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Enhanced Proliferation and Accelerated Adipogenic Differentiation of Mesenchymal Stem Cells via Fe_3O_4-Insulin Conjugates Stimulation

Jiao Lin[1], Ya Zhang[1], Xuewen Lu[1], Zhuo Xiao[1], Yinghui Zhang[2], Shuting Fang[2], Puchang Lie[1*] and Lingwen Zeng[1*]

[1]Key Laboratory of Regenerative Biology, South China Institute for Stem Cell Biology and Regenerative Medicine, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China
[2]School of Food Science and Engineering, Foshan University, Foshan, China

[*]**Corresponding authors:** Lingwen Zeng, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, 190 Kaiyuan Road, Guangzhou Science Park, Guangzhou, China, 510530, Tel: +86 2032015245; Fax: +86 2032015245; E-mail: zeng6@yahoo.com

Puchang Lie, Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences, 190 Kaiyuan Road, Guangzhou Science Park, Guangzhou, China, 510530, Tel: +86 2032015312; Fax: +86 2032015312; E-mail: liepuchang@163.com

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Abstract

The culture of human Umbilical Cord derived Mesenchymal Stem Cells (UC-MSCs), as well as the control of its differentiation toward different tissue lineages, is a very important part of regenerative medicine. In the present work, a systematic study of Fe_3O_4 nanoparticles and its insulin conjugates for growth and differentiation of UC-MSCs were carried out for the first time. The cell viability assay was performed using a number of analytical methods including MTT assay and cumulative population doubling time assay. Fe_3O_4-insulin conjugates were prepared and used for UC-MSC adipogenic differentiation. Results showed that the strong ability of Fe_3O_4-insulin conjugates allow it to act as a preconcentration platform for adipogenic induction, which accelerates MSC growing on it toward the adipogenic lineage. It was also found that the Fe_3O_4 nanoparticles had no effect on UC-MSC cellular phenotype. This work demonstrates that the biological activity of insulin is not affected when conjugated to Fe_3O_4 nanoparticles, which would help to enhance the growth and adipogenic differentiation of mesenchymal stem cells.

Keywords

Adipogenic Differentiation; Cell Proliferation; Fe_3O_4 Nanoparticles; Fe_3O_4-insulin Conjugates; Insulin; UC-MSCs

Introduction

Mesenchymal Stem Cells (MSCs) comprise a population of multipotent progenitors which can be originally isolated from bone marrow, adipose tissue, muscle, placenta, umbilical cord and umbilical cord blood [1,2]. Under appropriate microenvironments, MSCs can differentiate into multiple cell types, including adipocytes, chondrocytes, and cardiomyocytes, also into non-mesodermal-derived cells, including hepatocytes and neurons [3-6]. Recently, MSCs are being evaluated for their use in tissue engineering and cell-based therapies because of their no ethical restriction and low immunogenicity [7,8].

Obesity has been a common health problem worldwide and it is considered a major risk for a variety of metabolic disorders, such as diabetes mellitus, cardiovascular diseases, cholelithiasis, fatty liver disease and so on. Studies have shown that adipose differentiation and regulation disorders have been associated with the pathological processes involved in diabetes mellitus, fatty liver, hyperlipidemia and certain cancers [9]. Therefore, it's essential to study the mechanism and regulation of adipogenic differentiation for the prevention and treatment of these diseases and related drug screening. Furthermore, adipose tissue regeneration technology, which is a hot field of regenerative medicine, is required by the regeneration and repair of adipose tissue loss and damage due to

aging or pathological factors. And also, adipocytes are the source of seeding cells and the driving force of adipose tissue regeneration. Both of the above applications need effective and stable adipogenic differentiation method to obtain large quantity and high-quality adipocytes. However, the current differentiation strategy, which employs growth factor inducers, is of low efficiency and often needs weeks of culture for maturation of the specific lineage.

Recently, there are accumulating clues indicating that nanomaterials with particular properties and biocompatibility can modulate stem cell function, such as proliferation and differentiation [5,10]. For instance, nano-scaled materials such as polymeric, liposomal and silica nanoparticles act as potential carriers for delivery of genes, drugs or bioactive factor for stem cell differentiation [11]. With clear formation of neurites on layer-by-layer assembled single-walled carbon nanotube, mouse embryonic neural stem cells from the cortex were successfully differentiated into neurons, astrocytes and oligodendrocytes [12,13]. Dexamethasone-loaded carboxymethylchitosan/poly(amidoamine) dendrimer was designed to enhance the osteogenesis for rat bone marrow stromal cells *in vitro* [14,15]. Gold nanoparticles promote osteogenic differentiation of mesenchymal stem cells through a stress mechanism relied on p38/MAPK signaling pathway [16].

