using a pharmaceutical-grade glucometer (Accu-Check; Roche, Basel, Switzerland). The mice were considered diabetic and included in the groups' study when the glucose levels exceeded 300 mg/dl in two consecutive measurements. The study protocol was designed for the treatment groups (n=6/group). Diabetic model mice BALB/c were treated by injecting MSCs intraperitoneally 8 days after the last dose of STZ at doses of $1.5\times10^5$, $3\times10^5$, and $6\times10^5$ cells (represented as P1, P2, and P3, respectively). The control and sham groups received intraperitoneal injections of natrium chloride (NaCl).

## Blood glucose monitoring

Using the enzymatic colorimetric method, blood glucose levels were determined by blood samples from the orbital vein tail tip on day 2 and 44 using Accu-Check (Roche, Basel, Switzerland).

## Enzyme-linked immunosorbent assay (ELISA)

PDGF and VEGF levels released in the serum were measured by ELISA. Briefly, according to the manufacturer's instructions (Fine Test, Wuhan, China), the PDGF and VEGF levels were calculated on days 2 and 44, according to a standard curve constructed for each assay. The colorimetric absorbance was recorded at a wavelength of 450 nm.

## Histology analysis

Pancreatic tissue samples were immediately fixed in 10% neutral-buffered formalin, then embedded in paraffin (Thermo Scientific, Waltham, MA, USA). Next, the block paraffin pancreatic tissues were cut using a microtome with 5–10 µm thickness. Before staining, 5-µm sections were first deparaffinised and rehydrated using xylol and alcohol. The slides were stained with hematoxylin-eosin (H & E) and then analyzed under a light microscope.

## Data analysis

Data are shown as the means ± standard deviation (SD). The calculations were performed using SPSS 23.0 (IBM Corp., Armonk, NY, USA). After applying normality tests for the studied variables, the statistical significance of independent quantitative variables was assessed by the Kruskal-Wallis test, followed by Mann-Whitney post-hoc analysis. A $p$-value of $<0.05$ was considered significant.

# RESULTS

## Characteristics of UC-MSCs

MSCs isolated from the umbilical cord were analyzed based on their plastic adherent capability under standard culture conditions, antigen-specific surface markers, and differentiation capability after 5 passages. In this study, the cell morphology of MSCs at the fourth passage exhibited typical monolayers of spindle-shaped fibroblast-like cells, with adhering capability to the plastic flask **(Fig. 1a)**. To characterize MSCs surface antigens, we performed flow cytometry analysis as indicated by the International Society for Stem Cell Therapy. We found a high level of CD90 (96.7±1.3%), CD105 (67.1±0.5%), and CD73 (99.2±0.4%) regarding the International Society of Cellular Therapy (ISCT) **(Fig. 1b)**.[9]

## In-vitro differentiation

The ability of MSC to differentiate into osteogenic cells was analyzed by culturing the MSCs under an osteogenic medium for 20 days in which the calcium deposition was visualized as red colour after alizarin red solution ad-

