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Re-examination on the Preparation of Topical Hydrogels Containing Human Umbilical Cord Mesenchymal Stem Cell Exosomes on a Rat Animal Model

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ABSTRACT

Full thickness skin defects and its treatments are fields where new critical challenges remain unsolved. Recent discoveries on the therapeutic application of human umbilical cord derived exosomes (hUCMSCs) provides a new scope into facilitating the wound healing effect. Beyond an effective loading, a natural hydrogel is further employed by multiple studies. It is proved to extend the bioactivity of the exosomes and also to contribute the antibacterial and antioxidant effect using hydrogel. The combination of hydrogel and hUCMSCs might provide synergy benefits. Herein, we established a biocompatible, thermos-sensitive chitosan β-glycero-phosphate (CS/GP) hydrogel enclosing hUCMSCs which is performed on rat cutaneous wound sites. The material preparation and characterization, exosome derivation and characterization, and in vivo wound healing study are examined in this paper. The in vivo study demonstrates the effect of exosome and hydrogel as effective wound-healing promoters individually, implied by a faster and higher wound reduction rate. The study also shows that the effect of hydrogel loaded with exosome proves to need more clarifications and investigations to maximize their influence on wound sites healing.

KEYWORDS: *Topical Hydrogels; Human Umbilical Cord; Mesenchymal Stem Cell; Exosome; Rat Animal Model*

INTRODUCTION

The presence of skin protects organisms from pathogenic infections and reduces the direct damage done to our body system. However, as an outermost protection, dermal injuries happen frequently, caused by burns, vascular diseases, and diabetes[1]. There is an increasing research interest examining methods to maximize the healing effect of current applications dealing with both acute and chronic injuries.[2] Specifically, scientists focus on increasing the efficiency of wound debridement, preventing further infection of the injured sites, keeping optimal moisture balance, and removing excrescent exudates timely. Among all studies, stem cell derived exosomes applied with hydrogels has showed great potential in recent years.[3] [4]

Exosomes are 30–100 nm extracellular vesicles produced by cellular bodies, including all eukaryotes and prokaryotes. Initially, they are identified as a deliberate output of selected proteins, lipids, and RNAs from cells, like the rest of the extracellular vesicles. Recently, their potential value was investigated in further progressions associated with intercellular communication.[5] Specifically, people have drawn reasonable conclusions on the unique functions possessed by the exosomes derived from human umbilical cord mesenchymal stem cells(hUCMSCs) [6]. Known for their high differentiation, in vitro expansion, release of trophic materials, and immunoregulatory properties, mesenchymal stem cells (MSCs) are adult stem cells of particular importance in tissue repair and the treatment of various diseases. Among the universal traits possessed by all stem cells, huc-MSCs are ideal exosome sources due to their non-invasive isolation method, lower immunogenicity, and faster self-renewability etc. hUCMSC exosomes (hUCMSC EXO) conserve identical advantageous characteristics as their mother cell, with a lower immunogenicity and no tumorigenic outcomes. What's more, applying huc-MSC EXOs also maximizes the effect

1 Rani, Sweta, and Thomas Ritter. "The Exosome - a Naturally Secreted Nanoparticle and Its Application to Wound Healing."Advanced Materials, no. 27(2015): 5542–5552.

2 Tottoli, Erika Maria, et al. "Skin Wound Healing Process" Pharmaceutics, no. 8(2020):735.

3 Han, Chaoshan, et al. "Human Umbilical Cord Mesenchymal Stem Cell Derived Exosomes Delivered Using Silk Fibroin and Sericin Composite Hydrogel Promote Wound Healing." Frontiers in Cardiovascular Medicine.

4 Han, Chaoshan, et al. "Human Umbilical Cord Mesenchymal Stem Cell Derived Exosomes Encapsulated in Functional Peptide Hydrogels Promote Cardiac Repair." Biomaterials Science, no. 7(2019):2920–2933.

5 Van Niel, Guillaume, et al. "Shedding Light on the Cell Biology of Extracellular Vesicles." Nature Reviews Molecular Cell Biology, no. 4(2018): 213–228.