Fe_3O_4 nanoparticles, a member of Superparamagnetic Iron Oxide (SPIO) nanoparticles that are emerging as an ideal MRI probe for *in vivo* cell tracking and cellular imaging, have recently drawn attention as a means to increase cell growth, which can accelerate cell cycle progression by the free Iron (Fe) released from lysosomal degradation [17-19]. The research conducted by Song and Schafe respectively, showed that the cartilage and osteogenic differentiation of mesenchymal stem cells were not affected by SPIO [20,21]. However, whether Fe_3O_4 nanoparticles have effect on MSC adipogenic differentiation remains unclear. Herein, we reported a systematic study on the effects of the Fe_3O_4 nanoparticles and Fe_3O_4-insulin conjugates on the adipogenic differentiation of UC-MSCs. Adipogenic assays revealed that pure Fe_3O_4 nanoparticles had no effect on cellular ultrastructure and adipogenic differentiation. Under the optimized Fe_3O_4 nanoparticle concentration that has little cytotoxicity on UC-MSCs and no effects on the UC-MSC phenotype, Fe_3O_4-insulin conjugates were prepared and used for UC-MSC adipogenic differentiation. Results showed that the biological activity of insulin was not affected when conjugated to Fe_3O_4 nanoparticles, which enhanced the growth and adipogenic differentiation of UC-MSCs.

Materials and Methods

All experiments were performed in compliance with the relevant laws and institutional guidelines and Guangzhou Institutes of Biomedicine and Health Ethical Committee have approved the experiments. All volunteer mothers who donated umbilical cord samples have provided their written informed consent and the Ethics Committees have approved this consent procedure.

Materials

Indomethacin (Cat#:I7378), dexamethasone (Cat#:D4902), 3-Isobutyl-1-methylxanthine (IBMX, Cat#:I7018), insulin solution (Cat#:I9278) and Oil Red O (Cat#:O0625) were obtained from Sigma-Aldrich. Dulbecco's Modified Eagle's Medium-Low Glucose (DMEM-LG, Cat#:11885084), Fetal Bovine Serum (FBS, Cat#:10270-106), glutamine (Cat#:25030081) and Penicillin-Streptomycin (Cat#:15070063) were purchased from Gibco Corporation. Basic fibroblast growth factor (Cat#:RP-10915) was obtained from Invitrogen. Typan Blue Staining Cell Viability Assay Kit (Cat#:C0011) and MTT Cell Proliferation and Cytotoxicity Assay Kit (Cat#:C0009) were purchased from Beyotime Biotechnology.

Methods

Preparation of Fe_3O_4 nanoparticles and Fe_3O_4-insulin conjugates: Fe_3O_4 nanoparticles were prepared by chemical precipitation method [22]. Briefly, dissolving $FeCl_3 \cdot 6H_2O$ and $FeCl_2 \cdot 4H_2O$ iron salts separately in distilled water at 80°C to prepare 1M Fe^{3+} and 0.5M Fe^{2+} solution. Mixing the two solutions continuously and 25% ammonia water solution was added, following oleic acid addition 1 minute later. The reaction proceeded for 1h at 80°C. After then, the Fe_3O_4 nanoparticles were washed twice with ethanol and many more times with water until they reached a neutral pH level. Adding a water solution of $KMnO_4$ into the Fe_3O_4 nanoparticles and sonicated for 8h and washed with water. Finally, the Fe_3O_4 nanoparticles were separated through magnetic enrichment and the resulted Fe_3O_4 nanoparticles were carboxyl modified, which made them showed negatively charged.