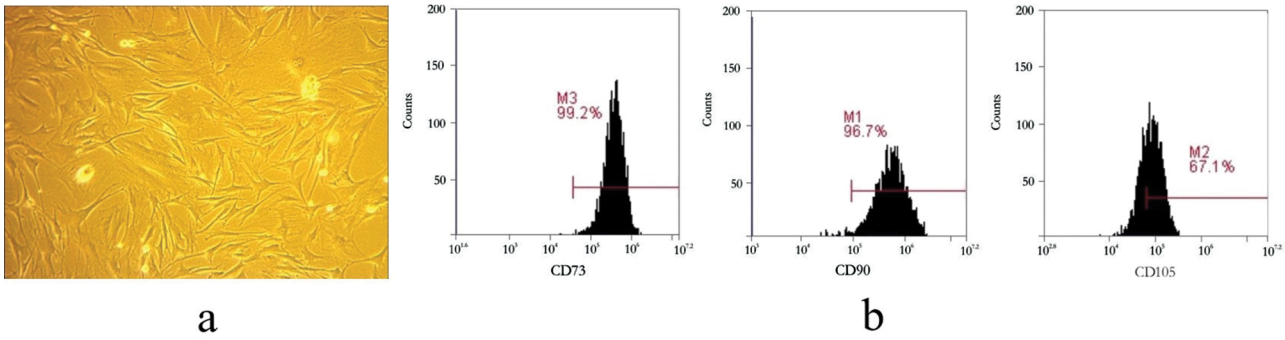


**Figure 1.** UC-MSCs candidate from the in vitro culture showed a spindle form such as fibroblast-like cells (×100 magnification) **(a)**; Flow cytometry characterization of UC-MSCs positively expressed CD90, CD105, and CD73 **(b)**.

ministration. To confirm the *in vitro* differentiation potential of MSCs, we used an osteogenic differentiation assay to demonstrate the ability of MSCs to differentiate into osteogenic cells. Under these osteogenic differentiation assays, we found red colour in most cell culture as calcium deposition that indicated that these MSCs had differentiated into osteogenic cells **(Fig. 2)**.

## MSCs increase and decrease PDGF and VEGF levels according to healing phases

The ability of MSCs to regulate PDGF and VEGF in accelerating an optimum healing process in the diabetic mouse model occurred either by increasing or decreasing their levels according to healing phases. Under the proliferation phases, MSCs increase PDGF and VEGF levels to recruit and proliferate endothelial cells for initiating neovascularization formations and regeneration of pancreatic islets.[19-21] To determine the role of MSCs in increasing PDGF and VEGF levels in the proliferation phase, we assessed those PDGF and VEGF levels using ELISA on day 2 following MSC administrations.

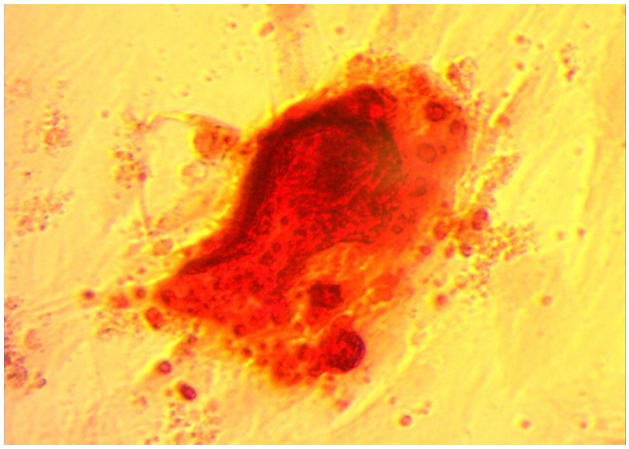

**Figure 2.** UC-MSCs were treated using osteogenic differentiation medium to assess the capacity of MSCs to differentiate into bone matrix. The calcium deposition appeared in red colour after alizarin red staining (×200 magnification).

We found that there was a significant increase of PDGF in P2 and P3 groups compared to the control group on day 2 ($p<0.05$), in which the P3 group, as the highest dose group of MSCs, showed the highest level of PDGF (288±35 pg/mL) **(Fig. 3a)**. We also found that VEGF's levels were significantly increased in group P3 compared to the control group on day 2 (127±26 pg/mL, $p<0.05$) **(Fig. 3c)**. Furthermore, to explore the prolonged effect of MSCs on the PDGF and VEGF associated with deleterious effects, we also assessed the PDGF and VEGF levels on day 44 in the remodelling phase in all study groups. Interestingly, we found no significant differences in the PDGF and VEGF levels for all the treatment groups versus the control group on day 44 **(Figs 3b, 3d)**, indicating that these PDGF and VEGF levels had gone into a normal metabolic level.

