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Original Article

Platelet-rich plasmagel combined with adipose stem cells in promoting wound healing in rats

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Abstract: Objective: To analyze the effect of platelet-rich plasmagel combined with adipose stem cells on wound healing of rats. Methods: 40 male Sprague-Dawley (SD) rats aged 6 weeks were enrolled and randomly divided into 4 groups, namely joint group (JG), gel group (GG), stem cell group (ScG) and control group (CG) to be treated and managed accordingly in a comparative analysis of wound healing. Results: (1) Negative CD34 and CD45, as well as positive CD29 and CD90 were indicated by flow cytometry. (2) All these groups at the specific time point of 7 d, and 14 d of treatment showed gradually reduced levels of type I collagen ($P<0.05$). (3) After 14 d of treatment, JG showed a higher occurrence of positive CD31 than the other groups ($P<0.05$). (4) There were significant differences between JG and any other group in terms of TGFβ1 levels ($P<0.05$). (5) Rats in JG spent the minimum time period in wound healing and those in CG spent the maximum one ($P<0.05$); GG and ScG did show obvious difference in the wound healing periods ($P>0.05$) but both were smaller than that in CG ($P<0.05$). Conclusion: The combination of platelet-rich plasmagel with adipose stem cells in wound healing of rats is conductive to accelerate the neovascularization and collagen synthesis, suggesting evident application values.

Keywords: Wound in rats, platelet-rich plasmagel, adipose stem cells, promotion, healing

Introduction

The recovery of wound in dermatic soft tissues is complicated and affected by multiple factors such as epithelization on the wound surface, granulation tissue hyperplasia, local inflammatory reactions, and local restructuring after wound healing, etc [1]. The crucial factors among these include the lack of cytokines which promote the healing, and insufficient sources of local stem cells. Theoretically, supplement of local stem cells and growth factors increases the recovery of wound in dermatic soft tissues [2].

Stem cells are generally divided into two types: embryonic stem cells isolated from primordial germ cells constituting early embryos; adult stem cells purified from various mature tissues of human body owning stem cell characteristics [3]. In practical, the application of embryonic stem cells involves problems in multi-aspects including immunological rejection and medical ethics, which are avoidable in the use of adult stem cells. Bone marrow mesenchymal stem cells (BMSCs) are capable of good self-renewing and proliferation *in vivo* and *in vitro*, and in some special cases differentiation over the boundaries of germ layers [4]. The sources of adipose stem cells are quite limited, and patients suffer a lot during the process of acquisition. Few stem cells could be acquired from the bone marrow tissues however [5]. Compared with bone marrow stem cells, adipose stem cells do not have the huge amount of hematopoietic stem cells but larger proportion of mesenchyme, which to a higher extent accelerates the revascularization of wider range of tissues with slight surgical trauma [6]. Thrombin activates platelets in platelet-rich plasma and the release of various growth factors of high concentrations. These factors interact and speed up the recovery [7].

In this study, the comparative analysis of platelet-rich plasmagel alone, adipose stem cells alone and platelet-rich plasmagel combined with adipose stem cells in 40 SD male rats randomly divided into 4 groups was arranged to find the differences in wound healing by these

protocols in hopes of providing more evidence to the effective treatment of dermatic tissue wounds.

Material and methods

Material

Experimental animals: 40 SD male rats (clean animals) aged 6 weeks, with a range of body mass 190 g-220 g.

Reagents: Anhydrous $CaCl_2$, thrombin, paraformaldehyde, chloral hydrate, & MEM/F12 (1:1) solution.

Apparatus: Refrigerator, sterilized surgical instruments, electronic balance, inverted phase contrast microscope, hgh-pressure steam sterilizer, 3 M membrane, high-speed centrifuge, and syringe (1 ml, 10 ml, & 20 ml).