The Fe_3O_4 nanoparticles were then incubated with insulin for 16h at 4°C and insulin was conjugated to the Fe_3O_4 nanoparticles through electrostatic adherence. In this case, the release of insulin is facile and faster in the biological system as reported previously [23]. Following repetitive washing of Fe_3O_4-insulin conjugates and removal of unconjugated insulin by magnetic enrichment, the Fe_3O_4-insulin conjugates were resuspended in PBS for future usage. The unconjugated insulin presented in the supernatant was determined using the Bradford method. The amount of conjugated insulin was calculated according to the formula below [24]: $q=(W_{ti}-W_{si})/W_{Fe_3O_4}$

where q was the amount of insulin conjugated onto a unit mass of Fe_3O_4 (mg/mg). W_{ti}, W_{si}, and $W_{Fe_3O_4}$ represented the total amount of insulin added, the amount of insulin in supernatant after conjugation, and the mass of Fe_3O_4, respectively.

Isolation and culture of UC-MSCs: Umbilical Cords (UCs) were aseptically collected from full-term cesarean-section patients with their consent at Guangzhou Red Cross Hospital. UC-MSC isolation was performed as described previously with some modifications. Briefly, Wharton's jelly was cut into 2 to 3 mm^3 pieces, and then cultured in a 37°C incubator in a 175 culture flask with growth medium containing DMEM-LG supplemented with 10%(v/v) FBS, 2mM glutamine, 5 ng/mL basic fibroblast growth factor, 100 U/mL penicillin, 100 mg/mL streptomycin, and 1 mg/mL amphoterin B. After 10-14 days of culture, UC-MSCs migrated from tissues. When cells reached 90-95% confluence, cells were harvested with 0.25% trypsin. The medium was changed every 3 days.

Cellular assays for UC-MSCs cultured with and without Fe_3O_4 nanoparticles: To test the effect of pure Fe_3O_4 nanoparticles and the optimum concentration on UC-MSC viability and proliferation, MTT reduction assay, cumulative population doubling time and

morphology tests were carried out using a method published previously [25]. Briefly, UC-MSCs were seeded in a 96-well tissue culture plate at the density of 4×10^4 cells per well and incubated for 3 days. After the addition of Fe_3O_4 nanoparticles at different concentrations (50, 100, 200, 300 and 400 nM), the cells were further incubated for 24h, 48h and 72h. Then, the adherent cells were harvested for MTT assay. Briefly, 20 µl of MTT (5.0 mg/ml in PBS) was added and incubated for another 4 h at 37°C. The supernatant was removed and DMSO was added and incubated for 10 min. After all crystals were dissolved, the optical density of each well was measured at 570 nm using a microplate reader. Cells without treatment were used as control.

Cells were cultured for 24h, 48h, and 72h, and were harvested with trypsin and counted after staining with trypan blue. The mean value of cell counts was calculated and mean Population Doubling time (PD) was obtained for each passage according to the formula: $PD=(lgN_t - lgN_0)/lg2$, where N_0 is the initial cell number and N_t is the harvested cell number.

In addition, the effects of pure Fe_3O_4 nanoparticles on the morphology of UC-MSCs were also investigated. Briefly, cells were cultured in a monolayer for 24 h and fed with pure Fe_3O_4 nanoparticles at different concentrations (50, 100, 200, 300 and 400 nM) for another 72 h. Then, the media was removed, and the cells were washed with PBS and observed under the microscope. Cells without treatment were used as control.

Adipogenic differentiation: Cells were induced to differentiate into adipocytes as described previously with some modifications [26]. Briefly, cells were cultured in a 24-well plate at a density of 1×10^4 cells/cm^2. After cells reached 70% confluence, the growth medium was changed to traditional adipogenic differentiation medium consisting of DMEM-LG supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 60 mM indomethacin, 1 mM dexamethasone, 0.5 mM IBMX, and 5 mg/mL insulin solution or adipogenic differentiation medium consisting of DMEM-LG supplemented with Fe_3O_4-insulin conjugates instead of insulin alone and other compounds same to the traditional adipogenic differentiation medium. Cells induced with traditional method and our new method which means the induction medium was supplemented with Fe_3O_4-insulin conjugates instead of insulin alone were incubated for indicated days, and the medium was changed every 3 days. Generation of neutral lipid vacuoles was visualized by staining with Oil Red O.

Real-Time Polymerase Chain Reaction (Real Time RT-PCR): Total RNA was isolated with RN easy mini kit following manufacturer's instructions and treated with DNase I. Complementary DNA synthesis was performed with 1 µg of total RNA using Prime Script RTase. Real-time PCR was conducted as follows: 95°C, 15 seconds; 60°C, 15 seconds; 72°C, 30 seconds for 40 cycles using ABI 7300 Real-Time PCR System. Expression level of b-actin gene was used as the internal control.