## MSCs increase the number of pancreatic islet cells

Along with the normal levels of PDGF and VEGF reached in the treatment groups on day 44, the optimal regeneration process of pancreatic islet β-cells was also completed. To explore the ability of MSCs in optimally regenerating the pancreatic tissue in T1D, we assessed the histopathological appearance on STZ-induced pancreatic tissue of mice following MSCs administration on day 44, the remodelling phase, using H&E staining **(Fig. 4)**. In this phase, we found a significant increase in the percentage of pancreatic islet cells in the P2 and P3 groups compared to the control group on day 44 ($p<0.05$), in which the highest percentage of pancreatic islet cells was seen in group P3, as the highest dose group of MSCs (87±12%) **(Fig. 5)**.

## MSCs decrease the blood glucose level from the proliferation to the remodelling phase

The increase of PDGF and VEGF levels in treatment groups indicated that MSCs gradually initiated the regeneration process of pancreatic islet β-cells. One of the important parameters to analyze the function of pancreatic tissues in diabetes is the serum glucose levels. To monitor

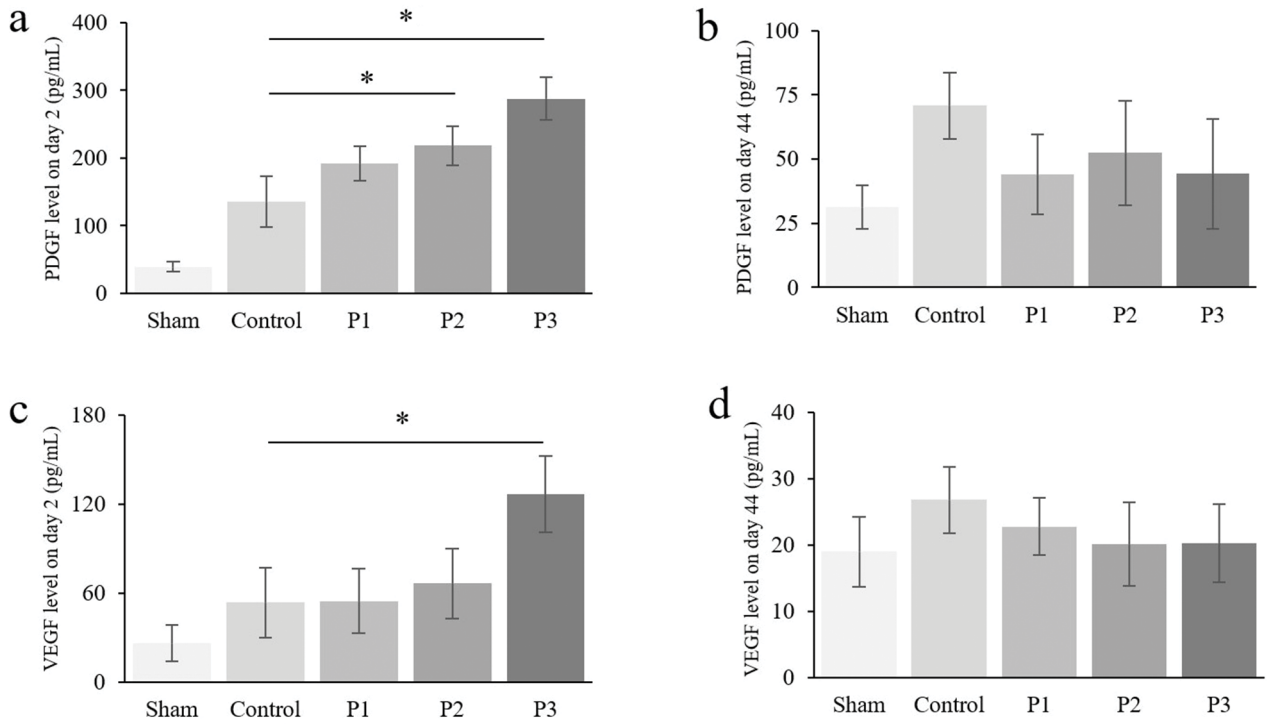


**Figure 3.** ELISA on day 2 showed a significant increase and optimum level of PDGF on P3 group **(a)**, whereas there were no significant differences of PDGF level on day 44 **(b)**. There was significant increase and optimum level of VEGF on P3 on day 2 **(c)**; however, there were no significant differences of VEGF level on day 44 **(d)**.