Methods

Identification of multipotential differentiation of adipose stem cells and cell surface marker in flow cytometry: The well-grown 3rd generation was digested and applied in a 6-well plate at the density of 5×10^4/ml, adipogenic and osteogenic differentiation inducing solutions were added for induced differentiation, and the inducing medium was changed every 3 days. The osteogenic differentiation group was subjected to alizarin red staining after 4 weeks of induced differentiation, whilst the adipogenic differentiation group was subjected to Oil Red O staining after 2 weeks of induction. The inverted microscope was then used for observing and identifying. Every procedure was approved by the Animal Care and Use Committee of Zhongnan Hospital of Wuhan University.

Gel preparation: The gel was prepared by modified Cascade-Esforax method, and PRP gels were prepared by calcium ion activation. 3 mL of venous blood was extracted from the rats, divided into two parts on average, and centrifuged for 10 min. 0.15 mL of calcium chloride (50 mg/mL) was added for activation. After standing, the middle layer of pale yellow gel was collected, which was PRP gel for future use.

Modeling: Rats were transabdominally anesthetized with fresh 7% chloral hydrate at a dosage of 0.4 ml/100 g. The hairs on both sides of the back spine were removed to make sure skin exposure. Fixed in prone position on the operating table, the rats were managed at the exposed area with sterilization by iodophor solution. Just at the site on both sides 2 cm away from the spine, a circular incision of 2 cm in diameter was made separately followed by sterilization with iodophor to the skin of back for three times. The 15th round knife was used to cut the skin along the marker into the surface of the muscular layer. So far, the dermatic wound of soft tissues was prepared.

Grouping and treatment: The wounds on both sides were subjected to subcutaneous injection in accordance with the 6 points equidistant to the wound edge, and intramuscular injection in accordance with the 2 points equidistant from the base. From each point, 0.1 ml of solution was applied. Rats in JG were locally injected with adipose stem cells and DMEM, coupled with platelet-rich plasmagel covering the wound surface. Those in ScG were locally injected with adipose stem cells and DMEM. Rats in GG were applied with local DMEM injection coupled with platelet-rich plasmagel covering the wound surface. The CG was applied with local DMEM injection alone. After the surgery, the wound surfaces in all groups were treated with chlortetracycline hydrochloride eye ointment, covered by 3 M membrane. The 4 groups of rats were raised apart. The 3 M membranes were removed after 3 days of feeding.

Observed indicators

On the 3rd, 5th, 7th, and 14th day of relevant treatment, two rats from each group were killed for isolating callus and adjacent tissues, which were then fixed with 4% formaldehyde before dehydration, waxing, embedding and slicing of about 5 μm. After hematoxylin-eosin (HE) staining, the healed wound tissues were subjected to histopathological observation. The density of newly developed microvessels was measured by CD31 immunohistochemical staining. Specific gravity of type I, III collagen was quantified by visual detection under polarized light after Sirius red staining where the type I collagenous fiber is normally yellow or red, while the type III is green. Ribonucleic acids (RNA) from callus of different temporal points were applied in the reverse transcription-polymerase chain reaction (RT-PCR) to determine

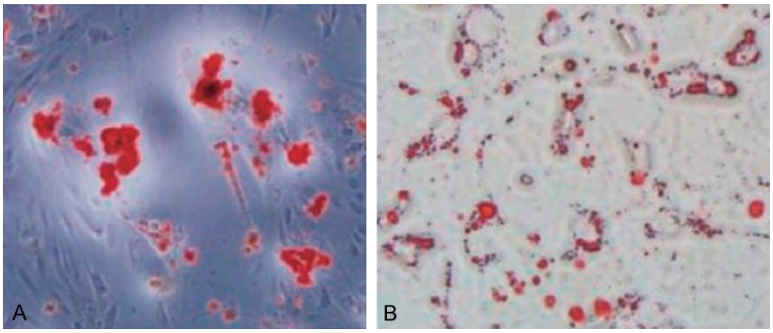

Figure 1. Induced multipotential differentiation of adipose stem cells and flow cytometry analysis *in vitro*. A. Alizarin red staining (×200) of adipose stem cells after 4 weeks of inducing osteogenic differentiation; B. Oil Red O staining (×200) of adipose stem cells after 4 weeks of inducing adipogenic differentiation.