The PCR primers and probes were as follows:

hPPAR-γ2 sense: 5'-GCGAGGATAGTTCTGGAA-3'

hPPAR-γ2 antisense: 5'-GGATAAGTCACCGAGGAG-3'

Image analysis: Image analysis was carried out following a previously published method [25]. Briefly, at least three random fields of each of triplicate wells of Oil Red O assay were photographed and the software Image J downloaded from National Institutes of Health was used for measurement of adipocyte percentage. Briefly, color images were converted to eight-bit gray-scale and binarized or thresholded to gain good contrast and brightness against the background. Adipocyte percentage was calculated according to the number of pixels in the thresholded images.

Statistical analysis: Data were expressed as mean±standard error of the mean. Statistical comparisons were performed using Student's t-test. * means p-value < 0.05 and were considered statistically significant, ** means $0.01 < p$-value < 0.05 and *** means $0.001 < p$-value < 0.01.

Results

Effects of Fe_3O_4 nanoparticles on UC-MSC proliferation

The principle of MSC induction was shown in figure 1. Fe_3O_4-insulin conjugates were synthesized according to the electrostatic adsorption. When added to the cultured cells, they entered the cells through endocytosis, then Fe^{2+} and Fe^{3+} produced by Fe_3O_4 nanoparticles in the cells can promote cell proliferation.

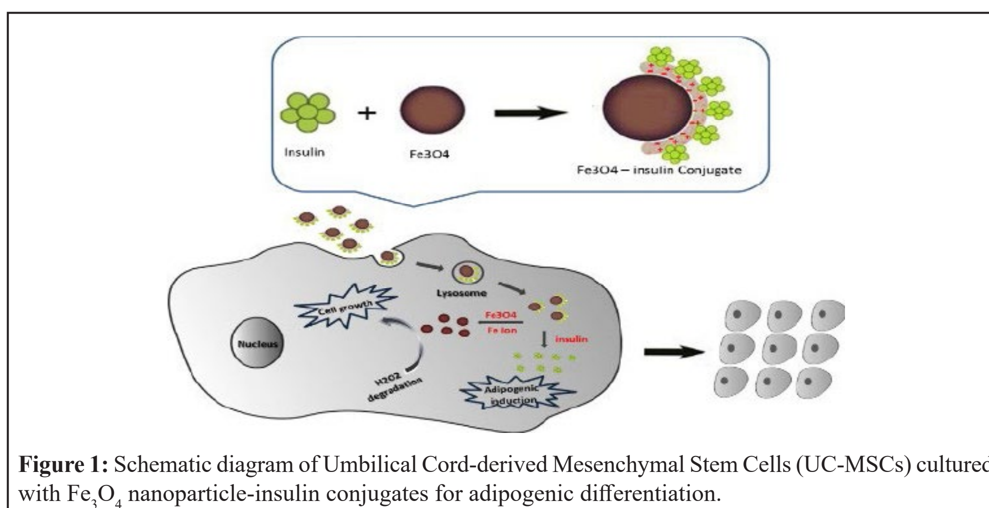

Figure 1: Schematic diagram of Umbilical Cord-derived Mesenchymal Stem Cells (UC-MSCs) cultured with Fe_3O_4 nanoparticle-insulin conjugates for adipogenic differentiation.

After cells migrated out from small pieces of umbilical cord Wharton's jelly during primary culture, they were cultured with and without Fe_3O_4 nanoparticles. As shown in figure 2A, both UC-MSCs cultured with and without Fe_3O_4 nanoparticles showed similar fibroblast-like morphology.

On the basis of the observed cellular effects and previous reports, we hypothesized that Fe_3O_4 nanoparticles may interact with cells by membrane adsorption and subsequent internalization through endocytosis, possibly serving as mechanical stimuli on UC-MSCs to activate mechanosensitive signaling pathway in the cells and thus induce adipogenic differentiation. Previous study showed that all cells require iron for the proper uptake of oxygen from their environment and mammalian cells require sufficient amounts of iron to satisfy metabolic needs or to accomplish specialized functions. Figures 2B and C shows that Fe stimulates growth-related enzyme activity in UC-MSCs.