6 Yaghoubi, Yoda, et al. "Human Umbilical Cord Mesenchymal Stem Cells Derived-Exosomes in Diseases Treatment." Life Sciences(2019)116733

of stem cell themselves by maintaining an adequate and longer-lasting biological activity, and ensuring successful and targeted delivery of substances. Thus, using hUCMSC EXO as an innovative cell-free therapy is blooming in recent years. Zhang et al have examined the influence of hUCMSC EXOs on angiogenesis using a rat burn model. The conclusion is that hUCMSC promotes the proliferation, migration, and tube formation of endothelial cells depending on doses. [7]

To amplify the advantages of hUCMSC EXOs, researchers explored better wound dressing to retain the medical effect of exosomes. One of the most promising wound dressing is hydrogel. Hydrogels are cross-linked networks of the same or different types of polymers with high water-absorbing capacity.[8] Profound research indicates the rising therapeutic value of hydrogel as wound dressing and pathways of drug delivery due to its distinct properties in antimicrobial trait, adhesion, hemostasis, anti-inflammatory, anti-oxidation, and substance delivery etc.[9,10,11] Especially, studies have shown that Chitosan/β-Glycerophosphate (CS/GP) Hydrogel is an eligible candidate among all dressings. Chitosan/β-Glycerophosphate (CS/GP) Hydrogel has an in situ thermal-sensitive gelling system. It is in fluid form at low temperature (room temperature or below) and becomes gel at around body temperature.[12] The positively charged chitosan and anionic GP are ionically linked together which makes it more thermal sensitive and non-toxic compared to covalently linked chitosan hydrogel. In all measurements, CS/GP hydrogel is a more biocompatible wound dressing option. However, most of the hydrogel applications are presented in injectable forms instead of topical forms. Moreover, how to implement it in real applications still needs more research.

Thus, in this study, I'm going to explore the detailed preparation procedure of exosome-loaded CS/GP topical hydrogel and its wound healing effect on a rat animal model.

MATERIAL AND METHODS

Materials

Chitosan CAS (viscosity: 100–200mPa.s, deacetylation degree ≥ 95%) was purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China); β-glycero-phosphate (Purity ≥ 97%) was purchased from Beijing Solarbio Science & Technology Co., Ltd (Beijing, China); the acetic acid (MW:60.05) was purchased from Sangon Biotech Co., Ltd. (Shanghai, China); the hUCMSCs supernatant were kindly provided by Guangzhou Doublle Bioproduct Co., Ltd. (Guangzhou, China)

Isolation and Identification of hUCMSC EXOs

The capsule filter (Anow Microfiltration, Hangzhou, China) was applied to isolate and purify the exosomes from hUCMSC culture supernatant. All equipments required need to be sterilized under high pressure beforehand, including a 0.45-0.65 μm filter, flasks and tubes. NaCl(0.9%) solution is prepared for further purification. And NaOH(0.5mol) solution is prepared to store and submerge the filter. 5000ml of frozen hUCMSC needs to be thawed before the experiment takes place. The procedures are then carried out according to the following steps: first, the thawed hUCMSC supernatant is filtered using a 0.45-0.65 μm filter that is further rinsed with the NaCl solution using a reflux system for 30 minutes; (This is to remove the cell debris and large molecules that is present in the supernatant.). Then the solution is collected using the sterilized flask; use the capsule filter to condense the pre-filtered supernatant to a desired concentration(about 10 times); use the saline solution to depigment the concentrated sample 4 times; finally, use a 0.22 μm Polyethersulfone (PES) syringe filter to pasteurize the concentrated and depigmented sample; divide the completed sample in sampling tubes and store them in -80°C conditions.

The surface characteristic markers of hUCMSCs derived exosomes were detected via Western Blotting method(WB), including CD81, CD63. The morphology of the exosomes was detected by TEM. The particle size and concentration of exosomes present in the supernatant was measured by the Nanoparticle Tracking Analysis (NTA).

Preparation of Chitosan-Based Hydrogel Containing hUCMSC EXOs

The thermosensitive chitosan based hydrogel is prepared through the crosslinking of chitosan and β-glycero-phosphate (CS/GP). Chitosan is first dissolved in acetic acid(0.1 $mol^{-1}L$) to make a solution of 2.5% concentration. The solution is stirred for at least 24 hours using the magnetic stirrer to ensure that chitosan is fully dissociated within the acid. Then β-glycero-phosphate solution (50.6%) is dissolved in water and mixed for at least 10 minutes. Transparent solution should be observed when ready. The chitosan acetate solutions would then undergo high-temperature (121°C) dissolution in the Ultrasonic Cleaner at 40 khz(SB-5200D,Scientz,

7 Zhang, Bin, et al. "Human Umbilical Cord Mesenchymal Stem Cell Exosomes Enhance Angiogenesis through the WNT4/β-Catenin Pathway." Stem Cells Translational Medicine,no. 5(2015):513–522.