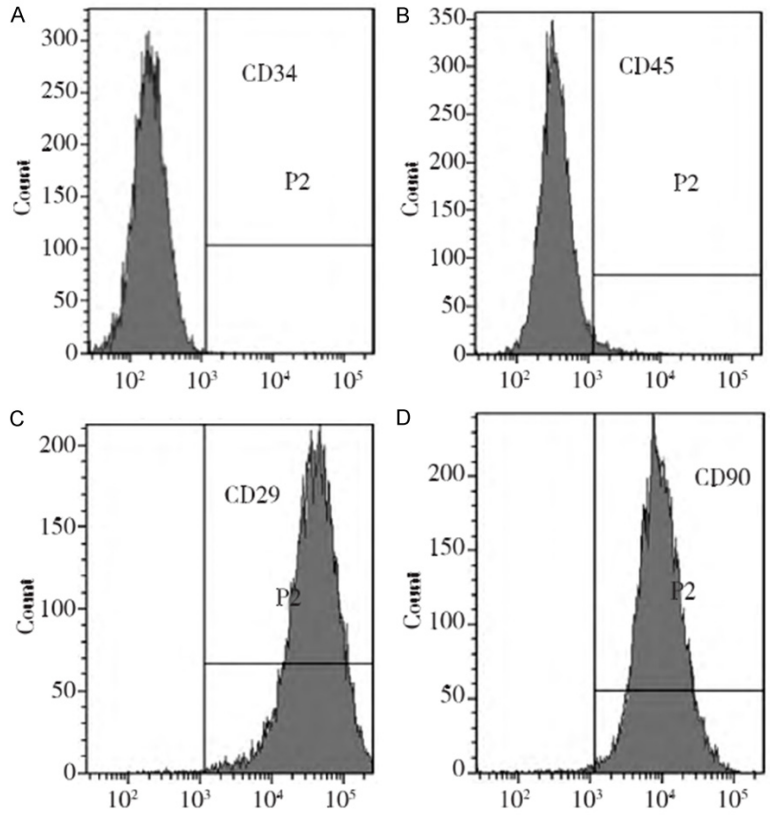

Figure 2. Phenotype Identification by flow cytometry. A. CD34 phenotype identification by flow cytometry showed negative; B. CD45 phenotype identification by flow cytometry showed negative; C. CD29 phenotype identification by flow cytometry showed positive; D. CD90 phenotype identification by flow cytometry showed positive.

expression of transforming growth factor-β (TGF-β1).

Statistics

Statistical analysis was performed in SPSS22.0 where measurement data were expressed as mean ± standard deviation and independent sample t-test was used for inter- & intra-group comparisons; enumeration data were expressed in [n (%)], inter- & intra-group comparisons were performed with X^2 test, and multi-points comparison in group was conducted with ANVOA. P<0.05 indicated significant difference(s).

Results

Induced multipotential differentiation of adipose stem cells and flow cytometry analysis in vitro

In the osteogenic differentiation medium after 4 weeks induction, it showed positive alizarin red staining and observed red calcium nodules, suggesting that these cells are capable of induced differentiation into osteoblasts. After 2 weeks of induction, the 3rd generation had markedly increased volume, the cytoplasm was filled with round lipid droplets like a cluster of grapes, and the positive Oil Red O staining could be observed as shiny red particles formed in round cells. These data suggest that cells used can differentiate into adipose cells. Flow cytometry revealed negative CD34 & CD45, and positive CD29 & CD90, which were in coincidence with the adipose stem cell phenotypes (**Figures 1**, **2**).