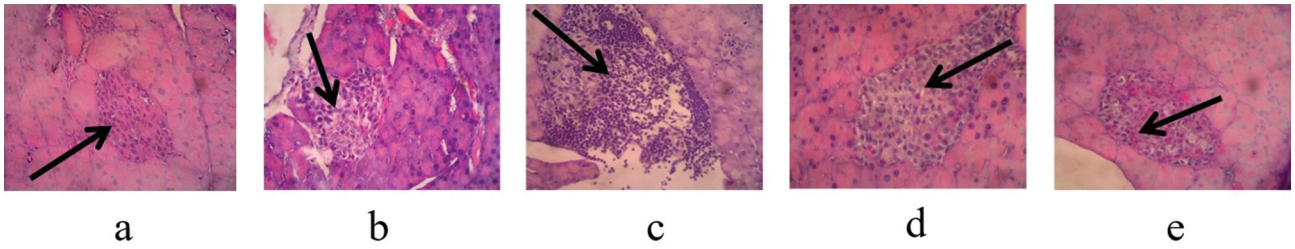


**Figure 4.** The H&E staining of STZ-induced pancreatic tissue in groups of sham **(a)**, control **(b)**, P1 **(c)**, P2 **(d),** and P3 **(e)** following MSCs administration on day 44.

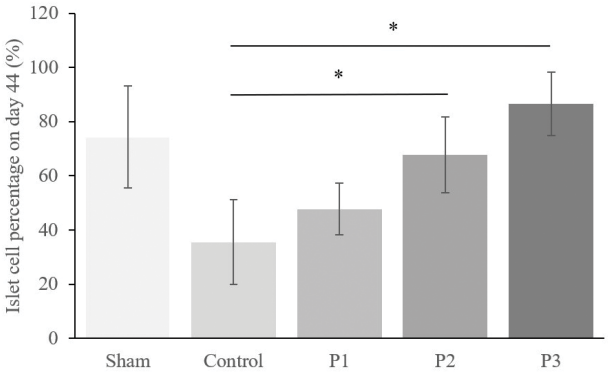


**Figure 5.** The percentage of islet cells on day 44 following treatments indicated the significant increase in groups P2 and P3.

the serum glucose level in all the study groups, we used the Accu-Check on day 2 and 44. In this study, we found a significant decrease in serum glucose level in group P3 on day 44 compared to controls with the highest decrease of blood glucose serum at P3 group (178±67 mg/dl, $p<0.05$) (**Fig. 6**).

## DISCUSSION

T1D, mostly caused by autoimmune disorders, may induce a prolonged chronic inflammation of islet β-cells leading to damage of pancreatic β tissues and metabolic dysfunction.[1] MSCs-based cell therapy is the most promising treatment approach to restore the pancreatic islets in diabetes, particularly in T1D, due to not only the ability of MSCs to differentiate and transdifferentiate into pancreatic β cells islets[25] but also their ability to trigger neovascularization for an