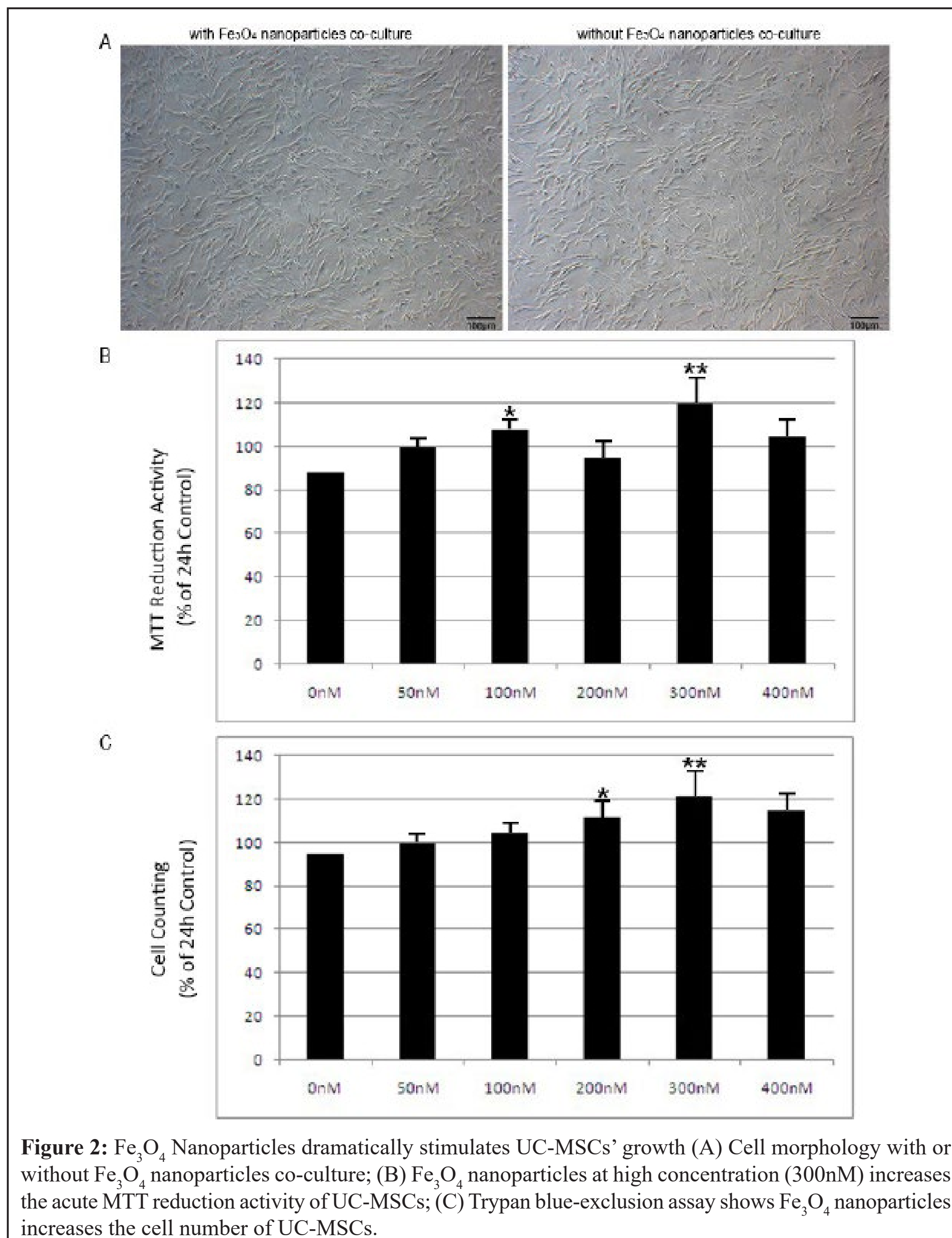

Figure 2: Fe_3O_4 Nanoparticles dramatically stimulates UC-MSCs' growth (A) Cell morphology with or without Fe_3O_4 nanoparticles co-culture; (B) Fe_3O_4 nanoparticles at high concentration (300nM) increases the acute MTT reduction activity of UC-MSCs; (C) Trypan blue-exclusion assay shows Fe_3O_4 nanoparticles increases the cell number of UC-MSCs.

