• Research Article •

Insulin promotes the osteogenic differentiation of umbilical cord mesenchymal stem cells

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Abstract

BACKGROUND: How to effectively and rapidly induce the osteogenic differentiation of human umbilical cord mesenchymal stem cells is the focus of the current stem cell research. Increasing evidence has demonstrated some growth factors, such as bone morphogenetic protein-2, have important effects on the transdifferentiation of umbilical cord mesenchymal stem cells into osteoblasts *in vitro*. However, widespread use of growth factors is limited because of high cost. Insulin is widely used in the cell culture and induction, but there is no report about the effect of insulin on the osteogenic differentiation of human umbilical cord mesenchymal stem cells.

OBJECTIVE: To observe the effect of insulin on osteogenic differentiation of human umbilical cord mesenchymal stem cells and to explore the feasibility of human umbilical cord mesenchymal stem cell transplantation in the treatment of diabetic delayed fracture healing.

METHODS: The passage 3 human umbilical cord mesenchymal stem cells were inoculated in two flasks, denoted as experimental group and control group. The insulin (10^{-7} mmol/L) was added to the experimental group but not to the control group. The proliferative capacity of human umbilical cord mesenchymal stem cells was evaluated by cell count kit-8 and alkaline phosphatase activity. The osteogenic differentiation capacity of human umbilical cord mesenchymal stem cells was evaluated by measuring the protein and mRNA expressions of type I collagen as well as osteocalcin mRNA level.

RESULTS AND CONCLUSION: After 1–2 weeks of induction, compared with the control group, insulin could significantly increase the number of human umbilical cord mesenchymal stem cells in the experimental group, the activity of alkaline phosphatase and expressions of type I collagen osteocalcin mRNA ($P < 0.05$). These data indicate that insulin can promote the proliferation and osteogenic differentiation of human umbilical cord mesenchymal stem cells.

Subject headings: Stem Cells; Tissue Engineering; Diabetes Mellitus; Cells, Cultured

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INTRODUCTION

Diabetes has become one of the most serious puzzles to global public health, the total number of people with diabetes is projected to increase to 366 million in 2 030[1]. Diabetes causes diminished bone formation and increases the risk of fracture[2]. Moreover, fracture healing is impaired in diabetic humans and in animal models[3]. Diabetes may affect differentiation and proliferation of osteoblasts or mesenchymal stem cells (MSCs) and the expression of growth factors that promote bone formation[4-5]. Research in the early 1 980 s revealed that the blood in the umbilical cord and placenta after the birth of a child was comparable to bone marrow for use in stem cell transplantation. Stem cell therapy to recover impaired osteoblast is an interesting project[6]. The mesenchymal stem cell transplantation has a promising therapeutic potential for fracture healing[7]. Over the past three decades, bone marrow mesenchymal stem cells (BMSCs) have been used as a

popular ce ll source for regenerative medicine research. However, the isolation of BMSCs frequently yields a low number of stem cells and the isolation procedure is invasive for donors or patients[8]. In contrast, umbilical cord mesenchymal stem cells (HUMSCs) are immune-privileged and immunosuppressive, which have a multipotent differentiation capacity and are readily available as a new cell source[9]. HUMSCs have been investigated in the treatment of Parkinson's disease[10], diabetes and spinal cord injuries[11-12]. However, the osteogenic differentiation capability of HUMSCs are lower than that of BMSCs[13].

MSCs differentiation into osteoblasts can be achieved by adding vitamin D3, ascorbic acid and β-glycerophosphate to the culture medium. Several laboratories use dexamethasone, a synthetic glucocorticoid, instead of vitamin D3. Dexamethasone appears to optimize the MSCs differentiation, but not specifically to the osteoblast lineage[14]. Several researches focus on HUMSCs transdifferentiating into osteoblasts *in vitro* with the effect of specific growth factors[15].

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes and characterized by a combination of insulin resistance and pancreatic beta-cell dysfunction. The mainstream therapies for T2DM include insulin sensitizers and exogenous supply of insulin. Recent reports suggest that stem cells may assist in improving metabolic control in subjects with T2DM[16]. Insulin is widely used in cell culture and induction; however, its effects on the osteogenic differentiation of HUMSCs have not been reported. In this study, we investigated the effect of insulin on the osteogenic differentiation of HUMSCs.

MATERIALS AND METHODS

Design

A randomized controlled cell experiment.

Time and settings

The experiment was completed at the Central Laboratory, the Affiliated Hospital of Qingdao University, China from August 2014 to June 2015.