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Zhejiang, China). The GP solution is then dropped into the fully dissolved chitosan solution that is bathed in ice while being stirred by the magnetic stirrer. The ideal proportion of CS and GP solution should be around 1ml: 60μl.

Rat Cutaneous Wound Modelling

Twelve specified pathogen free grade male rats(12 weeks) were purchased from Guangzhou Ruige Biological Technology Co.,Ltd (Guangzhou, China). The experimental animals are totally preparatory narcotized via the injection of Tribromethanol (0.2ml/10g). The dorsal hair of the rats is removed using a shaver, with the skin disinfected before the operation. The wound model includes four groups of round-shaped full-thickness dermal defect with the diameter of 9mm. The classified wound groups is one negative controlled group treated with phosphate buffered saline (PBS), one group treated with exosome-contained hydrogel(90μl hydrogel with 10μl exosome covered the wound), one group treated with pure hydrogel(100μl hydrogel covered the wound), and one group with pure exosome (90μl PBS solution with 10μl exosome). All treated rats are reared in different cages according to their groups in a SPF level animal laboratory. Treatments are given to the rats everyday for two weeks. The macroscopic effects of wound healing on rat models are carried out by taking photographs using phone cameras. Wound sizes (width and height) are measured using Vernier caliper every two days. Descriptive record regarding the detailed condition of injuries is written every day.

Mathematical model is used to examine the rate of reduction of wounds:

$$S = hw$$

$$R_n = \frac{S_1 - S_2}{S_1} \times 100\% \ (1 \leq n \leq 12)$$

$$R_f = \frac{(R_1 + R_2 + \cdots + R_{12})}{12}$$

S = size of the wound(mm^2)

h = height(mm)

w = width(mm)

Rn = daily reduction rate

Rf = final reduction rate

S1 = size of the wound of the previous day

S2 = size of the wound of the next day

Data Analysis

Data from at least 3 individual experiments are listed as mean ± standard deviation. One-way ANOVA test is applied to distinguish the significant difference and relationship between individual groups.

RESULTS

Characterization of huc-MSC Derived EXOs

The isolated and decontaminated exosomes derived from huc-MSCs are examined through WB method,TEM, and NTA (Figure 1). Through the WB analysis, high levels of CD81 and CD63 could be observed on the isolated exosomes (Figure1(a)). In Figure 1(b), the exosomes mostly have spherical morphologies in TEM tests. The range of diameters of isolated exosomes ranges from 10 to 200 nm based on the NTA results (Figure1(c)). The size of the highest distribution (1.75×10^7) is about 100 nm, which is within the expected range of the previous studies.[13]

Hydrogel Property

To testify the thermo-sensitive property of the prepared hydrogel, the well-blended CS/GP hydrogel is then divided into 1ml portions and is placed into sampling tubes at different temperature conditions 4℃, room temperature (26℃), and body temperature (37℃). 4group is at the thermostatic fridge; room temperature (26℃) group is in the room without additional temperature adjustments; body temperature (37℃) group is incubated in the thermostat water bath to simulate body conditions. Ideally, the hydrogel would gel works the most effectively under body temperature, according to existing studies[14]. Observing the photos of the samples that are placed horizontally on a flat platform taken after 30 minutes (Figure 2), the hydrogel extends the most and possesses high liquidity at 4. It however inherits great viscosity in the 37℃ condition, indicating its role as a longer-lasting carrier of exosomes. This observation matches the previous assumptions and fits the necessary functions as an epidermal coating.

