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Experimental study on repairing skin defect by tissue-engineered skin substitute compositely constructed by adipose-derived stem cells and fibrin gel

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Abstract. – OBJECTIVE: To study the application value of artificial skin substitute compositely constructed by adipose-derived stem cells and fibrin gel in skin defect.

MATERIALS AND METHODS: Adipose-derived stem cells (ADSCs) were obtained from healthy pure green fluorescent protein (GFP) transgenic mice and were proved to have multiple differentiation potentials. They compositely constructed artificial skin substitute in vitro with fibrin gel. 24 SD rats of either gender, with the gestational age of 3-5 weeks, were divided into four groups in the experiment, namely model group (autologous skin flap transplantation), adipose-derived stem cell transplantation group, fibrin gel transplantation group and compound transplantation group, 6 rats for each group. At the skin blood flow (measured by applying laser Doppler rheometer) and survival rate of the flap on 7d and 21d respectively, the materials were transplanted at back skin injury (1 cm×1 cm) to prepare tissue slice for routine HE staining. The conditions of wound healing were observed, and the angiogenesis of flap neovascularization was detected by the immunofluorescent assay.

RESULTS: The skin blood flow, the survival rate of flap and density of neovascularization 7d and 21d after transplantation were significantly higher than those of stem cell group, followed by the model group, with the lowest ones in the fibrin gel group. The differences had statistical significance ($p<0.05$). The wound healing time of compound group was significantly shorter than that of stem cell group, followed by the model group, with the longest one in the fibrin gel group. The differences had statistical significance ($p<0.05$).

CONCLUSIONS: The artificial skin substitute compositely constructed by adipose-derived stem cells and fibrin gel could significantly shorten healing time and improve the survival rate of the flap in skin defect, with better application value.

Key Words:
Adipose-derived stem cells, Fibrin gel, Tissue-engineered skin substitute, Skin defect.

Introduction

With the increase in occurrence rate of skin defects caused by various traffic traumas, burn, scald, pressure sores, surgeries and undesirable healing of diabetic skin, about 10-30% of patients have difficult-to-heal skin defects, which increases the rate of infection and cutaneous necrosis and lowers living quality[1]. Autograft of skin is always applied in the clinic, and it has the risks, such as low survival rate, long healing time, limited source and new defect of the donor site, which promotes the studies on tissue-engineered skin substitute[2]. The stem cells with pluripotent differentiation potential obtained from adipose tissue can directionally induce the differentiation and homing of vascular endothelial cells, fibroblasts, osteoblasts, chondroblasts, and neurocytes, which provides attractive future for repair materials in many fields[3]. Moreover, adipose-derived stem cells have rich sources and are simpler in isolation than bone marrow stem cell with higher safety[4]. As a stent material with good biocompatibility and degradability, fibrin gel can provide repair stent for adipose-derived

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stem cells after compounding with adipose-derived stem cells to provide support for cell metabolism, migration, and differentiation. Moreover, the fibrinogen and fibronectin it contained can stimulate extracellular matrix secretion and the regeneration ability of tissue[5,6]. After compounding with adipose-derived stem cells, it has fast molding speed and can be transplanted to skin defect directly, thus shortening treatment time and improving the survival rate of transplant, with better clinical application value[7,8]. On this basis, this work analyzed the application value of artificial skin substitute compositely constructed by adipose-derived stem cells and fibrin gel, providing reference basis for the reasonable clinical selection of transplants.

Materials and Methods

Isolation and Differentiation of Adipose-derived Stem Cells

Healthy pure green fluorescent protein (GFP) transgenic mice and SD rats (24 mice and rats) of either gender, with the gestational age of 3-5 weeks were provided by Sun Yat-sen University Animal Center. The study was approved by the Ethics Committee of the Weifang People's Hospital. They were fed normally and were used for the experiment after one week of adaptation.

The separation process of adipose-derived stem cells: After intraperitoneal anesthesia for transgenic mice, about 1 ml adipose tissue was taken from groin under aseptic conditions and washed with PBS cleaning solution containing mycillin (Invitrogen, Carlsbad, CA, USA). Adipose tissues were cut into pieces of 1 mm^3 and 2.5 ml collagenase I (Sigma-Aldrich, St. Louis, MO, USA) was added for digestion. After they were taken to shaking table (Beijing Liuyi Instrument Plant) to act at 37°C and 150 g for 30 min, they were centrifuged at 1000 g for 10 min. The supernatant was discarded and sediment was taken to prepare single-cell suspension by Dulbecco's Modified Eagle Medium (DMEM) medium containing 100 ml/L fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). After re-suspension, cells with the density of 2×105/ml were obtained. They were inoculated into culture flask and routinely cultured at 37°C with the content of 50 ml/LCO_2 (Bio-Rad, Hercules, CA, USA). The medium was changed after 24h of primary culture. When cell volume reached 80-85%, trypsin and EDTA digestion were used to continue subculture.