Materials

HUMSCs were obtained from the National Research Center for Stem Cell Engineering and Technology, China.

Methods

Group Information

HUMSCs were cultured in DMEM/F12 (1:1) with 10% fetal bovine serum, 100 U/mL penicillin at 37 ℃ in a humidified atmosphere containing 5% CO_2. The cells at passages 5−6 were used for the subsequent assays. HUMSCs cultured in osteogenic medium (50 mg/L ascorbate-2 phosphate, 10^{-8} mol/L dexamethasone, 10 mmol/L β-glycerophosphate). Insulin (10^{-7} mmol/L) was added to the culture medium as the experimental group. The cells in the osteogenic medium without any treatment were used as controls. The medium was changed every 2 days.

Cell proliferation assay

Cell counting kit-8 (CCK-8) was employed in this experiment to quantitatively evaluate HUMSC viability. Briefly, approximately 1×10^4 HUMSCs were seeded on each film placed in the 24-well plates for 12 hours, then the growth medium was replaced by 200 mL of osteogenic medium with insulin (10^{-7} mmol/L) in the experimental group, and the medium in the control group was replaced only by 200 mL of osteogenic medium at 37 ℃ in a humidified atmosphere containing 5% CO_2. Cells were harvested daily (6 wells/group) and enumerated for 7 consecutive days. Culture medium was removed every day and the cells were washed with PBS twice. Approximately 900 μL serum-free DMEM/F12 medium and 100 μL CCK-8 solutions were added, followed by incubation for 3 hours at 37 ℃. Supernatant was transferred to 96-well plates, and the absorbance value at 450 nm was determined using a microplate reader. Six parallel experiments in each sample were used to assess the cell viability.

Assay for the alkaline phosphatase (ALP) activity in HUMSCs

The ALP activity in HUMSCs was assessed at 7, 10, 12 and 14 days, using alkaline phosphatase activity colorimetric assay kit. The amount of p-nitrophenol was measured at 405 nm by STAKMAX™ microplate reader. Protein concentrations were measured using the Bio Rad protein assay kit with bovine serum albumin as a standard. The experiments were repeated three times.

Immunocytochemical staining of type I collagen protein

Following trypsinization, HUMSCs were seeded

onto glass coverslips with a density of 5×10^3 cells per well in DMEM/F12 (1:1) with 10% fetal bovine serum, 100 U/mL penicillin at 37 ℃ in a humidified atmosphere containing 5% CO_2. When 50%−70% confluence was reached, the medium was changed to the osteogenic medium with insulin or with no insulin. The medium was changed every 2 days. After 12 days, cells seeded on glass coverslips were washed with PBS and fixed with 4% formaldehyde (Unifix) for 15 minutes at room temperature. After fixation and washing in 0.01 mol/L PBS (pH 7.2), cells were permeabilized when needed with 0.05% Triton X-100 in PBS for 30 minutes at 4 ℃ prior to labeling and then washed again with PBS. Nonspecific binding sites were subsequently blocked with 3% normal goat serum (Dako Cytomation) in PBS for 20 minutes. After washing, cells were incubated with rabbit polyclonal antibody against type I collagen (diluted concentration of 1:100) overnight at 4 ℃. The cells were then washed again and incubated for 30 minutes with goat anti-rabbit horseradish peroxidase-conjugated secondary antibodies. After washing in PBS, a high-sensitivity diaminobenzidine chromogenic substrate system (DAB) and Mayer's hematoxylin were respectively used to visualize the peroxidase and for counter staining. After mounting in an aqueous mounting medium (Aquatex; Merck, Darmstadt, Germany), cell-seeded coverslips were examined using a photomicroscope equipped with an automated camera.

Real-time PCR

To assess the osteogenesis of HUMSCs, type I collagen protein and osteocalcin mRNA expressions were detected by real-time PCR. Total RNA was extracted from cultured HUMSCs with Trizol. The separation and precipitation of RNA were accomplished with chloroform and isopropyl alcohol. Total RNA (1 μg) was converted to cDNA and amplification was performed using One-Step qPCR Kit following the manufacturer's protocol. For determination of type I collagen and osteocalcin mRNA expression, SYBR Green detection was used and the values were normalized using glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Real-time quantitative PCR was performed in a 7300 Sequence Detection System (Applied Biosystems). Primers pairs used in this study are presented in **Table 1**.

