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Study on GelMA hydrogel loading small extracellular vesicles from human umbilical cord mesenchymal stem cells to promote wound healing

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Abstract. Experimental research methods were adopted. To investigate the effect of GelMA hydrogel loaded with small extracellular vesicles derived from human umbilical cord mesenchymal stem cells on wound repair.HucMSC-sEVs separated by ultra-high speed centrifugation showed a cup-shaped structure under transmission electron microscopy; hucMSC-sEVs express positive markers for small extracellular vesicles, namely CD9, CD63, and TSG101, but do not express negative markers for small extracellular vesicles, Calnexin. The results of the cell scratch experiment showed that 6 hours after scratching, the migration rates of HEK, HDF, and HUVEC in the sEVs group were significantly higher than those in the control group. After 12 hours and 24 hours of scratching, the migration rates of HEK, HDF, and HUVEC in the sEVs group were significantly higher than those in the control group. The Transwell experiment showed that after 12 hours of cultivation under the same conditions, the migration numbers of HEK, HDF, and HUVEC in the control group all were significantly less than the sEVs group、. The EdU staining results showed that the proportion of proliferating cells in the sEVs group of HEK, HDF, and HUVEC cells was significantly higher than that in the control group. SEM results showed that 15% GelMA hydrogel had regular three-dimensional network porous structure. The healing rate of full layer skin defect wounds in mice was statistically analyzed. The Gel+sEVs group showed a significant increase in wound healing speed compared to the sEVs group, Gel group, and blank control group.hucMSC-sEVs can be successfully extracted by ultrahigh speed centrifugation. HucMSC-sEVs can promote the migration and proliferation of HEK, HDF and HUVEC, and can be combined with GelMA hydrogel as wound dressing. GelMA hydrogel loaded with hucMSC-sEVs can significantly improve the speed of wound healing in mice.

Keywords: Mesenchymal stem cells, Small extracellular vesicles, Wound repair, GelMA hydroge.

1. Introduction

For a long time, wounds including burns and diabetic ulcers are often treated by debridement surgery, wound dressing exchange and negative pressure suction in clinics [1-3]. Because of the large area and deep degree of injury, even the skin tissue with strong regenerative ability is difficult to rely entirely on its own repair ability to make the wound heal by itself, and surgical treatments such as skin patch or flap

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transplantation are still needed, which brings great burden to the patients. Previous studies have shown that epidermal keratinocytes, dermal fibroblasts and vascular endothelial cells play important roles in epidermal formation, dermal tissue structure stabilisation and angiogenesis during wound healing [4, 5]. How to strengthen the activity of skin cells and enhance their regenerative function, and then promote wound healing is one of the current problems in the field of wound repair. With the development of regenerative medicine, stem cell therapy brings new hope for wound repair and regenerative healing [6, 7].

MSCs are a kind of pluripotent stem cells, which have the ability of self-renewal and multidirectional differentiation, and are one of the research hotspots in regenerative medicine, and are called “seed cells” because under ideal conditions, MSCs can ensure their self-replication and renewal ability through “endogenous asymmetric division” [7] which ensures their ability to self-replicate and renew themselves. MSCs come from a wide range of sources, including bone marrow MSCs, human umbilical cord MSCs and adipose-derived MSCs. Recent studies have found that MSCs exert their effects mainly through paracrine effects, in which extracellular vesicles are an important component of paracrine secretion.EVs are particles of approximately 40 - 5000 nm in size produced by cellular paracrine secretion, and small extracellular vesicles are a classification of EVs, denoting extracellular vesicles with a diameter of less than 200 nm [8]. Studies have shown that MSC-sEVs can promote epithelial cell proliferation and differentiation, induce angiogenesis, and promote functions such as nerve, bone, cartilage, and cardiac repair [9, 10], which is attributed to the fact that components such as proteins, microRNAs, long-chain non-coding RNAs, and lipids encapsulated in MSC-sEVs activate the relevant signalling pathways by acting directly or indirectly on the target cells, and thus exerting reparative and regenerative effects [11, 12]. In addition, MSC-sEVs have been shown to inhibit inflammation and attenuate apoptosis, and it has been demonstrated that sEVs produced by MSCs, such as BMSC-sEVs, hucMSC-sEVs, and ADSC-sEVs, can promote skin wound healing [13, 14]. However, sEVs directly applied topically or superficially injected into the wound will be recognised and cleared by the body’s immune system, and their short retention time in the wound cannot achieve the expected therapeutic effect, and increasing the dosage of topically or superficially injected sEVs does not significantly improve the above situation [15]. Therefore, how to increase the retention time of sEVs in the trauma to allow them to better exert their therapeutic effects is an urgent problem that needs to be solved.

