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Original Article

Adipose-derived SVFs with hyaluronic acid accelerate diabetic wound healing in diabetic porcine model

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Abstract: Objective: The impaired diabetic wound healing represents a significant clinical problem. Human stromal vascular fraction cells (SVFs) have been recognized as an attractive stem cell source. The aim of this study was to investigate the efficacy of adipose-derived SVFs with microneedles injection technology on diabetic wound healing. Methods: The diabetic porcine models were established on 4 female domestic pigs through injection of streptozocin (STZ) with their skin on the back being removed. The diabetic porcine pigs were randomly divided into 4 groups, i.e. PBS group (n = 12), HA group (n = 12), SVFs group (n = 12) and HA+SVFs group (n = 12). Thereafter, treatment with the corresponding drugs was carried out and the healing rate in each group was recorded and compared among groups. In addition, we also collected the tissue samples to perform histological analysis. Results: In HA+SVFs group, we found that wound healing rate was significantly accelerated in comparison with other three groups, and the HA and SVFs also have positive effect individually on wound healing. HA+SVFs had highest capillary density (31.83±3.19) compared with experimental groups (14.67±1.86, 21.83±2.56) and control group (8.16±1.17). The higher protein expression of VEGF (295.52±49.21 pg/ml) and bFGF (21.07±3.47 pg/ml) in group HA+SVFs was found than other groups. Conclusions: Local injection of HA+SVFs with microneedles injection technology can significantly accelerate the healing rates of wound on porcine models, and this may be an alternative method for treatment of wounds in diabetic patients.

Keywords: SVFs, HA, microneedle injection, healing, diabetic wounds

Introduction

Wounds in extremities of diabetes mellitus (DM) patients usually are refractory in clinical practices [1], and these patients usually suffer from the severe complications caused by various factors, such as peripheral vasculopathy, insufficiency of endogenous growth factors, etc. [2, 3]. Latest studies confirmed that tissue engineering technique is an effective approach to repair the diabetic wound [4, 5]. It is very important that seed cells must be easily obtained and amplified, and the vehicle must suitable for cell attachment and growth. Adipose-derived stromal vascular fraction cells (SVFs) can be further divided into several subgroups, including adipose-derived mesenchymal stem cells (ADSCs), etc. [6]. ADSCs are easily harvested and preserved, and, in specific medium, can recover the skin through the paracrine mechanism [7].

Hyaluronic acid (HA) is one of the main components of extracellular matrix and composed of glucuronic acid and N-acetylglucosamine. Current research shows that the addition of HA in the skin wound of diabetic rats can promote the skin epithelium, thus improve the quality of wound healing. Exogenous HA can be biodegradable *in vivo*, and it is an ideal biomaterial scaffold. HA has prominent cell affinity, it can interact with the binding protein, protein polysaccharide and growth factors [8, 9]. Microneedles are widely used in cosmetic surgery, it can import drugs into skin tissues accurately and well-distributed [10]. Although SVFs therapy has become an effective method in chronic wounds treatment, the effect of adipose-

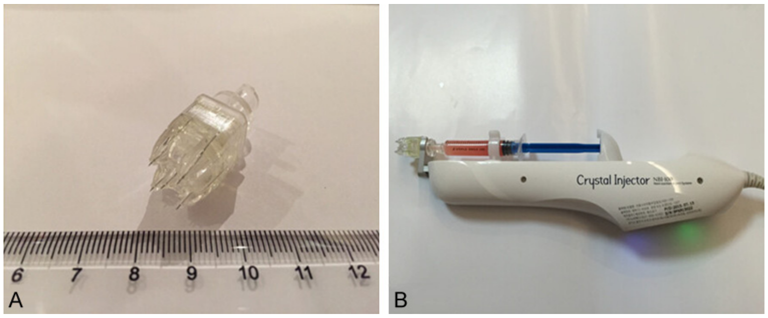

Figure 1. A. Microneedles. B. HA-SVFs mixture with microneedles injector. Isolated SVFs and HA solution (YVOIRE Hydro 10 mg/ml) that has been homogenized and made into HA-SVFs mixture (5 × 10^6/ml). In each group, the dissolved drugs were evenly injected into the granulation tissue of the wounds using microneedles injector (0.025 ml × 5 microneedles × 2 trigger = 0.25 ml, Crystal Multi Injector, Korea).

derived SVFs with microneedles injection technology on diabetic wound healing still remains unclear.

In this study, we aimed to investigate the efficacy of SVFs in combination with HA with microneedles injection technology on diabetic wound healing. The diabetic porcine model was used to determine the role of this novel method.