Table 1 Gene primer sequences

Gene	Primer sequence (5'−3')
OCN	F: TAG TGA AGA GAC CCA GGC GC
	R: CAC AGT CCG GAT TGA GCT CA
Type I collagen	F: TGA CCT CAA GAT GTG CCA CT
	R: ACC AGA CAT GCC TCT TGT CC
GAPDH	F: GGT ATC GTC GAA GGA CTC ATG AC
	R: ATG CCA GTG AGC TTC CCG TTC AGC

Note: OCN: Osteocalcin; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

The thermal cycling conditions for the real-time PCR were 95 ℃ for 2 minutes followed by 45 cycles of 94 ℃ for 15 seconds, 58 ℃ for 20 seconds and 72 ℃ for 30 seconds. Data were collected at 68 ℃. Relative expression levels for each primer set were normalized to the expression of GAPDH by the $2^{-\Delta\Delta Ct}$ methods[17].

Main outcome measures

(1) The proliferative capacity of HUMSCs; (2) ALP activity; (3) type I collagen protein expression; (4) the gene expression of type I collagen and osteocalcin.

Statistical analysis

All data were analyzed statistically using SPSS 21.0 statistical software. Each experiment was repeated at least three times, and representative data were reported. Continuous variables are expressed as mean±SD. Independent sample *t*-test was used to compare the continuous variables if these data normally distributed, which was confirmed with Kolmogorov-Smirnov test. The Wilcoxon signed-rank test was used to assess non-parametric data. A *P*-value of less than 0.05 was considered statistically significant.

RESULTS

Effect of insulin on HUMSC proliferation

The CCK-8 assay was employed to compare the HUMSC viability. The strongest cell viabilities were observed in the insulin group. Two groups of cells were in the incubation period at 1 day (**Figure 1**). In the insulin group, HUMSCs significantly increased and entered the exponential growth phase at 2 days, and cell proliferation reached a peak at 4 days and entered into the platform phase. Cell proliferation in the control group entered the exponential growth phase at 3 days and reached a peak into a platform phase at 5 days. The results demonstrated that insulin

significantly promoted cell proliferation compared with the control group ($P < 0.05$).

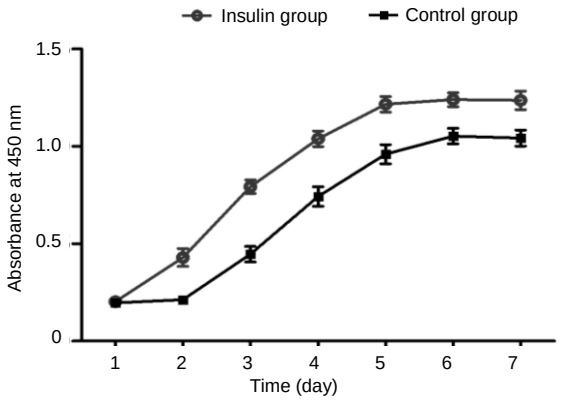

Figure 1 Proliferation curves of human umbilical cord mesenchymal stem cells
Note: The strongest cell viability was observed in the insulin group and significantly increased compared with the control group ($P < 0.05$).

Effect of insulin on ALP activity of HUMSCs

Osteogenic differentiation of HUMSCs cultured in the insulin group was investigated by the determination of the specific ALP activity. ALPase activity was initially detected on day 7 of cultivation. We found a clear increase of the ALP activity in the insulin-induced cells with a maximum of the ALP activity on day 10 of cultivation. The specific ALP activity of non-insulin induced HUMSCs, which served as control, showed a maximum at day 12. The maximum specific ALP activity in the insulin group was 1.3 times as high as that in the control group ($P < 0.05$) (**Figure 2**).

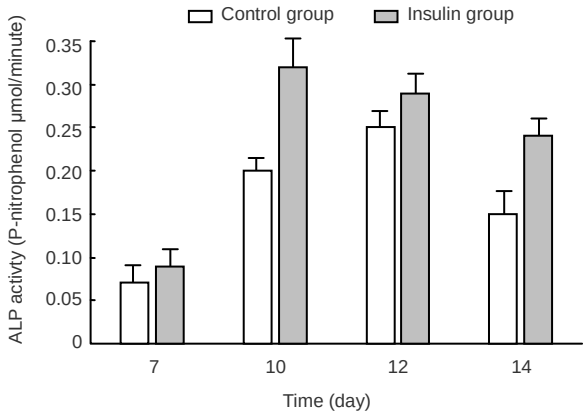

Figure 2 ALP activity of HUMSCs in the two groups
Note: The ALP activity of the insulin group was significantly higher than that of the control group ($P < 0.05$). ALP: Alkaline phosphatase; HUMSCs: human umbilical cord mesenchymal stem cells.

