Differentiation of human adipose-derived stem cells into neuron-like cells by *Radix Angelicae Sinensis**

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Research Highlights

1. *Radix Angelicae Sinensis*, which has antioxidant effects and no known toxicity, is used as an inducer to promote adipose-derived stem cell differentiation into neuron-like cells.

2. The results show that not only can *Radix Angelicae Sinensis* induce differentiation of human adipose-derived stem cells into neuron-like cells, it can also increase their survival rate, thus opening up new ideas for optimal induction methods of stem cells.

Abstract

Human adipose tissues are an ideal source of stem cells. It is important to find inducers that can safely and effectively differentiate stem cells into functional neurons for clinical use. In this study, we investigate the use of *Radix Angelicae Sinensis* as an inducer of neuronal differentiation. Primary human adipose-derived stem cells were obtained from adult subcutaneous fatty tissue, then pre-induced with 10% *Radix Angelicae Sinensis* injection for 24 hours, and incubated in serum-free Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 containing 40% *Radix Angelicae Sinensis* to induce its differentiation into neuron-like cells. Butylated hydroxyanisole, a common inducer for neuronal differentiation, was used as the control. After human adipose-derived stem cells differentiated into neuron-like cells under the induction of *Radix Angelicae Sinensis* for 24 hours, the positive expression of neuron-specific enolase was lower than that of the butylated hydroxyanisole-induced group, and the expression of glial fibrillary acidic protein was negative. After they were induced for 48 hours, the positive expression of neuron specific enolase in human adipose-derived stem cells was significantly higher than that of the butylated hydroxyanisole-induced group. Our experimental findings indicate that *Radix Angelicae Sinensis* can induce human adipose-derived stem cell differentiation into neuron-like cells and produce less cytotoxicity.

Key Words

neural regeneration; stem cells; human adipose-derived stem cells; *Radix Angelicae Sinensis*; neuron-like cells; differentiation; adipocytes; cytotoxicity; grants-supported paper; neuroregeneration

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Author contributions: Wang QZ and Zhou LL performed the majority of experiments. Guo Y, Liu GY and Cheng JY conducted the experiments and analyzed the data. Wang QZ wrote the manuscript. Yu H provided financial support, designed the study and revised the manuscript. All authors approved the final version of the paper.

Conflicts of interest: None declared.
INTRODUCTION

The discovery of neural stem cells has started an epoch of neural restoration research and there has been great progress in transplantation therapy for central nervous system diseases[1]. However, the use of neural stem cells is greatly limited because the majority of cells are derived from aborted fetuses. This gives rise to an ethical controversy[2]. Therefore, it is very important to find new sources of stem cells.

Mesenchymal stem cells derived from embryonic mesoderm are characterized by their multi-differentiation potential and capability of self-renewal. Mesenchymal stem cells can also be isolated from adult adipose tissues. As a new kind of seed cells, their application potential is broad and greatly eases the ethical dilemma. Adipose tissue can be easily obtained and sources are abundant[3-4].

In recent years, it has been found that adipose-derived stem cells have the potential to transform into neural stem cells and neurons[5]. The common induction method is to use a chemical agent or oxidizing agent, such as retinoic acid, dimethyl sulfoxide, β-mercaptoethanol and glycerin[5]. However, some antileptics cannot be widely applied to the human body because of certain toxicity. Currently, there are no reports on the induction effects of *Radix Angelicae Sinensis*, which is a newly emerged Chinese herb that has clinical curative effects on diseases of the nervous system[6].

In this study, we aim to explore the effects of *Radix Angelicae Sinensis* on human adipose-derived stem cell differentiation into neuron-like cells.

RESULTS

Morphology and identification of human adipose-derived stem cells

Cell morphology

Adipose-derived stem cells were successfully obtained and cultured from human adipose tissue. The cells had fibroblast-like morphology and rapidly proliferated *in vitro*. The proliferative phase of cultured cells was 7 days, and subsequently the cells entered the plateau phase on day 10. When primary cells reached 80–90% confluence (Figure 1), the cells spontaneously initiated adipogenic direction differentiation. Passage cells maintained their spindle shape without spontaneous differentiation.

Identification of immunophenotype

Immunocytochemical detection (Figure 2) of CD44- and vimentin-positive adipose-derived stem cells showed that the passage 3 cells highly expressed CD44 (85.4 ± 3.22%) and vimentin (97.1 ± 2.3%).