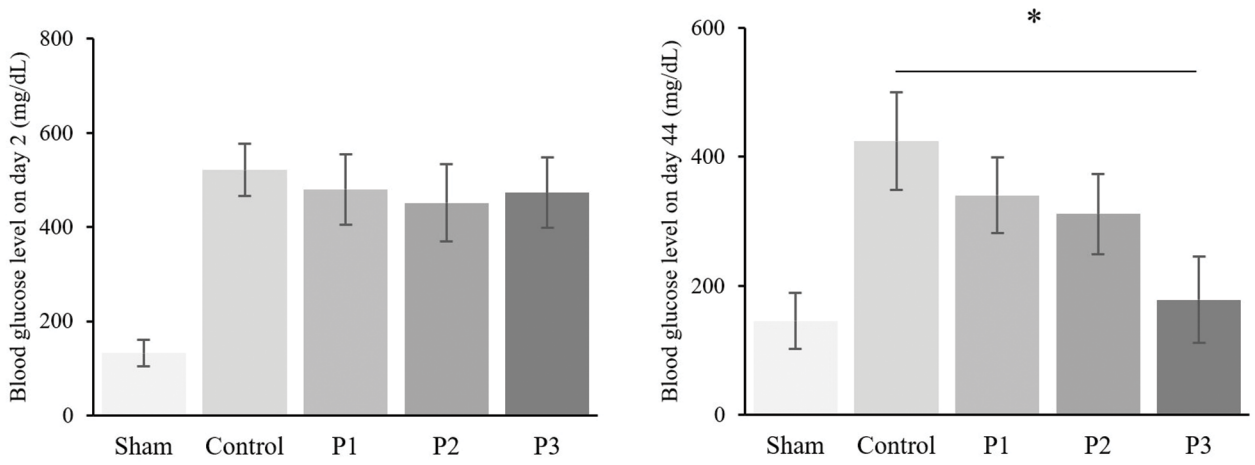


**Figure 6.** Blood glucose on day 44 showed a significant decrease on P3 compared to controls. Moreover, there was no difference between all groups in the glucose level on day 2.

optimal regeneration in addition to their capacity in controlling inflammation[26]. The release of VEGF and PDGF in injury areas have two opposite effects according to the phase of healing processes, in which the prolonged release of these VEGF and PDGF may induce fibrosis formation.[22,23] To investigate the role of MSCs in regulating the level of PDGF and VEGF either by decreasing their levels to normal levels in the remodelling phase that is associated with the increase of pancreatic islet β-cells and a normal plasma glucose level or by increasing their levels in the initial inflammation to the proliferation phase, we treated the T1D animal model using MSC at several doses as in a previous study.[18]

In this study, the significant increase of PDGF and VEGF following MSCs treatment on day 2 indicated that the inflammatory process in these diabetic mice was controlled. The controlled inflammation induces the healing phases transition from the inflammatory to proliferation characterized by a higher VEGF and PDGF release. The increase of PDGF and VEGF levels is a signal recruitment for most cells, including endothelial and endogenous MSCs, to homing into the damaged pancreatic tissues for initiating regeneration processes.[27] However, the MSCs migration is not induced by PDGF and VEGF only. The release of stroma derived factor 1 (SDF-1) and hepatocyte growth factor (HGF) by the damaged tissue, as well as by a previous proinflammatory milieu, is also initially attractive for MSCs migration.[28] The migrating MSCs actively inhibit surrounding inflammatory cells by releasing IL-10 and TGF-β and gradually initiating tissue regeneration **(Fig. 7)**.[6,26] This finding is supported by several studies reporting that MSCs could control the inflammatory milieu to induce a release of PDGF and VEGF for regeneration.[18-22] On the other hand, the regeneration failure in the control groups was assumed that the inflammatory milieu is still flaring up, thus inducing a release of proinflammatory cytokines to lyse and degrade PDGF and VEGF.[23]

The ability of MSCs to initiate the pancreatic islet cell formation occurred along with the increase of PDGF and VEGF in the proliferation phase and the decrease of those into the normal level in the remodelling phase once an optimal pancreatic islet cell formation was achieved. We found no significant difference in PDGF and VEGF levels on day 44 compared with the sham groups indicating the levels of PDFG and VEGF had a decrease in the normal metabolic levels. The decrease of PDGF and VEGF in those remodelling phases in this study suggests that MSCs are able to control the prolonged proliferation of the pancreatic islet cells that were potentially inducing fibrosis formation. The once pancreatic tissue formation is completely regenerated by MSCs, the proliferation phase comes into the stationary phase leading to the cellular microenvironment inactivation.[18] Furthermore, the increase of pancreatic islet cells following MSCs treatment is in line with restoring the normal function of pancreas tissue indicated by the normal blood glucose levels. These findings are consistent with a previous study reporting that high levels of PDGF and VEGF in the remodelling phase could trigger the accumulation of fibroblast cells in the injury site leading to tissue fibrosis.[20,29]