Methods

Animal models and surgical procedures

In our previous experiment, we had plenty of experience in making the animal models [11]. Four female domestic pigs (21-25 kg) were selected as the subjects and acclimatized for one week before the experiment. With the prepared diabetic models, we detected the level of serum glucose once per day. After subcutaneous injection of insulin (Neutral Insulin Injection, Wan bang, Jiangsu, China), the blood glucose was maintained between 250 and 500 mg/dl. Criteria for successful model establishment were that models exhibited significant symptoms of hypoglycemia initially, and then the level of blood glucose could be sustained over 250 mg/dl. The diabetic wound models were made by removal of full thickness skin (1.5 cm × 1.5 cm) on the pigs' back under general anesthesia. Four pigs were as cell donors for the preparation of adipose-derived SVFs. Finally, 12 wounds were made on each pig, the 12 wounds cover four groups on one pig (3*4), and those wounds were randomly divided into four groups: PBS group (Group A, n = 12); HA group (Group B, n = 12), SVFs group (Group C, n = 12) and HA+SVFs group (Group D, n = 12).

SVFs isolation and HA+SVFs preparation

On the day of HA administration (3 days after operation), tissues collected from the neck region were rinsed in PBS (GIBCO), and cut into small pieces followed by digestion. After filtration, cell suspension was centrifuged for collecting the pellets, which were then placed into lysis buffer containing 154 mM NH_4Cl, 10 mM $KHCO_3$ and 0.1 mM ethylene diamine tetra-acetic acid. After being washed with PBS twice, we obtained the SVFs [11]. Isolated SVFs and HA solution (YVOIRE Hydro 10 mg/ml, LG) were then homogenized and made into HA-SVFs mixture (5 × 10^6/ml).

Method of administration

Three days after surgical procedures, PBS and HA were injected into the wounds in Groups A and B, respectively. SVFs suspension was injected into the models in Group C. HA-SVFs mixture (5 × 10^6/ml) were injected into the models in Group D. Drug solution was evenly injected into the granulation tissue of the wounds using microneedles injector (0.025 ml × 5 microneedles × 2 trigger = 0.25 ml, Crystal Multi Injector, Korea) (**Figure 1**). Five minutes after injection of the drugs, all wound were covered with the sterilized vasline gauze.

All procedures were performed in strict accordance with the guidelines of the Chinese Ministry of Public Health (CMPH) under aseptic condition.

Measurement of healing rate

Wound healing rates were detected respectively at the postoperative 7^{th}, 14^{th} and 21^{st} days. With the photographs taken at each time point, we calculated the healing rates with Adobe Photoshop 9.0 software in following formula: Healing rate = [(original size - non-healing area)/original area] × 100%.

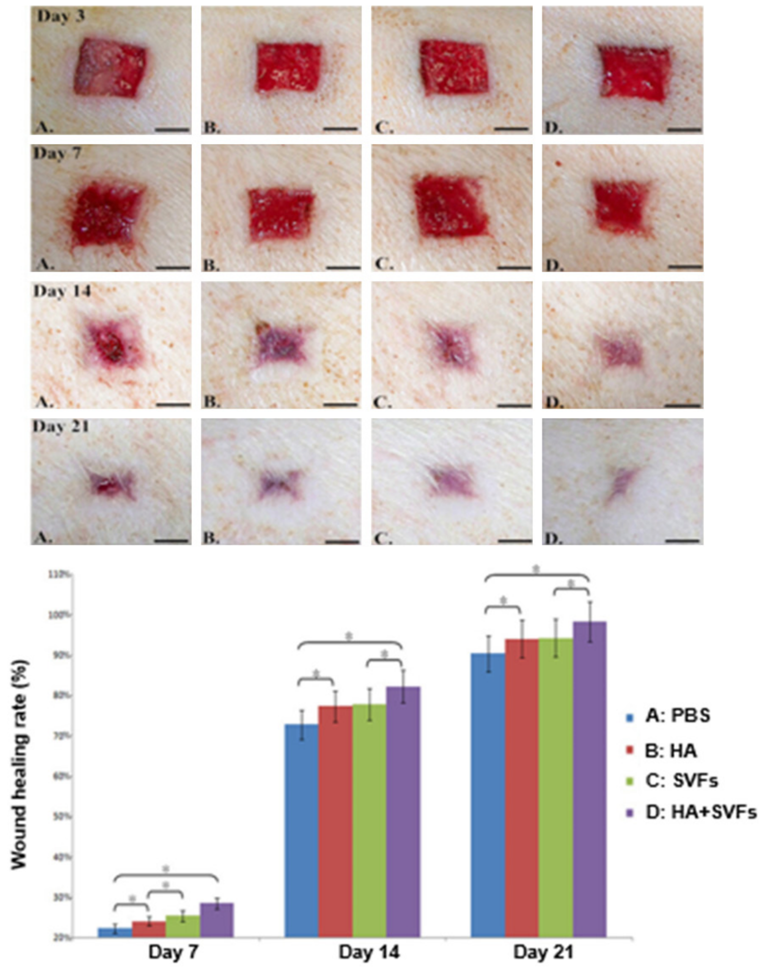

Figure 2. In Group D (HA+SVFs), wound healing rate is the highest among all groups at all time points, while the rate in Group C is significantly higher than Group A and B (P<0.05); in Group B, the rate is significantly higher than Group A (P<0.05) on the day 7. On the day 14 and 21, the rates have no significant difference between Group B and C (P>0.05), but their healing rate are still higher than Group A (P<0.05).