Hydrogel is a three-dimensional reticulated gel formed by physical or chemical cross-linking of hydrophilic polymers with good biocompatibility [16]. Gelatin is a product of partial denaturation of type I collagen, which retains the natural arginine-glycine-aspartic acid peptide of collagen, which facilitates cell and tissue adhesion [17]. The amino group of gelatin can react with methacrylic anhydride to synthesise methacrylic anhydride gelatin, which generates free radicals and polymerises and cross-links under the action of photoinitiators and 405 nm blue light.The GelMA hydrogel solution has good fluidity at room temperature and can be added dropwise to the wound site, and then is able to cross-link rapidly under UV illumination, it can form a gel in-situ, to adapt to wounds of different shapes and depths [18].

In this study, we verified that hucMSC-sEVs can promote the migration and proliferation of skin cells associated with wound healing, and developed a GelMA hydrogel loaded with hucMSC-sEVs, which can be used to form a gel to cover the wound in situ at the skin defect, and to promote the healing of skin wounds through the action of hucMSC-sEVs in the wound microenvironment.

2. Methods

2.1. Extraction and identification of hucMSC sEVs

hucMSC-sEVs were extracted from human umbilical cord mesenchymal stem cells using ultracentrifugation. The morphology of hucMSC-sEVs was observed under transmission electron microscopy, and surface markers (CD9, CD63, TSG101, and Calnexin) were identified by Western blotting.

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2.2. Cell migration and cell proliferation detection

Human epidermal keratinocytes, human dermal fibroblasts, and human umbilical vein endothelial cells were divided into a control group and an experimental group, respectively. The NC group cells were cultured in complete medium, while the sEVs group cells were cultured in complete medium and 100%, respectively Cultivate hucMSC-sEVs at a concentration of 100μg/mL. Verify the migration ability of each group of cells using cell scratch experiments and Transwell experiments, and detect the proliferation ability of each group of cells using 5 - ethynyl - 2 '- deoxyuridine (EdU) staining.

2.3. Animal experiments

15% GelMA hydrogel was prepared, and the microstructure of the hydrogel was observed under scanning electron microscope. To establish a full-thickness skin defect wound model in mice, the mice were randomly divided into control group, hucMSC-sEVs group, GelMA hydrogel group and hucMSC-Gel+sEVsMA hydrogel group. The NC group mice were injected with PBS solution around the wound edge, the sEVs group mice were injected with the same volume of sEVs suspension, the Gels group was injected with 15% GelMA solution on the wound surface, and the Gels+sEVs group was injected with the same volume of 200% GelMA solution on the wound surface 15% GelMA solution of 200μg/mL sEVs was fully crosslinked with ultraviolet light, and the wound healing rate was observed and counted on days 0, 4, 8, and 12 after modeling.

3. Results

3.1. Characterisation of small extracellular vesicles of MSC origin

The results showed that hucMSC-sEVs showed a cup-shaped structure under the scanning electron microscope; the results of WesternBlot experiments showed that the isolated sEVs were enriched with positive markers for small extracellular vesicles, i.e., CD9, CD63, and TSG101, whereas the sEVs-negative marker, Calnexin protein, was almost absent in the isolated sEVs, and the results suggested that the hucMSC-sEVs were successfully extracted after ultrahigh-speed centrifugation successfully extracted hucMSC-sEVs. see Figure 1.