Cell morphology after neurogenic differentiation

Angelica-induced group: After adipose-derived stem cells were pre-induced for 24 hours, cell morphology showed no significant change. After being induced for 12 hours, a few cells retracted towards the nucleus. At 24 hours of induction, cell bodies extended short processes, displayed neuronal morphologies and formed bipolar or multi-polar processes (Figure 3A). At 48 hours of induction, the number of neuron-like cells did not significantly increase, and some of them intersected with others in the form of a network (Figure 3C).

Butylated hydroxyanisole-induced group: After adipose-derived stem cells were pre-induced for 24 hours, cell morphology showed no significant change. After being induced for 6 hours, some cells increased refraction
and extended short processes. At 12 hours, cells displayed neuronal morphologies. At 24 hours, small particles appeared in the cytoplasm of neuron-like cells (Figure 3B). At 48 hours, the number of neuron-like cells did not increase, whereas the number of shrinking cells increased, and some cells began to detach (Figure 3D).

Control group: The cells showed no significant proliferation and maintained a flat shape.

**Neuron-specific enolase and glial fibrillary acidic protein expression after neurogenic differentiation**

After the cells were induced for 24 and 48 hours, neuron-specific enolase (a specific marker of neurons)-positive cells were visible under a fluorescence microscope (Figure 4A). Glial fibrillary acidic protein showed no positive expression in all three groups (Figure 4B). After 24 hours of induction, the percentage of neuron-specific enolase-positive cells was lower in the angelica-induced group than that of the butylated hydroxyanisole-induced group, but after 48 hours, the percentage was significantly higher in the angelica-induced group ($P < 0.05$; Table 1).

**Survival rate of cells after neurogenic differentiation**

Trypan blue staining showed that the cell survival rate in the angelica-induced group was significantly higher than the butylated hydroxyanisole-induced group ($P < 0.05$) after 24 and 48 hours of induction (Table 2).

![Figure 3](image)

**Table 1** Effect of angelica induction on the percentage (%) of neuron-specific enolase positive cells in human adipose-derived stem cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Induction 24 hours</th>
<th>Induction 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angelica-induced</td>
<td>24.82±3.21&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.59±2.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>BHA-induced</td>
<td>38.21±3.32&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.33±2.61&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>2.83±0.79</td>
<td>1.82±0.80</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, $n = 5$ in each group at the same time point, and the experiment was repeated three times. *$P < 0.05$, vs. butylated hydroxyanisole (BHA)-induced group; †$P < 0.05$, vs. control group using analysis of variance followed by least significant difference test.

**Table 2** Effect of angelica induction on cell survival rate (%) in human adipose-derived stem cells

<table>
<thead>
<tr>
<th>Group</th>
<th>Induction 24 hours</th>
<th>Induction 48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angelica-induced</td>
<td>82.77±2.38&lt;sup&gt;ab&lt;/sup&gt;</td>
<td>82.29±4.73&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>BHA-induced</td>
<td>36.82±4.52&lt;sup&gt;b&lt;/sup&gt;</td>
<td>6.59±1.29&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Control</td>
<td>93.27±0.76</td>
<td>94.36±3.38</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD, $n = 5$ in each group at the same time point, and the experiment was repeated three times. *$P < 0.05$, vs. butylated hydroxyanisole (BHA)-induced group; †$P < 0.05$, vs. control group using analysis of variance followed by least significant difference test.

**DISCUSSION**

Safford and colleagues<sup>[5]</sup> reported that both human and mouse adipose-derived stem cells can differentiate into neuron-like cells, and confirmed the expression of nestin,
glial fibrillary acidic protein, and neuronal nuclear protein. Yang et al.\(^7\) and Zheng et al.\(^8\) also reported adipose-derived stem cell differentiation into neural cells in low-serum-concentration medium, and expression of nestin and neuron-specific enolase. To date, no group has produced evidence that adipose-derived stem cells are capable of differentiating into mature, functional neuronal cells in vitro\(^5\). However, in vivo studies with adipose-derived stem cells are promising. Adipose-derived stem cells have been therapeutically evaluated in animal models of cerebral ischemia\(^9\), spinal cord injury\(^10\) and stroke\(^11\).

Inducers such as butylated hydroxyanisole and dimethyl sulfoxide are toxic chemicals for in vitro differentiation of adipose-derived stem cells. These inducers are not suitable for clinical application. Previous studies addressing Chinese medicine induction of mesenchymal stem cell differentiation into neuron-like cells usually adopt baicalin\(^12\) or Salvia\(^13\) as the inducer. However, roles of angelica on the induction of human adipose-derived stem cell differentiation are rarely reported.