Visual inspection of the wound surfaces

Visual inspection performed 3 days after treatment found that, in JG and GG there were light red blood crusts newly developed at the sites covered by platelet-containing plasmagel, and the dryness of the wounds in the two groups was better than that in ScG or CG. 1 week after treatment, also by visual inspection, the size

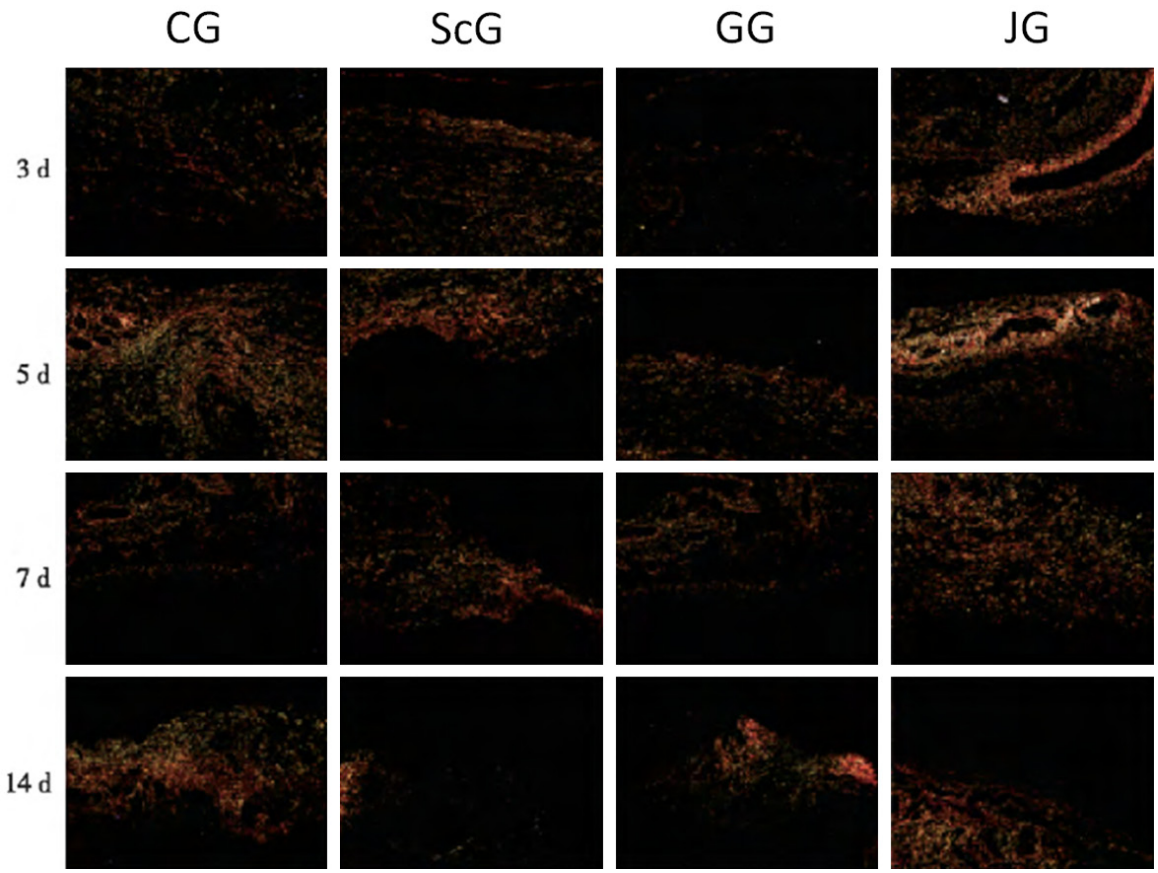

Figure 3. Sirius Red staining (×100) of wound biopsies from different temporal points. After 3 days of treatment, JG's type I collagen level was superior to those in GG, ScG and CG (P<0.05); as compared with the CG, type I collagen levels in JG, GG as well as ScG were much higher (P<0.05). The trends after 5 days of treatment were the same as those 3 days after. 7 days and 14 days after treatment, all groups had gradually reduced type I collagen levels (P<0.05).

and depth of wound were successively smaller in CG, ScG, GG, and JG on a statistically significant basis. 2 weeks after treatment, all groups of rats were generally healed.