Effects of Fe_3O_4-insulin conjugates on adipogenic differentiation of UC-MSCs

As previously reported, insulin is one of the important supplements of adipogenic induction and adipogenic maintenance media [27-29]. In the present study, the effects of Fe_3O_4-insulin conjugates on UC-MSC adipogenic differentiation have been investigated The human umbilical cord derived MSCs were cultured with traditional adipogenic induction medium and basal medium supplemented with Fe_3O_4-insulin conjugates, respectively. Figures 3 (A-C) shows Oil Red O staining of adipocytes derived from human umbilical cord MSCs cultured with traditional adipogenic induction

culture medium or basal medium supplemented with Fe_3O_4-insulin conjugates. The results demonstrate that Fe_3O_4-insulin conjugates promote the differentiation of UC-MSCs toward adipocytes with increased oil formation (Figure 3C).

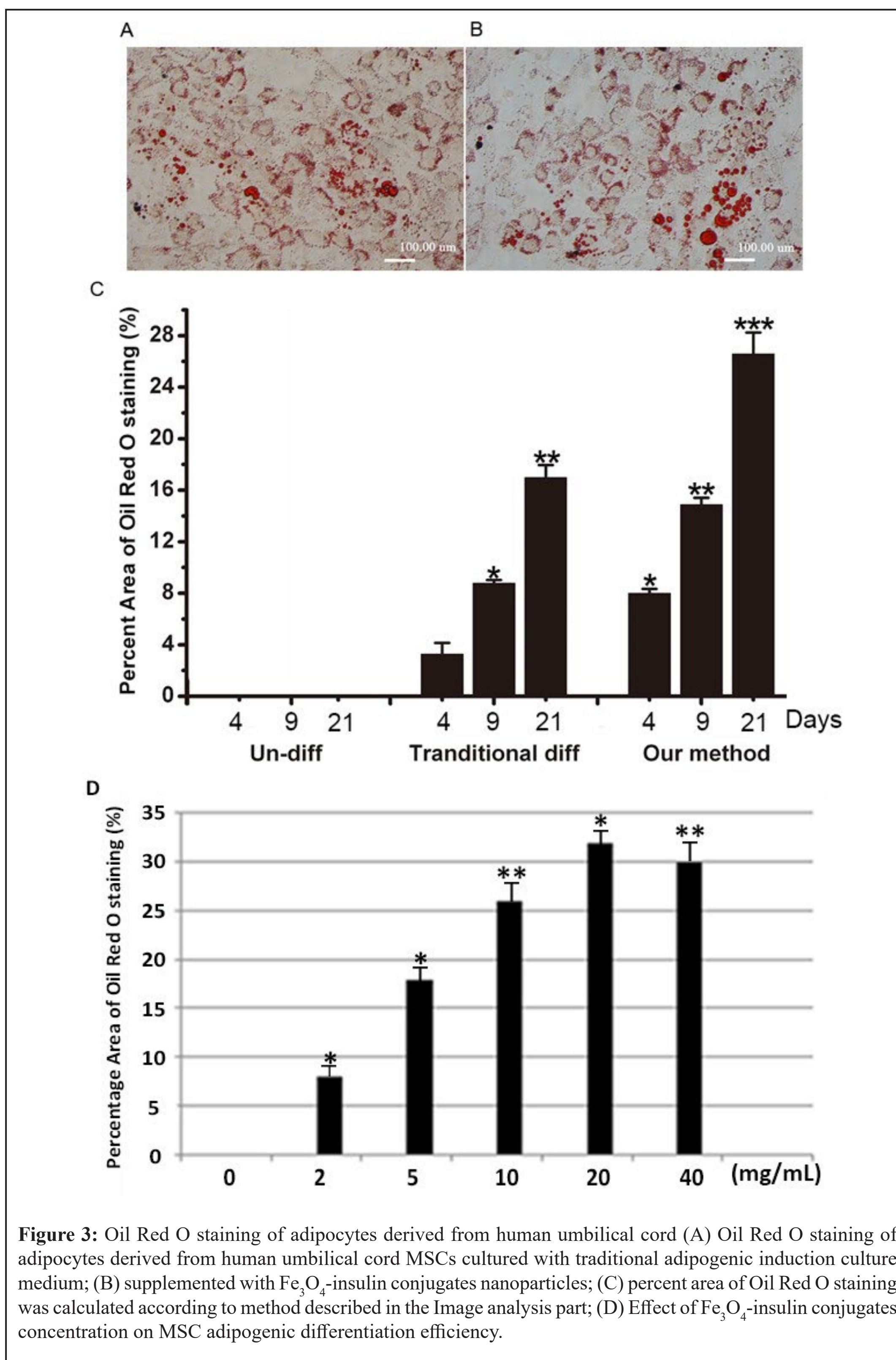

Figure 3: Oil Red O staining of adipocytes derived from human umbilical cord (A) Oil Red O staining of adipocytes derived from human umbilical cord MSCs cultured with traditional adipogenic induction culture medium; (B) supplemented with Fe_3O_4-insulin conjugates nanoparticles; (C) percent area of Oil Red O staining was calculated according to method described in the Image analysis part; (D) Effect of Fe_3O_4-insulin conjugates concentration on MSC adipogenic differentiation efficiency.

The percent area of Oil Red O staining (+) were also calculated to further evaluate the effects of Fe_3O_4-insulin conjugates on adipogenic differentiation of UC-MSCs, after being cultured in different culture medium for different periods of time, the cells were treated with Oil Red O staining of the lipid vacuoles and the percent area of Oil Red O staining of UC-MSCs were calculated. When cultured with traditional method, 18% of UC-MSCs formed adipocyte colonies, while 26% of UC-MSCs formed adipocyte