Radix Angelicae Sinensis, the dried root of Angelica sinesis (Dan-gu-si), is a herb used in traditional Chinese medicine to enrich blood, promote blood circulation, treat and modulate blood deficiency patterns such as menstrual disorders, and modulate the immune system\(^14\). The most active ingredients of Radix Angelicae Sinensis are polysaccharides, Z-Stillicide, and ferulic acid\(^14\). Kang and Kim\(^5\) found four kinds of major neuroprotective effects of coumarin in angelica. Radix Angelicae Sinensis extract decreases β-amyloid-induced neurotoxicity and tau phosphorylation in cultured cortical neurons\(^15\). It also improves learning and memory capability of rats with dementia\(^16\). These indicate that Radix Angelicae Sinensis can be applied to treat nervous system diseases.

In this study, we cultured and identified human adipose-derived stem cells. The cells were obtained from human adipose tissue by collagenase digestion, had fibroblast-like morphology and rapidly proliferated in vitro. Currently, there are no specific signs of adipose-derived stem cells. The cell surface phenotype of human adipose-derived stem cells is quite similar to that of mesenchymal stem cells. Adipose-derived stem cells and mesenchymal stem cells display numerous molecules including CD29, CD44, and CD49\(^17\). Immunohistochemical staining shows that more than 85% of the subcultured cells expressed CD44; and the positive expression rate of vimentin, a specific marker of mesenchymal cells, was up to 97%, suggesting that cells obtained from human adipose tissue are mesenchymal stem cells.

After adipose-derived stem cells were induced with Radix Angelicae Sinensis for 24 hours, some cells began to differentiate into neuron-like cells; after being induced for 48 hours, a small number of neuron-like cells intersected each other in the form of a network. Immunofluorescence staining detected that, after the cells were induced for 24 hours, the percentage of neuron-specific enolase positive cells in the angela-induced group was less than the butylated hydroxyanisole-induced group. These results suggest that adipose-derived stem cells under the induction of Radix Angelicae Sinensis show differentiation changes at both morphological and molecular levels. Glial fibrillary acidic protein expression was negative, indicating that the cells have not differentiated into glial cells.

According to a recent analysis of Radix Angelicae Sinensis, it contains a variety of phospholipids including ferulic acid and other substances\(^18\). A study showed that ferulic acid reduced cerebral infarct area and neurological deficit scores in rats with transient middle cerebral arterial occlusion\(^19\). The main reason is that ferulic acid enhances the activity of superoxide dismutase, and decreases lipid peroxide levels. It has strong antioxidant properties and scavenges oxygen free radicals. Radix Angelicae Sinensis has potential therapeutic effects on diabetic peripheral neuropathy, and the mechanism may be related to a direct increase in nerve growth factor expression and direct or indirect increase in brain derived neurotrophic factor expression\(^18\). Therefore, the mechanism underlying Radix Angelicae Sinensis’ induction of adipose mesenchymal stem cell differentiation into neuron-like cells may be related to its antioxidant effects and ability to improve cytokine expression. The exact mechanism has yet to be further explored.

Radix Angelicae Sinensis injection can be directly applied in vivo, so it offers a convenient method for inducing adipose-derived stem cells in clinical application. Our findings suggest that adipose-derived stem cells could differentiate into neuron-like cells in vitro, with the induction of DBT injections, and present no impact on cell viability. However, the differentiation rate is low. Therefore, increasing the percentage of cell differentiation could be an exciting topic for further research. For example, a combination of Radix Angelicae Sinensis with cytokines to induce human adipose-derived stem cells
should be investigated.

MATERIALS AND METHODS

Design
A comparative observation regarding cell culture and induction in vitro.

Time and setting
Experiments were performed at the Medical Molecular Biology Laboratory of Luzhou Medical College in China from April 2009 to August 2010.

Materials
Adipose tissue was obtained from seven healthy women who underwent subcutaneous liposuction surgery in the Affiliated Hospital of Luzhou Medical College, China. The experiments were carried out in accordance with the Declaration of Helsinki, and ethically approved by Ethics Committee of Luzhou Medical College in China. All donors signed informed consents.