Collagen synthesis analysis

By Sirius red staining, Type I collagen was shown as yellow and red and held the top position in content among the 4 groups; type III collagen of lower level showed green color. Based on the 14-day continuous treatment, the type I collagen level was positively proportional to the period of treatment. After 3 days of treatment, ScG and GG did not reveal remarkable differences (P>0.05); JG's type I collagen level was superior to that in ScG (P<0.05) - the same was true between JG and GG (P<0.05); while as compared with the CG, JG, GG as well as ScG showed much higher type I collagen levels (P<0.05). As of 5 days of treatment, the difference between ScG and GG was not significant (P>0.05); JG's type I collagen level was superior to that in ScG (P<0.05) - the same was true between JG and GG (P<0.05); as compared with the CG, JG, GG as well as ScG showed much higher type I collagen levels (P<0.05). 7 days and 14 days after treatment, all groups had gradually reduced type I collagen levels, with differences statistically significant (P< 0.05) (**Figure 3**).

Observation of CD31 staining for microvascular density

In all groups, the occurrence of positive CD31 increased with the extension of time. 14 days after treatment, the level in JG was apparently higher than that in GG, ScG or CG (P<0.05) (**Figure 4**).

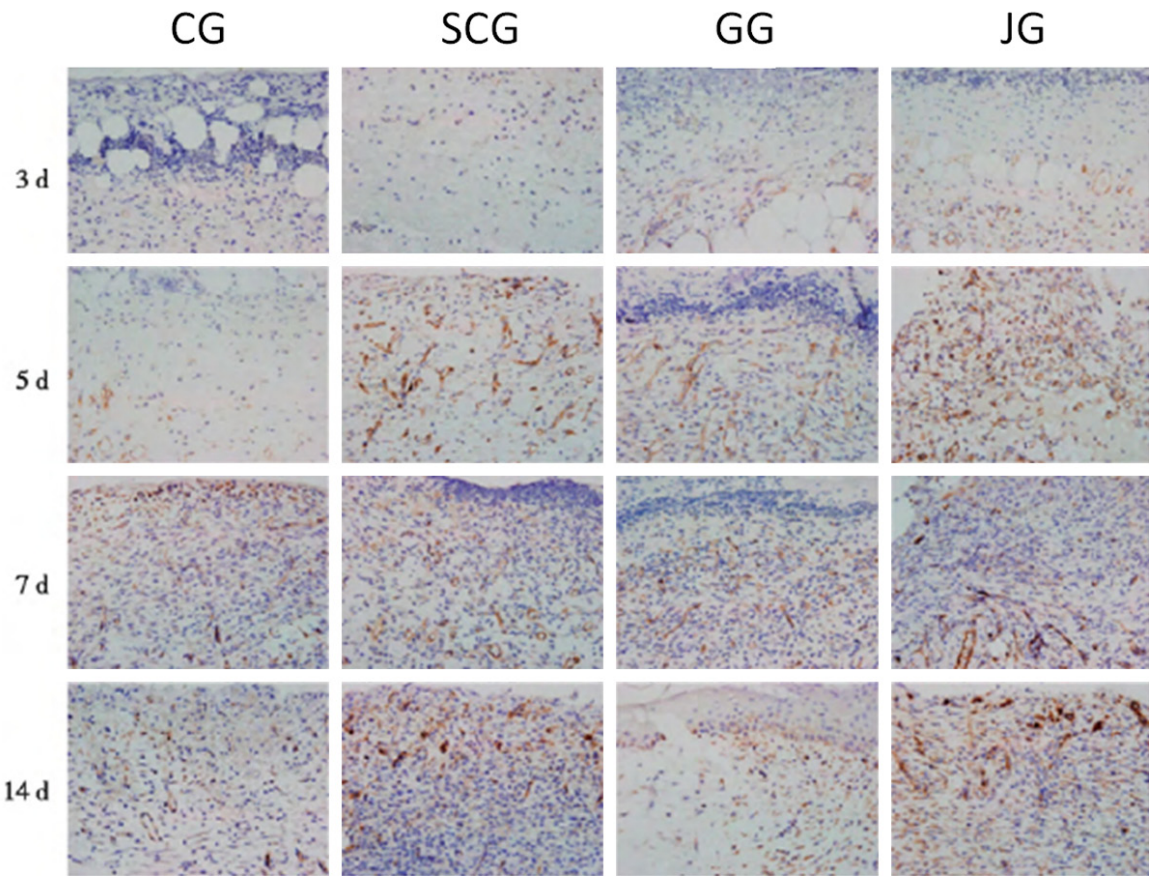

Figure 4. CD31 staining (×100) of wound biopsies from different temporal points. 3, 5, 7, and/or 14 days after, as compare with that in CG, the GG, ScG and/or JG showed significantly higher rates of positive CD31 respectively (P<0.05 for each).