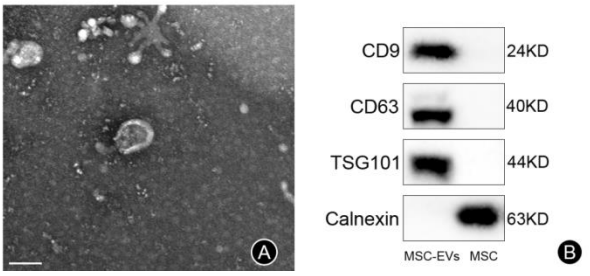

Figure 1. Identification of hucMSC-sEVs. 1A. Identification of hucMSC-sEVs morphology Transmission electron microscopy Scale bar is 100 nm; 1B. Identification of characteristic markers of sEVs, protein blotting method

3.2. Cell migration

3.2.1. Cell scratch test. At 6, 12 and 24 hours after scratching, the HEK migration rates in the NC group were (2.0±2.4)%, (14.0±5.8)% and (58.1±2.9)%, respectively, which were significantly lower than those in the sEVs group, which were (51.4±3.3)%, (88.2±2.0)%, and (95.7±1.4)% (all P<0. 001; t-values were 20.81, 20.98, and 20.04, respectively).

The HDF migration rate in the NC group was (9.3±3.4)%, (19.5±3.5)%, and (47.8±5.7)% at hours 6, 12, and 24, respectively, which was significantly lower than that in the sEVs group, which was (19.8±4.6)%, (35.4±3.4)%, and (62.2±1.0)% (p-values 0.033, 0.004, and 0.013, respectively; t-values 3.18, 5.68, 4.28, respectively).

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The HUVEC migration rate in the NC group was (7.2±2.9)%, (10.1±1.7)%, and (39.1±7.2)% at hours 6, 12, and 24, respectively, which was significantly lower than that in the sEVs group, which was (15.1±1.2)%, (38.9±0.6)%, and (56.7±2.6)% (p-values of 0.012, <0.001, and 0.016, respectively; (t-values of 4.32, 19.33, 4, respectively). See Figure 2.

The results indicated that sEVs could significantly promote the migration of keratinocytes, fibroblasts and vascular endothelial cells in the horizontal direction.

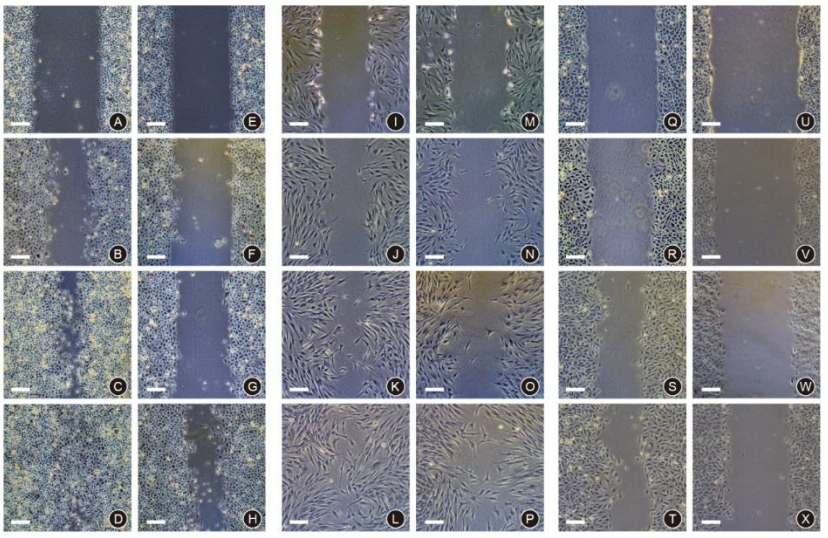

Figure 2. Cell migration area at each time point after scratching of HEK, HDF and HUVEC in 2 groups

3.2.2. Cell Transwell Experiment. After the two groups of cells treated by different methods were cultured under the same conditions for 12h, the numbers of HEK, HDF and HUVEC migrated in the NC group were (91.0±20.0), (48.3±12.2) and (39.0±2.0), respectively; all of them were significantly less than those in the sEVs group (193.7±17.2), (215.7±9.1) and (84.7± (3.5) ones (p-value 0.003, <0.001, 0.002, t-value 6.75, 19.57, 7.66, respectively). See Figure 3 and Table 2.