Effect of insulin on the protein expression of type I collagen in HUMSCs

Following 7 days of osteoinduction, type I collagen protein in both groups was positive for immunohistochemical staining, and the cytoplasm was stained brown. The staining of type I collagen protein in the control group was weaker than that in the insulin group (**Figure 3**).

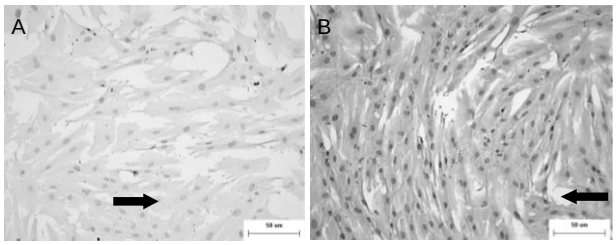

Figure 3 Immunolocalization of type I collagen of HUMSCs
Note: (A) Control group; (B) insulin group. The staining of type I collagen protein in the control group was weaker than that in the insulin group (the arrows show the brown-stained cytoplasm). HUMSCs: Human umbilical cord mesenchymal stem cells.

Effect of insulin on the mRNA expressions of type I collagen and osteocalcin in HUMSCs

Real-time PCR was used to detect the mRNA expression of type I collagen and osteocalcin after 14 days of induction. Insulin-treated HUMSCs appeared to have a significant increase in osteocalcin and type I collagen mRNA compared with the control group ($P < 0.05$).

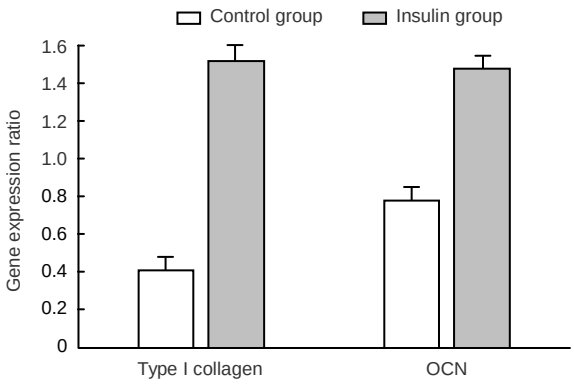

Figure 4 The mRNA expression of OCN and type I collagen in human umbilical cord mesenchymal stem cells
Note: The mRNA expression of OCN and type I collagen in the insulin group was significantly higher than that in the control group ($P < 0.05$). HUMSCs: Human umbilical cord mesenchymal stem cells; OCN: osteocalcin.

DISCUSSION

Studies over the last decade have demonstrated that cells with characteristics similar to bone marrow mesenchymal stem cell reside in all postnatal organs as well as in extra-embryonic tissues available after childbirth[18-19]. Especially, the umbilical cord has been already considered as a useful alternative to the bone marrow[20]. HUMSCs are highly proliferative, possess

distinct osteogenetic and chondrogenic differentiation potential and synthesize a broad spectrum of trophic factors. Therefore, HUMSCs are considered to be the prime candidate for future applications in the regenerative medicine.

Insulin can promote the proliferation and differentiation of osteoblasts and improve the metabolic status of diabetes patients. It can accelerate diabetic fracture healing[21-22]. A new research direction in the treatment of fracture nonunion of diabetic patients is the potential differentiation of HUMSCs to osteoblasts[23]. There are a variety of factors regulating proliferation and differentiation of HUMSCs[24]. Insulin-like growth factor-1 can increase cell proliferation and migratory effects of MSCs and there are some common functions between insulin and insulin-like growth factor-1[25-26]. Consistent with previous results, our experimental data further confirm that insulin promotes HUMSCs proliferation and enhances cell number in cultured HUMSCs models.

ALP is an outcome variable of early osteoblast differentiation or prompt bone calcification[27]. In this study, ALP in HUMSCs of the insulin group was higher than that of the control group, suggesting that insulin could significantly accelerate osteoblast differentiation of HUMSCs. Type I collagen provides the structural framework for connective tissues and plays a key role in the cascade of events leading to the formation of new bone from stem cells or precursor cells[28]. Osteocalcin exhibits hard tissue-specific expression and binding activity to hydroxyapatite. Therefore, to measure secreted osteocalcin is useful for evaluating osteoblastic differentiation in the regenerative bone[29].