Histological analysis on HE staining

The sections made of tissues healed uncovered clear gradation of gland-like structures in JG, a large number of new vessels, as well as collagenous fiber arrangement that comes near healthy skin tissues. Although the ScG developed many gland-like structures, the amount of new blood vessels and the orderliness in collagenous fiber arrangement were not as good as the JG. In GG, there were many new capillaries and ordered collagen fibers, but fewer gland-like structures. In CG, except newly formed blood vessels, the field of view was filled with disordered collagenous fibers, and only a small amount of gland-like structures was visible (**Figure 5**).

Comparison of changes in TGF-β1 levels

TGFβ1 levels in JG, ScG, & GG gradually increased beyond that in CG (*P*<0.05) after treatment, and in each of the three groups the multi-points comparison revealed significant differences (P<0.05). In particular, the increase in JG was the most evident with respect to any temporal points with statistical significance as compared with the other groups (P<0.05) (**Table 1**).

Days for complete wound healing

The days for complete wound healing in JG, GG, ScG and CG were 16.12±1.53, 19.86±1.34, 19.79±1.40 and 23.31±1.92, respectively. The difference between GG and ScG-both groups had longer days for healing than the JG (P<0.05) - was not distinct (*P*>0.05), unlike those obvious ones between the max. (23.31±1.92) and the min (16.12±1.53) (*P*<0.05) (**Figure 6**).

Discussion

At present, cellular therapies are widely used in clinical practices for trauma repair. Cellular therapies largely expedite the establishment of