colonies when cultured with Fe_3O_4-insulin conjugates (Figure 3C). As shown in figure 3C, the UC-MSCs had large quantity of lipid vacuoles after induction for 9 days whereas the traditional differentiation method needed 21 days to reach similar amount of lipid vacuoles.

To address the effect of Fe_3O_4-insulin conjugates on MSC adipogenic differentiation, we also did experiments with different Fe_3O_4-insulin conjugates concentration and to Figureure out how they affect cell differentiation efficiency. According to the calculating method showed in the method part, the amount of conjugated insulin was 400ng (insulin)/ng (Fe_3O_4). Considering that high concentration of Fe_3O_4- (as high as 400nM) do harm to the cells, we evaluated the effects of different concentration Fe_3O_4-insulin on adipogenic differentiation ranging from 0 to 40ug (insulin)/100ng (Fe_3O_4). Because 100ng/mL Fe_3O_4 almost equal to 400nM Fe_3O_4. In the experiments, we use insulin concentration to show the concentration of Fe_3O_4-insulin conjugates, and the results showed that after 21 days' induction and Oil Red O staining, percentage area of the staining was increase following the increase of insulin concentration until 20mg/ml (Figure 3D). And when it comes to 40mg/mL, the efficiency is lower than that of 20mg/mL. The results indicated that 20mg/mL of Fe_3O_4-insulin concentration produces the highest efficiency in MSC adipogenic differentiation induction.

Expression analysis of adipogenic-specific marker by real-time RT-PCR

The messenger RNA expression of the adipogenic-specific marker *hPPAR-γ2* was analyzed by real-time RT-PCR. The marker *hPPAR-γ2* gene was not expressed before MSCs were incubated in the adipogenic differentiation medium. Expression was detected after the cells were incubated in the adipogenic differentiation medium for different periods of time, and the expression of this marker was several times higher in Fe_3O_4-insulin conjugates containing medium than in the traditional differentiation medium (Figure 4).