This study showed that MSCs could regulate PDGF and VEGF levels in the inflammatory and remodelling phases associated with the pancreatic islet β-cells formation in diabetic model mice (*Mus musculus*). The initial increase of PDGF and VEGF levels accelerated the optimum point in the proliferative phase. However, those molecules gradually decreased toward normal metabolic levels in the remodelling phase to achieve the homeostasis of the dynamic state of equilibrium. In this study, we did not investigate the pancreatic β cells marker, thus the exact mechanism of MSCs in β pancreas regeneration remains unclear. We also did not analyse several anti-inflammatory cytokines such as IL-10 and attractant molecules for homing MSCs such as SDF-1; thus, the roles of paracrine mechanism and migration of MSCs were also unclear.

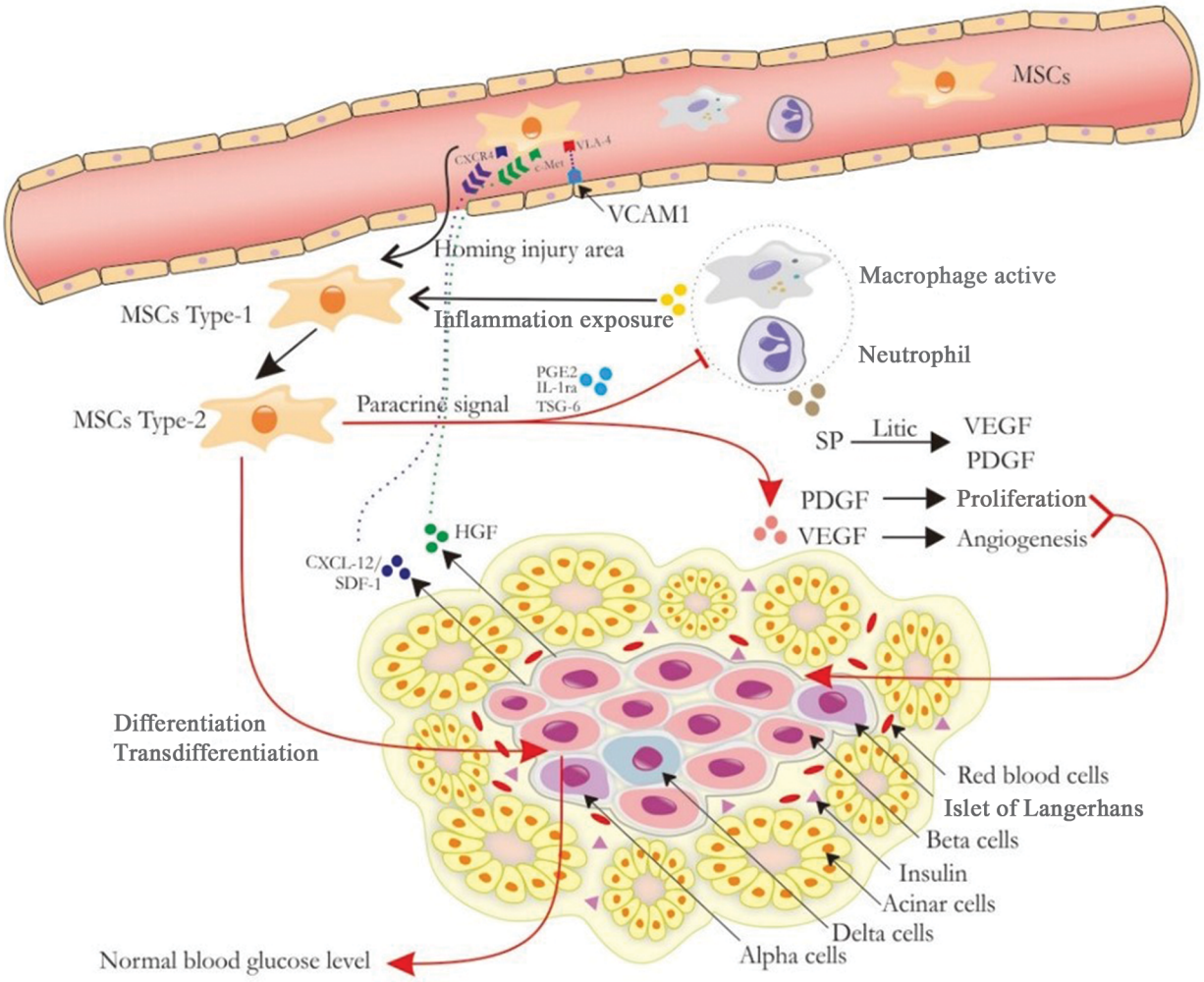


**Figure 7.** Schematic illustration of homing mechanism of MSCs to injured pancreatic area. The interactions of receptor VLA-4 and VCAM-1, receptor CXCR4 and SDF-1, and receptor cMet and HGF trigger the transmigration of MSCs to pancreatic tissue injury. MSCs release anti-inflammatory cytokines to inactivate the inflammatory cells leading to MSCs polarization from type 1 to type 2. The release of VEGF and PDGF under controlled inflammation initiates islet cells proliferation correlated with the optimal healing of pancreatic islet cells at the remodelling phase. In line with these mechanism, the VEGF and PDGF levels decreased gradually during the remodelling phase. The complete healing of pancreatic islet was characterized by normal blood glucose levels.

## CONCLUSIONS

Based on this study, we concluded that MSC could regulate PDGF and VEGF levels in wound healing phases and remodel pancreatic islet β-cells regeneration to control blood glucose levels in diabetic model mice.

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# Роль мезенхимальных стволовых клеток в регуляции тромбоцитарного фактора роста и фактора роста эндотелия сосудов во время регенерации островковых клеток поджелудочной железы на модели животных с диабетом