The process of osteogenic and adipogenic differentiation: GFP-ADSCs cells of 3rd-4th generation were taken to inoculate on a 6-pore plate and cultured by DMEM medium containing 100 ml/L FBS. When cell volume reached 80-85%, induction and differentiation experiment was conducted. Osteogenic induction liquid (DMEM medium containing 50 μmol/L ascorbic acid, 10 mmol/Lβ-glycerophosphate, 0.01 μmol/L vitamin D3 and 100 ml/L fetal bovine serum (FBS – Sigma-Aldrich, St. Louis, MO, USA) was used and changed once every 3-5d. After being cultivated for 14d, cell masses were taken to conduct Type I collagen immunocytochemical staining (the kit was purchased from R&D Systems, Minneapolis, MN, USA). After being cultivated for 21d, von Kossa staining (the kit was purchased from R&D Systems, Minneapolis, MN, USA) was conducted to observe the formation of mineralized nodules. Adipogenic induction liquid (DMEM medium containing 1 μmol/L dexamethasone, 10 μmol/L bovine insulin, 200 μmol/L indomethacin, 0.5 μmol/L IBMX and 10% FBS, Sigma-Aldrich, St. Louis, MO, USA). After 21d, 4% paraformaldehyde was used for fixation, and oil red 0 was used for staining and observation.

Construction of Compound of Adipose-derived Stem Cells and Fibrin Gel

ADSCs of 3rd-4th generation in good growth condition were selected to be conducted flow cytometry (Applied Biosystems, Foster City, CA, USA) sorting and the positive rate of surface marker molecules, including CD44, CD90, and CD29 could reach 99%. 5×10^6/ml resuspension cell of trypsin and EDTA digestive juice were applied to mix with 0.15 ml fibrin gel main body liquid (Applied Biosystems, Foster City, CA, USA). 0.15 ml catalyzer was added to form 0.3 ml membrane gel.

Experimental Grouping

The experiment was divided into four groups, namely, model group (autologous skin flap transplantation), adipose-derived stem cell transplantation group, fibrin gel transplantation group and compound transplantation group, 6 rats for each group. The transplantation was conducted at back skin injury, and the transparent dressing was used to paste temporarily.

Observation Indexes

At the skin blood flow (measured by applying laser Doppler rheometer, GE, Fairfield, CT,

USA) and survival rate of the flap on 7d and 21d respectively, the materials were taken to prepare tissue slice for routine HE staining. The conditions of wound healing were observed, and the angiogenesis of flap neovascularization was detected by the immunofluorescent assay. Among which, the calculation of survival rate of the flap was conducted by shooting flaps with a digital camera (Canon, Tokyo, Japan) and Image-Pro Plus.v6.0 image analysis software (Microsoft, USA), and survival rate of flap = (survived flap area/design flap area)×100%. Immunofluorescent assay: after passing dewaxing and rehydration, slices were fixed with acetone for 10 min and, then, placed in a wet box to be incubated with 5% sheep serum for 15 min. Rabbit anti-mouse GFP protein primary antibody and anti-CD31 multiple antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added. After being hatched at 4°C overnight, they were washed with PBS for three times and TRITC marked goat anti-rabbit second antibody IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added. After being hatched for 1h, cell nucleus was dyed with DAPI. After being washed with PBS for three times, Clearmount was used to seal slices. Observation and analysis were conducted through fluorescence microscope (Olympus, Tokyo, Japan) and vascular densities were compared by calculating the average value of the number of vessels in 5 random fields of view on each slice.

Statistical Analysis

The SPSS20.0 software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis, measurement data were expressed by the mean ± standard deviation, comparisons among groups were analyzed by One-way ANOVA, the comparison between two groups was tested by LSD method, comparison within the group was tested by *t*-test. $p<0.05$ indicated that the difference was statistically significant.

Results

Comparison of Skin Blood Flow

The skin blood flow of the compound group 7d and 21d after transplant was significantly higher than that of the stem cell group, followed by the model group, with the fibrin gel group the least, the difference was statistically significant ($p<0.05$). Please refer to Table I.

Table I. Comparison of skin blood flow (PU).