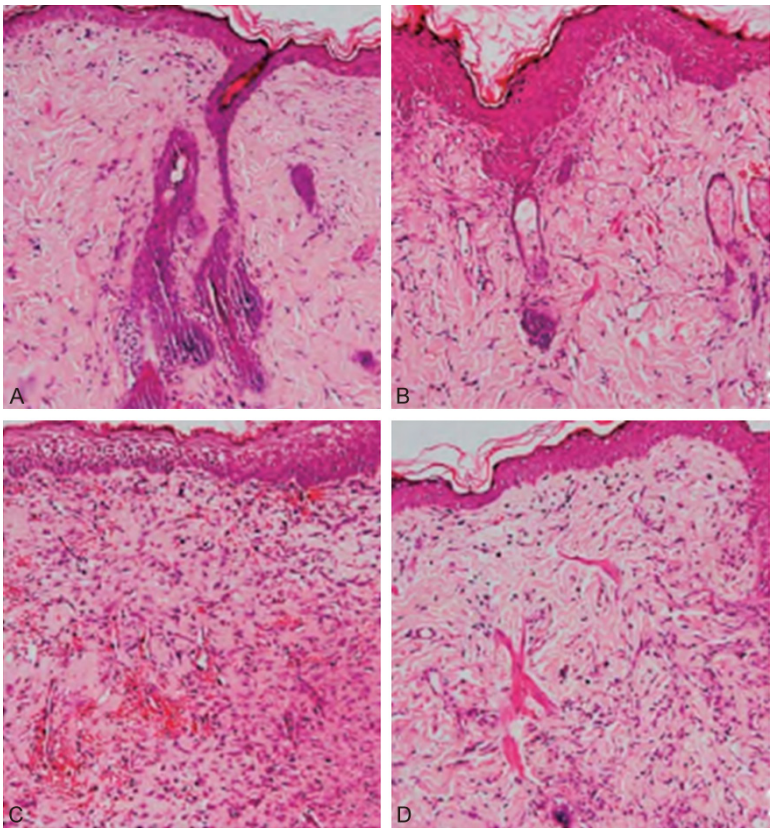

Figure 5. HE staining (×100) in 4 groups after 14 days of treatment. A. Joint group; B. Gel group; C. Stem cell group; D. Control group.

Table 1. Changes in TGFβ1 levels after treatment in all groups ($\bar{x}$ ± sd, β-actin)

Group	Before	3 days after	5 days after	7 days after	14 days after
JG (n=10)	0.31±0.05	0.42±0.10	0.46±0.06	0.51±0.06	0.55±0.06
GG (n=10)	0.32±0.06	0.40±0.09	0.44±0.11	0.48±0.10	0.51±0.13
ScG (n=10)	0.32±0.05	0.37±0.06	0.41±0.08	0.44±0.09	0.46±0.09
CG (n=10)	0.31±0.07	0.35±0.07	0.40±0.06	0.42±0.06	0.45±0.10

local microcirculation, shorten the period of hypoxia, drive the growth of tissues, and cut the days required for complete healing [8, 9]. In cellular therapies, adult stem cells are important seed cells sourced from mature organs and tissues. Tissues and cells induced by adult stem cells step away from immunological rejection, matching, ethics problems, and transplanted adult stem cells in the body are capable of persistent existence as well as proliferation even [10, 11].

Also, adipose stem cells are important seed cells for cell therapies by accelerating angiogenesis and tissue regeneration coupled with blood supply improvement [12]. Shyh-Chang N et al [3] indicated that hematopoietic stem cells were obtained from bone marrow sourced stem cells, while mesenchyme of high purity was acquired from those adipose-derived stem cells. Mens MMJ et al [13] found that adipose stem cells could differentiate into vascular endothelial cells and were involved in angiogenesis. They believed that adipose stem cells are better in function of tissue regeneration or angiogenesis than bone marrow stem cells, and in cell therapy the former is preferable for faster revascularization of local tissues. Heo SJ et al [14] used adipose stem cells in local fat transfer and detected significantly improved survival of free grafted fat. Miki T [15] concluded that it was the adipose stem cells that made the blood supply improved in his local injection of adipose stem cells in flap of rats that uncovered a higher flap survival. In this paper, the eight points from which the rats were subcutaneously injected with drugs were designed to ensure fully exploited adipose stem cells in wound healing. Compared with the CG, well healed wounds with new rosy and dense granulation tissues, without any infection, in rats in the ScG confirmed the facilitation in healing through actions of adipose stem cells.

Previously, wound repair was on a more frequent basis treated with single growth factor, which only promote the recovery at a certain phase [16]. Many studies have found that the combination of various growth factors offers greater conformity with the biological processes of wound healing that produces the optimum [17, 18]. Passaretti F et al [19] pointed out that activated platelets release different growth fac-