Histological analysis

The samples (granulation tissue of the wound) were collected under general anesthesia (day 7 after operation). Angiogenesis in sample tissues was measured by immune-labelling method [11]. In 6 randomly selected fields, two independent researchers were designated to count the capillaries in sample tissues (400 ×).

Expression of VEGF and bFGF in sample tissues

VEGF and bFGF are critical to the wound healing process, and, thus, were detected by ELISA. On 7th day after operation, 0.3 g specimen tissues were collected for homogenization. After centrifugation, we measured the levels of VEGF and bFGF using ELISA kit (CUSABIO, China), and also detected the optical density in a microplate reader (Biotek Instruments, USA) [11].

Statistical analysis

Data were expressed as mean ± standard deviation, and analyzed with SPSS 15.0 (SPSS Inc., Chicago, IL). One-way analysis of variance was carried out to perform intergroup comparison. *P*<0.05 suggested that the difference had statistical significance.

Results

Wound healing rate

In our study, all of the diabetic porcine models were successfully established. As for the wound healing rate on day 7 after operation, the rate in Group D (HA+SVFs) was the fastest in all groups (P<0.05, **Figure 2**) followed by the rates in Group C, Group B and Group A (*P*<0.05). On the day 14 and 21, comparison of the wound healing rate between Group B and C showed no statistically significant difference (P>0.05), but their healing rate remained still higher than Group A (PBS) (P<0.05), suggesting that the HA+SVFs could obviously accelerate the process of wound healing, and the HA and SVFs also have positive effect individually on wound healing (**Figure 2**).

HA+SVFs group showed great vascularization in wound

Group D (HA+SVFs 31.83±3.19) had highest capillary density in all experimental groups and control group. Compared with Group A (PBS 8.16±1.17), Group B (HA 14.67±1.86) and C (SVFs 21.83±2.56) had higher capillary density, Group C (SVFs) had better performance in capillary density than Group B (HA) (**Figure 3**).

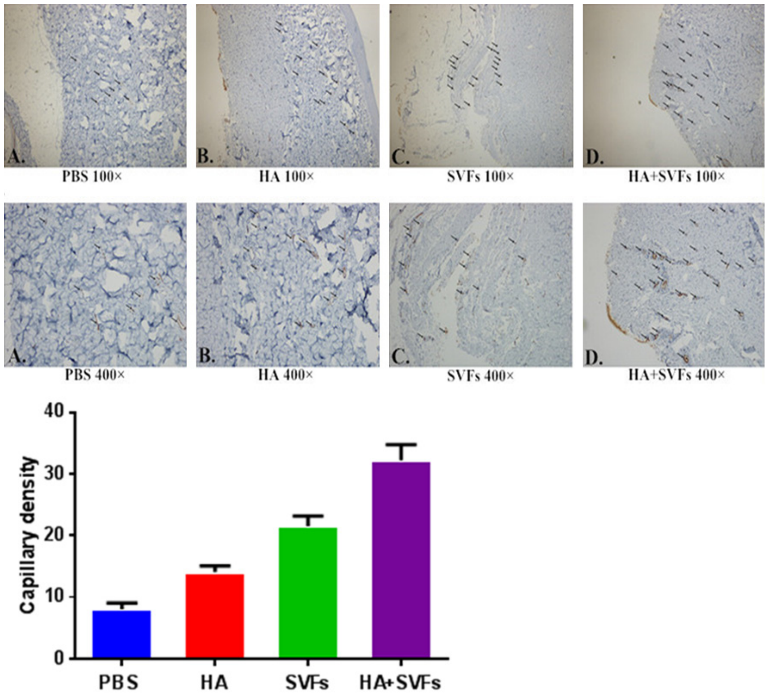

Figure 3. On day 7 after operation: Group D (HA+SVFs 31.83±3.19) had highest capillary density in all experimental groups and control group. Compared with Group A (PBS 8.16±1.17), Group B (HA 14.67±1.86) and C (SVFs 21.83±2.56) had higher capillary density, Group C (SVFs) had better performance in capillary density than Group B (HA).