Methods
Isolation and culture of human adipose-derived stem cells
Human adipose tissues were collected during skin grafting. The tissue samples were repeatedly washed with Hank’s balanced salt solution to remove any remaining blood. Blood vessels and fibrous material were cut off and discarded. Then, specimens were digested with an equal volume of 0.1% collagenase type I (Gibco, Gaithersburg, MD, USA) in a shaker incubator at 37°C for 90 minutes. After digestion, the cells were filtered through a mesh and centrifuged for 10 minutes at 107.3 × g. The sedimented cells were resuspended in an osmotic buffer for 10 minutes to lyse contaminated erythrocytes. The cell pellets were collected by centrifugation, at a final concentration of 2 × 10⁴ cells/mL in a 75 cm² culture flask using Dulbecco’s modified Eagle medium (DMEM; Gibco) containing 10% fetal bovine serum (Gibco) at 37°C with 5% CO₂. After induction for 24 hours, cultures were washed twice to remove unattached cells and then fresh medium was replaced.

Fresh medium was replenished every 3 to 4 days. After cells reached 70–80% confluence, the cells were passaged by 0.25% trypsinization and replated at 1:2 dilution.

Cell phenotype detected by immunocytochemistry
Morphological changes and proliferation characteristics of the cultured cells were observed under an inverted phase microscope (Olympus, Tokyo, Japan). Immunocytochemistry was used to determine the expression of the surface molecule CD44 and vimentin (a marker of mesodermal cells) on passage 3 cells. According to conventional immunocytochemistry steps, we used mouse anti-human CD44 monoclonal antibody and mouse anti-vimentin polyclonal antibody (Beyotime Biotechnology, Beijing, China).

Neurogenic differentiation
Passage 3 human adipose-derived stem cells were seeded onto a slide at 1 × 10⁵ cells/mL. When the cells attached to the wall, DMEM containing 10% fetal bovine serum (FBS) was replaced with serum-free DMEM/Nutrient Mixture F12 (F12; Gibco), containing 10% Radix Angelicae Sinensis (250 g/L; Department of Pharmacy, Hubei University Second Hospital, China). Then cells were induced with serum-free DMEM/F12 containing 40% Radix Angelicae Sinensis, as the angelica-induced group. Butylated hydroxyanisole-induced group and control group were performed at the same time. In the butylated hydroxyanisole-induced group, human adipose-derived stem cells were induced with the pre-induction medium (containing 1 mmol/L β-butyral hydroxyanisole (Sigma) + DMEM/F12) for 24 hours, then were induced with the induction medium (containing 200 μmol/L butylated hydroxyanisole + 2% DMSO + DMEM/F12). The control group had no inducers. The changes of cell morphology during the induction process were observed under an inverted phase microscope.

Immunofluorescence staining of neuron-specific enolase and glial fibrillary acidic protein expression
After human adipose-derived stem cells were induced for 24 and 48 hours, cells were washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 30 minutes; rinsed three times with PBS and incubated with blocking solution containing 0.4% Triton X-100 in PBS at room temperature for 1 hour. The cells were then incubated with mouse monoclonal primary antibodies against neuron-specific enolase and glial fibrillary acidic protein (1:200; Zhongshan Golden Bridge Biotechnology, Beijing, China), diluted in blocking solution at 4°C for 24 hours. The negative control cells were incubated without primary antibody. The cells were then washed thrice with PBS and incubated with secondary anti-mouse antibody labeled with Cy3 (1: 1000; Beyotime Biotechnology, Beijing, China) diluted in blocking solution at room temperature for 1 hour in darkness. The cells were then washed thrice with PBS and slides were mounted in Vectashield Hard Mounting Medium with 4’,6-diamidino-2-phenylindole (DAPI; Beyotime Biotechnology). DAPI stains nuclei
specifically, with little or no cytoplasmic labeling. Its blue fluorescence stands out in contrast to green, yellow, or red fluorescent probes of other structures, which is conducive to cell count. The cells were analyzed under a fluorescence microscope (Olympus).

**Cell viability detected by trypan blue staining**

The dye exclusion test was used to determine the number of viable cells present in a cell suspension. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue. After cells were induced for 24 and 48 hours, they were gathered and prepared into a cell suspension, then stained with 0.4% trypan blue solution (Sigma) for 2 minutes. The cell survival rate was calculated as previously described.[20]

**Statistical analysis**

Data were statistically processed using SPSS 13.0 software (SPSS, Chicago, IL, USA) and expressed as mean ± SD. One-way analysis of variance followed by least significant difference test were used for statistical analysis. A P < 0.05 was regarded as significant.

## REFERENCES


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