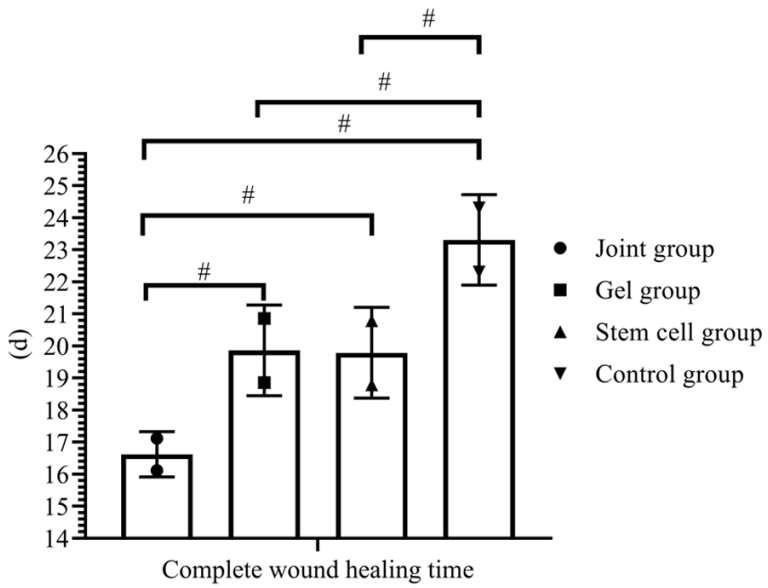

Figure 6. Days required for complete healing in 4 groups. Compared with those in GG, ScG or CG, JG showed shorter days required for complete healing (P<0.05). The days required in GG were shorter than that in CG (P<0.05), similar to that between ScG and CG (P<0.05). #P<0.05 in comparison of two groups.

tors close to those in the body for a period of time. Platelet-rich plasma made of platelets with different methods serves the effective growth factor supply.

In the past, platelet-rich plasma prepared by centrifugation is liquid before it is activated and cannot be covered on wound surface. Besides, the plasma if not activated does not release a large amount of growth factors in a longer period of time [20]. In this study, the platelet-rich plasma was mixed with thrombin and calcium ions to ensure a gelatinous status, i.e. platelet-rich plasmagel, which once activated by the activator releases different growth factors of high concentration that of course are synergistically proportional. These factors possess various mechanisms in wound healing and interact in certain cases to offer the best effects [21]. Platelet-rich plasmagel offers advantages over single growth factor, including more proper types of growth factors, concentration ratio that is close to that in the body, and synergistic effects produced [22]. Platelet-rich plasmagel is autogenous. It kicks off immunological rejections that may be caused by heterogeneous or allogenic growth factor [23]. A large number of white blood cells with close platelet sedimentation coefficient and monocytes in gel reduce the risk of infection [24].

In this study, the 4 groups of rats were prepared with wounds by the same method and treated differently. Based on visual inspection, there was light red blood crust developed at sites covered by platelet-rich plasmagel in JG and GG after 3 days of treatment; the wound dryness of rats in the two groups were better than those in ScG or CG. 1 week later, the size and depth of wounds in all groups improved, and generally healed after 2 weeks of treatment, suggesting that platelet-rich plasmagel was as useful as adipose stem cells in wound repair. Following the treatment of 3 days, 5 days, 7 days and 14 days, TGFβ1 levels increased gradually and the level in JG was the topmost among the 4 groups, indicating that the combination of platelet-rich plasmagel with adipose stem cells offers elevated TGFβ1 than platelet-rich plasmagel or adipose stem cells alone. With respect to days for complete healing, the differences between GG and ScG-both groups had superior days for healing than the CG (P<0.05) - were not distinct (*P*>0.05), unlike those obvious ones between the JG (the Max., 23.31±1.92) and the CG (the Min., 16.12±1.53) (*P*<0.05). Although platelet-rich plasmagel and adipose stem cell alone promotes wound healing, the combination significantly speeded up the recovery and shortened the days required.

In conclusion, platelet-rich plasmagel combined with adipose stem cells offer better wound healing effects with shortened days required as compared with either of them. The research in this paper put more emphasis on pathology results instead of interpretation of indicators. The biased findings could not be representative. Broader and in-depth analyses for more scientific treatments in wound healing are warranted.

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Disclosure of conflict of interest

None.

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