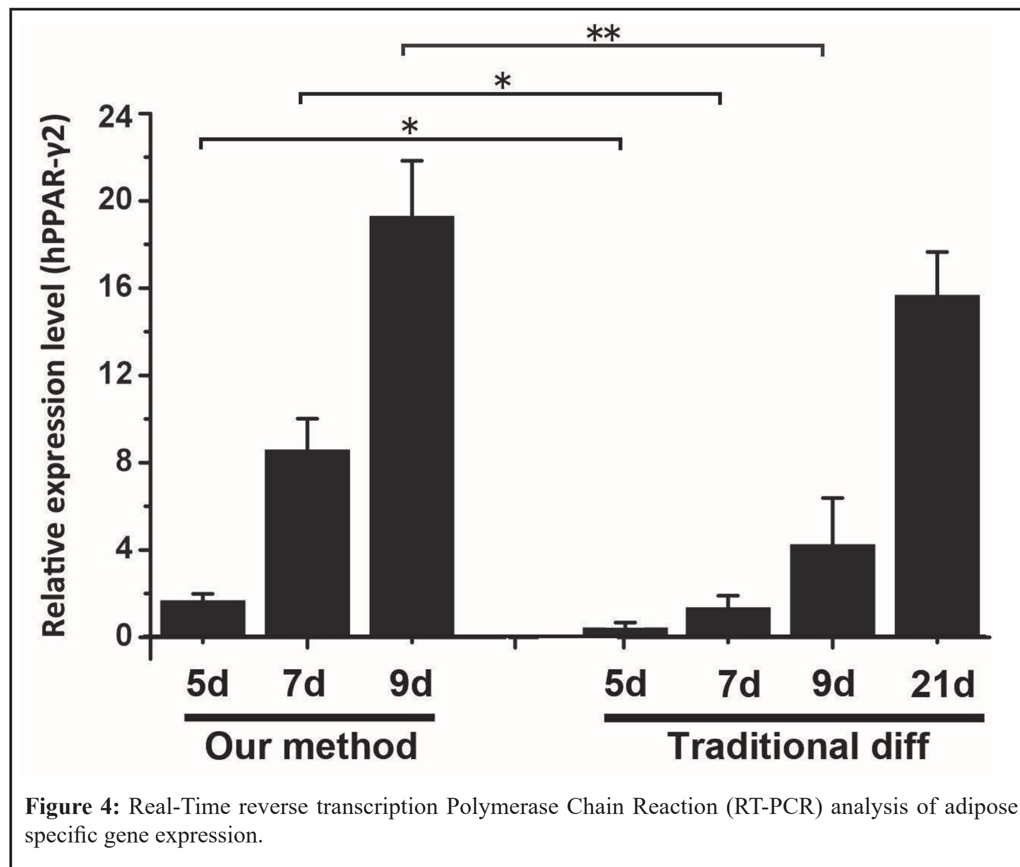

Figure 4: Real-Time reverse transcription Polymerase Chain Reaction (RT-PCR) analysis of adipose specific gene expression.

Discussion

When given suitable stimulations, the human UC-MSCs in both Fe_3O_4-insulin conjugates containing culture medium and transitional induction medium can differentiate into adipocyte-like cells. However, the present study demonstrates that when UC-MSCs cultured with Fe_3O_4-insulin conjugates, almost every cell in the spheroids contained Oil Red O positive lipids, whereas only a small number of the traditional induction cultured cells stained positively. Fe_3O_4 nanoparticles has no effect on UC-MSC cellular phenotype and Fe_3O_4-insulin conjugates accelerate UC-MSC growing on it toward the adipogenic lineage, which would help to enhance the growth and adipogenic differentiation of mesenchymal stem cells. When cultured in traditional method, 18% of UC-MSCs formed adipocyte colonies. However, 26% of UC-MSCs formed adipocyte colonies when cultured with Fe_3O_4-insulin conjugates. The UC-MSCs had large quantity of lipid vacuoles after induction for 9 days whereas the traditional differentiation method needed 21 days to reach similar amount of lipid vacuoles. Real Time (RT) PCR revealed that the expression of adipocyte specific marker was

several times higher in Fe_3O_4-insulin conjugates induction method than in the traditional differentiation method demonstrating that Fe_3O_4-insulin conjugates can dramatically accelerate adipogenic differentiation of UC-MSCs. According to the previous report [23], we hypothesized that cells uptake Fe_3O_4-insulin conjugates through endocytosis and the efficiency is higher than that of insulin alone, and the release of insulin from Fe_3O_4-insulin conjugates in cells are easy for the conjugates are formed through electrostatic adherence. In conclusion, mesenchymal stem cells can get enough insulin at a short time to promote adipogenic differentiation when incubated with Fe_3O_4-insulin conjugates, which result in higher adipogenic differentiation efficiency. The short induction period can satisfy the scientific utility and can get to the wanted adipogenic differentiation state within a short time, which will promote the adipogenic-related study. So the method is time saving and may also promote the clinical study of some disease which need adipose tissue transplantation.

Conclusion

In our work, a systematic study of Fe_3O_4 nanoparticles and its insulin conjugates for growth and differentiation of UC-MSCs were carried out for the first time. And we verified that Fe_3O_4-insulin conjugates promote adipocyte differentiation of UC-MSCs better than that of traditional induction medium. The underlying mechanism maybe that cells are more easily to uptake Fe_3O_4-insulin conjugates through endocytosis and the insulin can release from the conjugates to play roles in cell differentiation in the cells [23].

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