Group	7d	21d
Model group	67.6±12.5	102.6±15.6
Stem cell group	154.8±43.8	269.8±54.8
Fibrin gel group	32.5±10.4	35.6±12.4
Compound group	256.3±56.2	421.5±62.8
F	156.524	172.342
p	<0.001	<0.001

Comparison of Survival Rates of Flaps

The skin flap survival rate of the compound group 7d and 21d after transplant was significantly higher than that of the stem cell group, followed by the model group, with the fibrin gel group the least, the difference was statistically significant ($p<0.05$). Please refer to Table II.

Wound Healing Condition

In vitro cultivation and observation of ADSCs showed that stem cells presented small polygon and mononuclear and some of them had processes and could give out green fluorescence. A large number of fat granules were found in the cytoplasm after passages; however, autonomous calcium nodules and mature adipocyte differentiation were not found. Under the condition of the induction, a large number of black calcium nodules and orange lipid droplet granules could be detected. A large number of GFP-ADSCs positive cells were found in the compound group in the shape of a long spindle. They differentiated to dermal fibroblasts and transformed into endothelial cells to form a tube-like structure. The time of wound healing for the compound group was significantly shorter than that of stem cell group, followed by the model group, and that of fibrin gel group was the longest. Their differences had statistical significance the sum of (10.3±2.6), (16.3±4.2) and (20.9±3.6) was more than 21d, F=34.526, $p<0.001$.

Table II. Comparison of skin flap survival rate (%).

Group	7d	21d
Model group	16.5±7.2	23.4±9.3
Stem cell group	20.3±6.4	36.8±7.3
Fibrin gel group	3.4±1.2	3.6±1.3
Compound group	42.6±13.6	65.8±20.5
F	26.354	32.527
p	<0.001	<0.001

Angiogenesis Condition

Angiogenesis density of the compound group 7d and 21d after transplant was significantly higher than that of the stem cell group, followed by the model group, with the fibrin gel group the least, the difference was statistically significant (p<0.05). Please refer to Table III.

Table III. Angiogenesis density (number/field).

Group	7d	21d
Model group	1.2±0.6	2.0±0.9
Stem cell group	2.0±0.6	3.7±1.3
Fibrin gel group	0.3±0.1	0.4±0.2
Compound group	3.5±0.9	5.6±1.2
F	16.532	22.524
p	<0.001	<0.001

Discussion

Tissue engineering technology had a very important value in the aspects of constructing and repairing seed cells and stent materials. After induction differentiation, adipose-derived stem cells, as a skin substitute, showed a favorable response to the aspects of blocking bacterial invasion, preventing water loss, inducing neovascularization, providing nutrients and oxygen, improving microcirculation and reducing the seepage, etc. It also had better application value in adding skin blood flow and improving the survival rate of flap[9,11].

This study showed that the transgenic mice could express abundant adipocytes. After their stem cells were extracted and *in vitro* isolation and induction differentiation was carried out, they were proved to have better osteogenesis and lipoblast capability[12]. The adipocytes were transplanted at the sites of transplanted skin lesions after compounding with fibrin glue, the skin blood flow, survival rate of flap and density of neovascularization 7d and 21d after transplantation were significantly higher than those of stem cell group, followed by the model group, with the lowest ones occurred in the fibrin gel group. Their differences had statistical significance. The wound healing time of compound group was significantly shorter than that of stem cell group, followed by the model group, with the longest one occurred in the fibrin gel group. Their differences had statistical significance. The simple fibrin gel group could be used as blank control, which indicates that the composite group had better skin healing capability than that of the single stem cell group. As good structure support, fibrin gel provided stable microenvironment and activity place for directional differentiation, migration and repair functions of stem cells[13,14]. Compared with the autogenous skin grafting of model group, the effects on the repair of skin lesion were better, which proved that the repairing substitute provided by engineering technology had better safety and effectiveness[15].

At present, the repairing materials provided by tissue engineering technology had more application experiences in orthopaedics, stomatology and burn repair[16]. The rejection reaction generated by purification and allosome implanting after the separation of adipose-derived stem cells[17] needed to be further studied. The efficiency[18], transplantation path and dosage[19,20] of the recombination of autologous stem cells and fibrin gel recombination needed to be further explored.

Conclusions

The artificial skin substitute compositely constructed by adipose-derived stem cells and fibrin gel could significantly shorten healing time and improve the survival rate of flap in skin defect, with better application value. Its application potential could be continuously evaluated in clinical studies.

Conflict of interest

The authors declare no conflicts of interest.

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