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**Дата получения:** 15 августа 2020 ♦ **Дата приемки:** 13 января 2021 ♦ **Дата публикации:** 31 декабря 2021

**Образец цитирования:** Putra A, Suwiryo ZH, Muhar AM, Widyatmoko A, Rahmi FL. The role of mesenchymal stem cells in regulating PDGF and VEGF during pancreatic islet cells regeneration in diabetic animal model. Folia Med (Plovdiv) 2021;63(6):875-83. doi: 10.3897/folmed.63.e57636.

## Резюме

**Введение:** Диабет относится к гетерогенной группе метаболических заболеваний, характеризующихся повышенным уровнем сахара в крови из-за аутоиммунного заболевания или сочетания инсулинорезистентности и дефицита инсулина. Фактор роста эндотелия сосудов (VEGF) и тромбоцитарный фактор роста (PDGF) являются основными факторами регенерации повреждённой ткани поджелудочной железы. Однако длительное высвобождение этих молекул может вызвать образование волокон. Мезенхимальные стволовые клетки (МСК) обладают высоким потенциалом регенерации повреждённой ткани поджелудочной железы, секретируя PDGF и VEGF.

**Цель:** Целью этого исследования было изучить влияние МСК на уровни PDGF и VEGF на 2-й и 44-й дни у мышей с диабетом и определить количество островковых клеток поджелудочной железы и уровень сахара в крови.

**Материалы и методы:** В этом исследовании использовался метод контрольной группы и животных, разделённых на пять групп: плацебо, контрольная и три обработанные группы (P), которым вводили МСК в дозах $1.5\times10^5$, $3\times10^5$, и $6\times10^5$ клеток. Уровни PDGF, VEGF и сахара в крови измеряли с помощью ELISA, а количество островковых клеток поджелудочной железы анализировали с помощью окрашивания ХЕ.

**Результаты:** Это исследование обнаружило значительное увеличение уровней PDGF и VEGF на второй день и значительное увеличение процента островковых клеток на 44 день наряду со снижением уровня сахара в крови. Однако не было обнаружено разницы между уровнями VEGF и PDGF на 44 день.

**Заключение:** МСК регулируют уровни VEGF и PDGF в фазах заживления ран и ремоделируют регенерацию β-клеток для контроля уровня сахара в крови на модели диабета у мышей.

## Ключевые слова

диабет, МСК, PDGF, VEGF