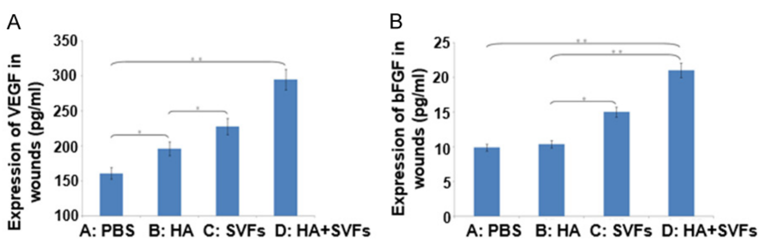

Figure 4. On the day 7 after drug administration: (A) The protein expression of VEGF in Group D (HA+SVFs) (295.52±49.21) pg/ml is the highest in all groups. Similarly, as for the expression of bFGF, Group D (HA+SVFs) shows a higher level (21.07±3.47) pg/ml in the diabetic wound granulation zone than the other three groups (*P<0.05*) (B). The levels of VEGF and bFGF in Group B (HA) and C (SVFs) are higher than Group A (PBS). In Group C (SVFs), the expressions of VEGF and bFGF in wound granulation zone are higher than those in the Group B.

Levels of VEGF and bFGF in wound tissues

On the day 7 after drug administration, we found that the protein expression of VEGF in Group D (HA+SVFs) (295.52±49.21) pg/ml was the highest in all groups (P<0.05) (**Figure 4A**). Similarly, as for the expression of bFGF, Group D (HA+SVFs) showed a higher level (21.07±3.47) pg/ml in the diabetic wound granulation zone than the other three groups (*P<0.05*) (**Figure 4B**). The levels of VEGF and bFGF in Group B (HA) and C (SVFs) were higher than Group A (PBS). In Group C (SVFs), the expressions of VEGF and bFGF in wound granulation zone were higher than those in the Group B.

Discussion

Adequate blood supply and a good local environment are necessary for healing [12]. It has been shown that diabetic wounds are usually caused by the surrounding cytokines in low levels [13]. In previous study, we have confirmed that SVFs has a significant promoting effect on diabetic wound healing [11]. In this study, we used adipose-derived stromal vascular fraction cells (SVFs)+ hyaluronic acid (HA) with microneedles injection technology in diabetic porcine model, the HA+SVFs with microneedles injection technology group showed a faster rate in wound healing and vascularization compared with other groups. We used HA as seed cells carrier, combined with micro needle injection technology, which can make the seed cells be delivered to the granulation tissue accurately, and the HA can keep the wound in a relatively humid environment. Our results indicate that local injection of HA+SVFs with microneedles injection technology can significantly accelerate the healing rates in wound of porcine models, and this may be an alternative method for treatment of wounds in diabetic patients.

The SVFs were easily obtained from subcutaneous fat, and, thus, adipose-derived SVFs without culture *in vitro* are effective choice for the clinical treatment of diabetic wounds. It has been reported that SVFs are difficult to be retained in the wound tissues since they are quite easily to be diffused into extracellular fluids after seeding [14]. Thus, we use HA as the carrier of seed cells and obtained the better effect. In the process of wound healing, the extracellular matrix is an essential component, which is composed of glycoprotein and proteoglycan, glycosaminoglycan and other large biological molecules [15]. The main component is the glycosaminoglycan, and the highest content of glycosaminoglycan is HA. HA is one of the main components of extracellular matrix and composed of glucuronic acid and N-acetylglucosamine [16]. It has been shown that the addition of HA in the skin wound of diabetic rats can promote the skin epithelium, thus improve the quality of wound healing [17]. Exogenous HA can be biodegradable *in vivo*, and it is an ideal biomaterial scaffold. HA has prominent cell affinity, it can interact with the binding protein, protein polysaccharide and growth factors [18]. In consistent with previous studies, our results also showed the above relative characteristics.

In recent years, microneedles are widely used in cosmetic surgery, it can import drugs into skin tissues accurately and well-distributed [10, 19]. The most representative application of the technology was inject HA into the dermis of the skin [20]. In our study, we apply this technology to the wound treatment creatively, and achieved better therapeutic effect. We showed that the HA and SVFs have positive effect individually on wound healing. HA+SVFs had highest capillary density. The protein expression of VEGF and bFGF in Group HA+SVFs was highest in all groups.

Previous study also showed that pluripotent mesenchymal stem cells, surrounding the adipose-derived SVFs, can be differentiated into endothelial cells, produce high levels of VEGF/bFGF and promote the angiogenesis *in vitro* [21]. Although new treatment method is effective on diabetic wound healing, it still has its limitations. The most suitable proportion and the frequency of administration of HA and SVFs still need to be further studied. In some cases, we found there was a slight bleeding and pain from the wound; we will improve the method of administration in further study.

In conclusion, local injection of HA+SVFs with microneedles injection technology promotes the healing process of diabetic wound in porcine model, which could potentially provide a better therapeutic option for diabetic wounds.

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Disclosure of conflict of interest

None.

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