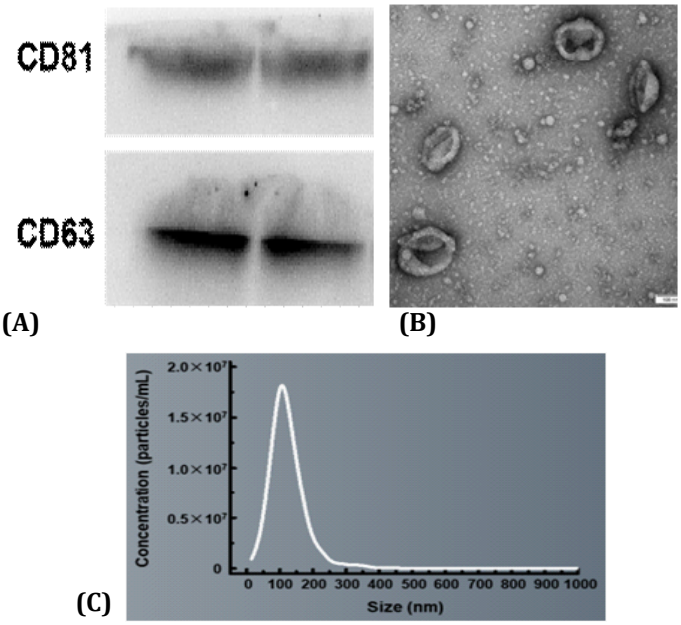

Figure 1. Identification and characterization of hucMSC-derived exosomes: (A)detection of biomarker (CD81,CD63) expressions by western blot analysis; (B)TEM image of exosomes, 100μm; (C) particle size distribution of exosomes.

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14 Wenquan, Liang, et al. Temperature Sensitive Chitosan Hydrogel. 24 Aug. 2011.

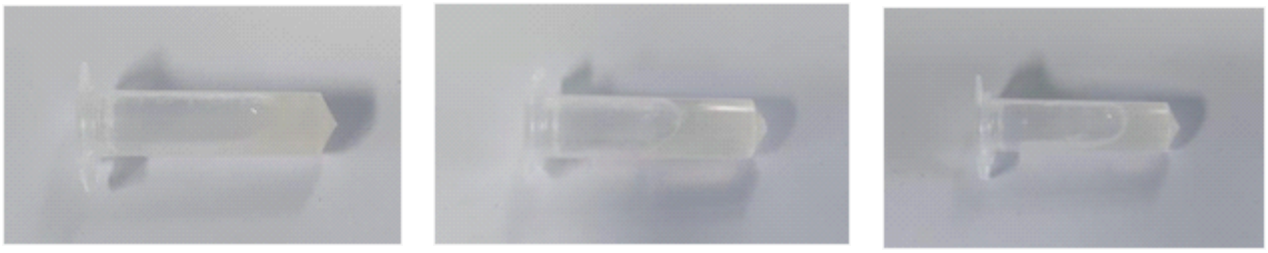


Figure 2. CS/GP Gelling Effect under different temperatures(4, 26,37)

In Vivo Wound Healing Study

Figure 3 has shown the macroscopic wound site of the full cutaneous impaired models. Each group is treated with PBS (control), pure exosome supernatant, pure hydrogel, and exosome loaded hydrogel. All groups are observed visually with a great reduction in the size of wounds after 14 days. There was 1 infection case in the Exo-hydrogel group at Day 8, observed with yellow pus. The EXO hydrogel and hydrogel group have shown a slower healing rate at Day 4 and Day 7 than other treated groups. The pure exosome and control groups have shown the most efficient wound repair effect with no obvious signs of cutaneous injuries by the 14th day while the rest of the groups presented traces of white peeling skin.

