Enhanced Proliferation and Accelerated Adipogenic Differentiation of Mesenchymal Stem Cells via Fe$_3$O$_4$-Insulin Conjugates Stimulation

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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$_3$O$_4$ 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$_3$O$_4$-insulin conjugates were prepared and used for UC-MSC adipogenic differentiation. Results showed that the strong ability of Fe$_3$O$_4$-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$_3$O$_4$ 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$_3$O$_4$ nanoparticles, which would help to enhance the growth and adipogenic differentiation of mesenchymal stem cells.

Keywords

Adipogenic Differentiation; Cell Proliferation; Fe$_3$O$_4$ Nanoparticles; Fe$_3$O$_4$-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 non-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$_3$O$_4$ 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$_3$O$_4$ nanoparticles have effect on MSC adipogenic differentiation remains unclear. Herein, we reported a systematic study on the effects of the Fe$_3$O$_4$ nanoparticles and Fe$_3$O$_4$-insulin conjugates on the adipogenic differentiation of UC-MSCs. Adipogenic assays revealed that pure Fe$_3$O$_4$ nanoparticles had no effect on cellular ultrastructure and adipogenic differentiation. Under the optimized Fe$_3$O$_4$ nanoparticle concentration that has little cytotoxicity on UC-MSCs and no effects on the UC-MSC phenotype, Fe$_3$O$_4$-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$_3$O$_4$ 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#:17378), 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$_3$O$_4$ nanoparticles and Fe$_3$O$_4$-insulin conjugates:** Fe$_3$O$_4$ nanoparticles were prepared by chemical precipitation method [22]. Briefly, dissolving FeCl$_3$·6H$_2$O and FeCl$_2$·4H$_2$O iron salts separately in distilled water at 80℃ 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℃. After then, the Fe$_3$O$_4$ 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$_3$O$_4$ nanoparticles and sonicated for 8h and washed with water. Finally, the Fe$_3$O$_4$ nanoparticles were separated through magnetic enrichment and the resulted Fe$_3$O$_4$ nanoparticles were carboxyl modified, which made them showed negatively charged.

The Fe$_3$O$_4$ nanoparticles were then incubated with insulin for 16h at 4℃ and insulin was conjugated to the Fe$_3$O$_4$ 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$_3$O$_4$-insulin conjugates and removal of unconjugated insulin by magnetic enrichment, the Fe$_3$O$_4$-insulin conjugates were suspended 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]:

\[
q = \frac{(W_{ins} - W_{ins}^\prime)}{W_{Fe_3O_4}}
\]

where $q$ was the amount of insulin conjugated onto a unit mass of Fe$_3$O$_4$ (mg/mg). $W_{ins}$, $W_{ins}^\prime$, 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$_3$O$_4$, 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℃ 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$_3$O$_4$ nanoparticles:** To test the effect of pure Fe$_3$O$_4$ nanoparticles and the optimum concentration on UC-MSC viability and proliferation, MTT reduction assay, cumulative population doubling time and
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 Fe₃O₄ nanoparticles on UC-MSC proliferation

The principle of MSC induction was shown in figure 1. Fe₃O₄-insulin conjugates were synthesized according to the electrostatic adsorption. When added to the cultured cells, they entered the cells through endocytosis, then Fe²⁺ and Fe³⁺ produced by Fe₃O₄ nanoparticles in the cells can promote cell proliferation.
After cells migrated out from small pieces of umbilical cord Wharton’s jelly during primary culture, they were cultured with and without Fe$_3$O$_4$ nanoparticles. As shown in figure 2A, both UC-MSCs cultured with and without Fe$_3$O$_4$ nanoparticles showed similar fibroblast-like morphology.

On the basis of the observed cellular effects and previous reports, we hypothesized that Fe$_3$O$_4$ 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$_3$O$_4$ Nanoparticles dramatically stimulates UC-MSCs’ growth (A) Cell morphology with or without Fe$_3$O$_4$ nanoparticles co-culture; (B) Fe$_3$O$_4$ nanoparticles at high concentration (300nM) increases the acute MTT reduction activity of UC-MSCs; (C) Trypan blue-exclusion assay shows Fe$_3$O$_4$ nanoparticles increases the cell number of UC-MSCs.

**Effects of Fe$_3$O$_4$-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$_3$O$_4$-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$_3$O$_4$-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$_3$O$_4$-insulin conjugates. The results demonstrate that Fe$_3$O$_4$-insulin conjugates promote the differentiation of UC-MSCs toward adipocytes with increased oil formation (Figure 3C).

The percent area of Oil Red O staining (+) were also calculated to further evaluate the effects of Fe$_3$O$_4$-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

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$_3$O$_4$-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$_3$O$_4$-insulin conjugates concentration on MSC adipogenic differentiation efficiency.
colonies when cultured with Fe₃O₄-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 Fe₃O₄-insulin conjugates on MSC adipogenic differentiation, we also did experiments with different Fe₃O₄-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 (Fe₃O₄). Considering that high concentration of Fe₃O₄ (as high as 400nM) do harm to the cells, we evaluated the effects of different concentration Fe₃O₄-insulin on adipogenic differentiation ranging from 0 to 40ug (insulin)/100ng (Fe₃O₄). Because 100ng/mL Fe₃O₄ almost equal to 400nM Fe₃O₄. In the experiments, we use insulin concentration to show the concentration of Fe₃O₄-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 Fe₃O₄-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 Fe₃O₄-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 Fe₃O₄-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 Fe₃O₄-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. Fe₃O₄ nanoparticles has no effect on UC-MSC cellular phenotype and Fe₃O₄-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 Fe₃O₄-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 Fe₃O₄-insulin conjugates induction method than in the traditional differentiation method demonstrating that Fe₃O₄-insulin conjugates can dramatically accelerate adipogenic differentiation of UC-MSCs. According to the previous report [23], we hypothesized that cells uptake Fe₃O₄-insulin conjugates through endocytosis and the efficiency is higher than that of insulin alone, and the release of insulin from Fe₃O₄-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 Fe₃O₄-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 Fe₃O₄ nanoparticles and its insulin conjugates for growth and differentiation of UC-MSCs were carried out for the first time. And we verified that Fe₃O₄-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 Fe₃O₄-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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