The results showed that sEVs could significantly promote the migration of keratinocytes, fibroblasts and vascular endothelial cells in the vertical direction.

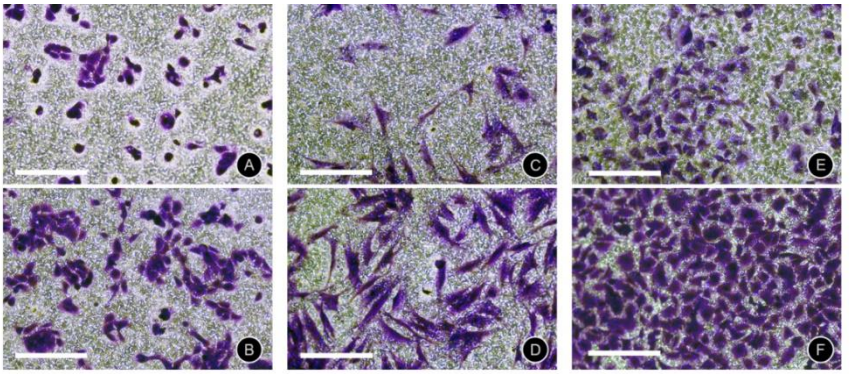

Figure 3. Migration of HEK, HDF and HUVEC Transwells in 2 groups Crystalline violet × 200, scale bar is 100 μm.

3.3. Cell proliferation

EdU staining and Hoechst staining were performed after cell culture until the density reached 80%-90%, and the number of EdU-stained positive cells of the three types of cells in the sEVs group was seen to

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be significantly higher than that of the NC group, as shown in Fig. 4. The statistical proportions of the proliferating cells were (48.7±0.8%), (63.9±0.9)%, and (35.9±2.2)%, respectively, all of which were significantly higher than that of the NC group (all P< 0.001, t-values 22.00, 13.82, and 32.32, respectively).

Note: The lower left small image in each figure is the Alexa Fluor 488 labelled EdU stained (green, indicating proliferating cells) image; the lower right small image is the Hoechst 33342 stained nucleus (blue); the upper large image is the composite image of the lower 2 small images, with the green and the blue superimposed in cyan.

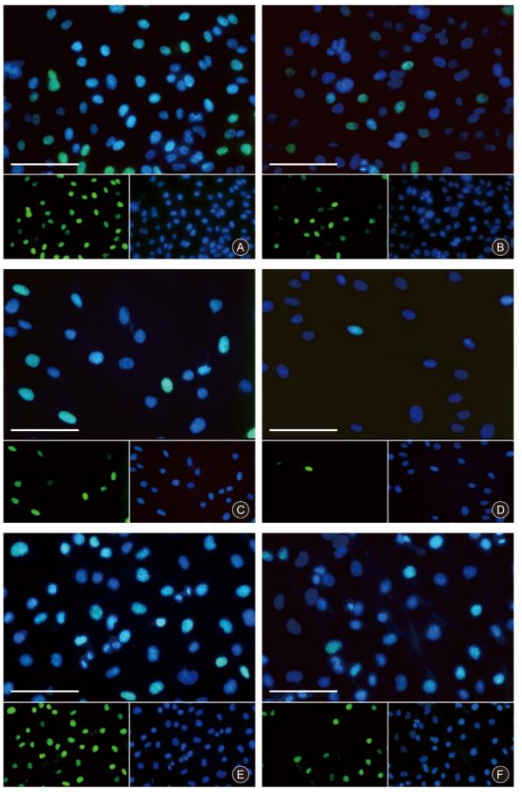

Figure 4. Proliferation of HEK, HDF and HUVEC in 2 groups Alexa Fluor 488, Hoechst 33342 × 200, scale bar 100 μm.