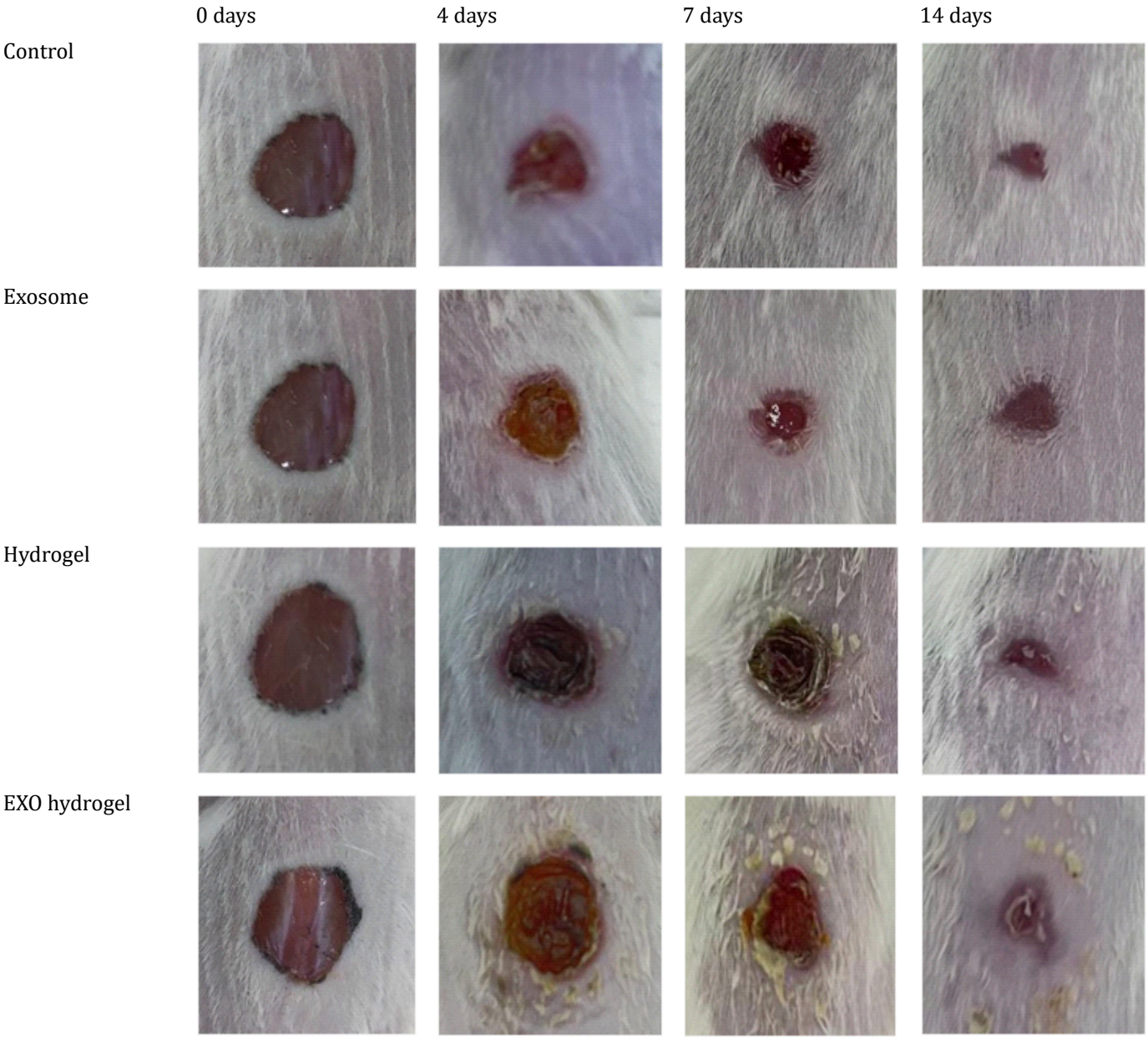
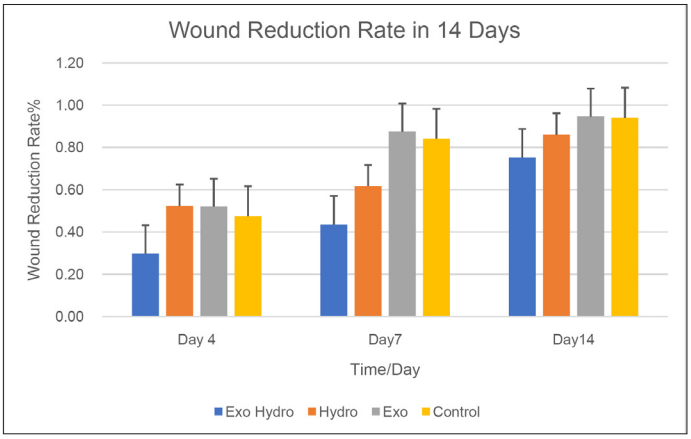


Figure 3 (a). Rat wound sites treated with hydrogel and exosome facilitated application in 14 days