Recent studies have showed that insulin can promote type I collagen and osteocalcin expression of osteoblasts[30-31]. Similarly, we found that insulin also accelerate HUMSC osteogenic differentiation. This was manifested by increased type I collagen protein and mRNA, ALP activity and osteocalcin mRNA expression. We speculated that continuous exposure of cells to insulin might induce insulin-like growth factor 1 receptor signaling, activating a variety of downstream genes linked to “the Wnt (a portmanteau of Wingless and integration 1) family and the bone morphogenetic proteins (BMPs)”. Insulin is able to contain the deleterious effect of high concentrations of glucose on BMSC-derived osteoblast proliferation and function and promote the differentiation of BMSCs into osteoblasts[32]. Regrettably, our data fail to provide the molecular mechanism of insulin that accelerates HUMSC osteogenic differentiation.

Our experimental data indicate that insulin can promote HUMSCs proliferation and osteogenic differentiation. Temporally specific addition of insulin for HUMSCs transdifferentiating into osteoblast *in vitro* appears to be an important strategy to enhance osteogenesis. The insulin-treated HUMSCs will be expected as a feasible and powerful tool for osteogenic differentiation of HUMSCs in the treatment of fracture nonunion in diabetic patients.

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胰岛素促进脐带间充质干细胞成骨分化的作用

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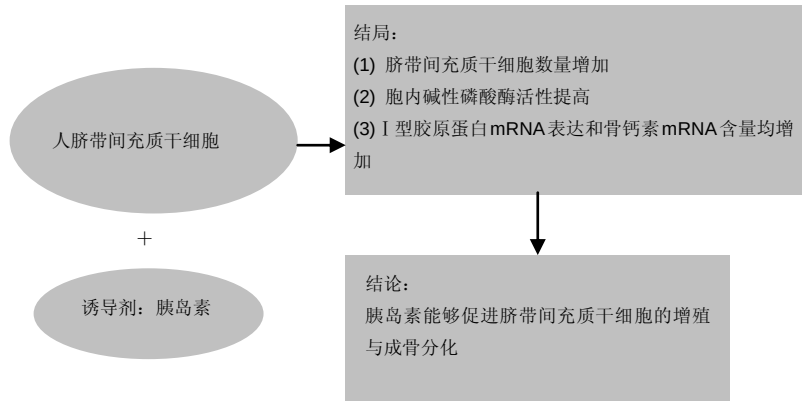
文章快速阅读：

自体骨髓间充质干细胞复合富血小板纤维蛋白促进拔牙窝创口愈合的修复作用



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文题释义：

细胞分化的本质：是基因组在时间和空间上的选择性表达，通过不同基因表达的开启或关闭，最终产生标志性蛋白质。

细胞成骨分化的方法：在细胞培养基中添加维生素D3，抗坏血酸和β甘油磷酸可以促进间充质干细胞分化为成骨细胞。传统的诱导成骨分化的方法效率较低，虽然添加一些生长因子可以促进成骨分化，但是生长因子的费用过高，不适合普遍应用。

摘要

背景：如何有效快速的诱导脐带间充质干细胞成骨分化是当前干细胞研究的重点。既往研究表明生长因子如骨形成蛋白2对脐带间充质干细胞的分化有重要作用，但是生长因子费用较高，不利于普遍使用。胰岛素在细胞培养及诱导过程中广为使用，胰岛素对脐带间充质干细胞有何作用当前未见研究。

目的：观察胰岛素对脐带间充质干细胞成骨分化的影响，探讨脐带间充质干细胞治疗糖尿病性骨折愈合延迟的可行性。

方法：将第3代人脐带间充质干细胞分别接种于2个细胞培养瓶中，分为实验组与对照组。实验组加入 10^{-7} mmol/L 胰岛素的培养液，对照组未加胰岛素培养，采用细胞增殖活性检测试剂盒分别绘制两组细胞的增殖曲线来评价增殖能力，采用细胞碱性磷酸酶活性比色法定量检测碱性磷酸酶的活性，采用免疫细胞化学方法与实时荧光定量聚合酶链反应方法检测Ⅰ型胶原蛋白、mRNA的表达及骨钙素mRNA含量。

结果与结论：诱导一两周后，同对照组相比，实验组脐带间充质干细胞的数量增加；胞内碱性磷酸酶的活性提高，Ⅰ型胶原蛋白mRNA表达和骨钙素mRNA含量增加($P < 0.05$)。结果说明胰岛素能够促进脐带间充质干细胞的增殖与成骨分化。

关键词：

干细胞；脐带脐血干细胞；胰岛素；糖尿病；成骨分化；骨折不愈合；脐带间充质干细胞；Ⅰ型胶原蛋白；骨钙素；细胞增殖；碱性磷酸酶

主题词：

干细胞；组织工程；糖尿病；细胞，培养的

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