3.4. Characterisation of GelMA hydrogels loaded with MSC-derived extracellular vesicles

The GelMA hydrogels showed a loose and porous sponge-like structure inside with a uniform and smooth gel composition. See Figure 5.

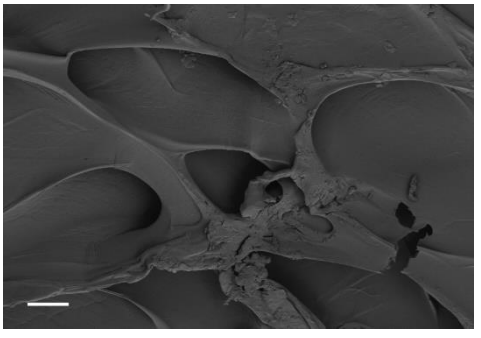

Figure 5. Microstructure of GelMA hydrogel, scale bar 20 μm.

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3.5. Skin wound healing in mouse whole skin trauma model

In the mice whole skin trauma model, no statistically significant difference was observed in the trauma area of each group when measured in the immediate postoperative period. On the general observation, on the 4th postoperative day, obvious redness, swelling and secretion could be seen on the wound in the NC group, while no obvious signs of wound inflammation and infection could be seen in the remaining three groups. On postoperative day 8, the Gel+sEVs group had the smallest residual wound, the best degree of skin keratinisation at the healed site, and good hair follicle activity. On the 12th postoperative day, the skin wounds in the Gel+sEVs group were completely healed, while the remaining three groups still showed different degrees of unhealed wounds, and the wound healing rates of the sEVs group, the Gel group, and the NC group were (97.84±0.18)%, (93.73±0.45)%, and (78.09±1.09)%, respectively. Compared with the NC group, the wound healing rate was significantly higher in the sEVs group, suggesting that the use of sEVs alone had a promoting effect on skin wound recovery. Compared with the sEVs group, the Gel+sEVs group had faster wound healing, as shown in Figure 6 and Table 4.The results indicated that hucMSC-sEVs or GelMA hydrogel alone could improve the wound healing rate of whole skin defects in mice to a certain extent, whereas the pro-healing effect of GelMA hydrogel loaded with MSC-sEVs was the most pronounced for the wound healing of mouse skin.

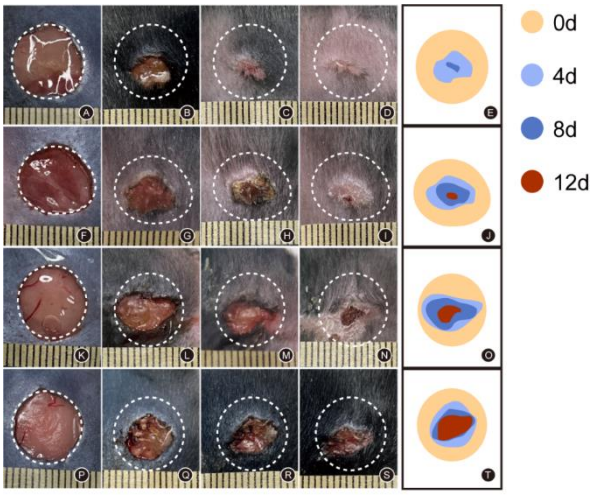

Figure 6. Healing of the whole skin defect wounds on the back of mice in 4 groups at various time points. Note: The white dashed circle in each figure shows the shape of the wound in the immediate postoperative period in that group.

4. Conclusion

In this study, we constructed a GelMA hydrogel loaded with hucMSC-sEVs, which prolonged the presence of hucMSC-sEVs on the wounds, and promoted the proliferation and migration of wound-healing-related cells, thereby promoting the healing of skin wounds. The results of this study provide a certain theoretical basis for the clinical treatment of skin wounds, further proving that the novel biomaterials can be combined with the traditional wound treatment modalities, and provide new ideas for the future treatment of clinical wounds.

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