In accordance with the visual observation, the quantitative wound reduction rate calculated by excel shows that the Exo and control group achieved the highest increase of wound reduction rate on Day 14 compared to the other groups, with similar final rates of 94.67% ± 22.84%and 94.11% ± 24.6%. The data indicates a similar wound healing effect of pure exosome and saline. Their reduction rates also achieved a higher level faster in the first 7 days among all groups, indicating their efficiency as wound dressings. The reduction rate of the Hydro group and Exo Hydro group kept advancing until Day 14, but reveals however less dramatic changes than the other two on the final day. Although the rate of Hydro group has shown a less significant effect (86% ± 17.4%) by Day 14, it is still respectively high. However, the Exo Hydro group doesn't show the predicted wound reduction effect in this experiment as much research has suggested. It has revealed the lowest rate of wound reduction during the whole process, with the final rate of 75.24% ± 23.3%. There's also a case of infection during the experiment. And this phenomenon is most likely related to the preparation and application method of the Exo hydrogel. As research has demonstrated, the effect of different hydrogel is largely dependent on their types and their ways of application, making them hard to perform well in all aspects at the same time. In this case, the CS/GP hydrogel didn't show excellent anti-bacterial property. Overall, the Exo and control group obtained the most significant healing effect, which satisfies the visual observation.



DISCUSSION

Exosomes have showcased their valuable properties both in therapeutic and diagnostic fields. Yet due to the physical constraints of sustaining the activity of these isolated extracellular vesicles on wound sites, researchers have developed means of elongating their wound-healing effects. And there is already lots of existing research which dives deeply into the specific wound scenarios which applies the hydrogel as a drug carrier. However, few of them are done with topical hydrogels. Moreover, most of them lack a fully implemented examination of the possible factors which could affect the overall result when the experiments are repeated. Thereby, I am intrigued by a more detailed procedure of developing such hydrogel and the possible precautions that could be done to ensure the efficiency of the experiment.

As shown by the result, it is inconsistent with some assumptions that exosome hydrogel could have an impressive impact on improving the overall wound recovery, indicating yet fulfilled system of preparing the experiment. And there are several factors that we found during the procedure could be decisive for the overall result.

Though topical medication is a well-practiced form of treatment, there could still be some disadvantages regarding smaller and more active experimental targets. Within the 14 days of the study, we observed that though the hydrogel dressing could have an impact on the wound sites, their effect is not maximized due to the constant movements and the self-nibbling behavior of the rats. Most exosomes may not be able to fully operate before being rubbed off by the rats' active interactions with their surroundings. Thereby, an injectable hydrogel which obtains low off-target leakage is more suitable in this setting according to the study conducted by Bertsch et al[15]. However, in reality, most patients would not expose their wound sites in frequent contacts. Thereby, there is a potential that the Exo hydrogel may present a better image in non-experimental settings.

The biocompatibility and biodegradability of hydrogels is also important when serving as a scaffold in resemblance to the natural ECM. In replacement of the original complicated bio-structure, hydrogel needs to have an appropriate 3D structure and pore size that matches the target cell.[16] As Nahhidi has stated, biocompatibility refers to the ability of scaffolds to provide enough space for the cells to migrate and expand. Thereby, if the pores are not large enough to allow the fundamental diffusion and transport of substances, then the biocompatibility would vary accordingly.[17] In terms of natural hydrogels, they possess great biocompatibility, however, are difficult to process and control. Research suggested that methods that aims to mitigate the biodegradability would often result in a lower biocompatibility.[18]

One more hypothesis is that the higher moisture level of the hydrogel caused the slower wound recovery. It is a study we can conduct in the future to change moisture levels and understand the impact.

The current studies are abundant regarding the therapeutic application of hydrogel. Despite cases of successful delivery of drugs through hydrogel in vivo, its practical value is still undetermined. More in vivo tests and experiments need to be carried out to specify the scenarios where the target cell, loaded substance, and the artificial scaffold could invoke each other's most potential.

15 Bertsch, Pascal, et al. "Self-Healing Injectable Hydrogels for Tissue Regeneration." Chemical Reviews, 2022, https://doi.org/10.1021/acs.chemrev.2c00179.

16 Naahidi, Sheva, et al. "Biocompatibility of Hydrogel-Based Scaffolds for Tissue Engineering Applications." Biotechnology Advances, vol. 35, no. 5, 2017, pp. 530–544., https://doi.org/10.1016/j.biotechadv.2017.05.006.

17 ibid

18 Ibid

CONCLUSION

In this study, we demonstrated the effect of hUCMSCs Exo hydrogel on rat cutaneous wound sites. The result is not promising. But as individual treatments, hUCMSC exos and hydrogels both present their potential as candidates of wound healing promotion. Despite the result shown in the study, which is most likely caused by systemic errors, exo hydrogel possess valuable clinical uses in general. However, a more detailed and intricate system is necessary to prepare a more biocompatible and readily wound dressing.

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