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EXPLORATION OF APPLICATION OF GUILIN WATERMELON FROST IN PULP REVASCULARIZATION OF IMMATURE PERMANENT TEETH WITH PERIAPICAL PERIODONTITIS

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Abstract: Purpose: To explore the possibility of applying Guilin watermelon frost in pulp revascularization of young permanent teeth with periapical periodontitis. Material and methods: Divided the stem cells from human exfoliated deciduous teeth (SHED) into A. B. C and D four groups. A without any treatment, B. C. D groups were treated with calcium hydroxide, triantibiotic intracanal dressing and Guilin watermelon frost separately for one hour after treatment of conventional irrigation of 1% sodium hypochlorite and normal saline. Results: Compared to group A, cell growth was found to be slower down in group B. C. and D. No statistic differences were found. While after special treatment, cell survival rate decreased significantly, group D decreased more than group C, and group B survived more than group C. Conclusion: Guilin watermelon frost is not fit for pulp revascularization of immature permanent teeth with periapical periodontitis temporarily.
Keywords: Watermelon Frost; Stem cells from human exfoliated deciduous teeth (SHED); Periapical diseases in immature permanent teeth

1. INTRODUCTION

The traditional therapy of periapical periodontitis in immature permanent teeth is apexification. However, it usually fails to thicken the root canal wall and lengthen the root after treatment, which leads to high probability of teeth fracture in the long term or the failure to correct the wrong crown-root ratio (CR). The results of current basic and clinical studies indicate that pulp revascularization in infected root canal system is possible. Pulp revascularization (PR) for treatment of periapical diseases in immature permanent teeth has a good clinical application prospect and can replace traditional apexification in appropriate cases[1] with the in-depth understanding of pathogens in the root canal necrosis infected by pulpitis and periapical periodontitis, with regard to the selection of root canal disinfection drugs during the Pulp revascularization (PR), some scholars believe that the combination of metronidazole, minocycline and ciprofloxacin can effectively kill the common pathogens in the root canal. Full sterilization of the infected root canal and offering the substrate for the development of new tissue as well as effective coronal closure can provide the necessary biological environment for successful angiogenesis. There have also been studies with conflicting opinions on the biocompatibility of the paste as a root canal disinfectant. Da Silva LA et al. believed that the triple

antibiotic paste had cytotoxicity to tissues[2]. In addition, the possibility of bacterial resistance in the triple antibiotic paste was one of the disadvantages of this efficient antibiotic agent. Minocycline in the triple antibacterial paste could also lead to staining in teeth. Guilin Watermelon Frost spray is a compound preparation developed from 14 traditional Chinese medicines, such as watermelon cream, borax (calcination), scutellaria, rhizoma coptidis, cortex phellodendron, vietnamese sophora root, bulbus fritillariae thunbergii, rhizoma belamcandae, natural indigo, borneol, and soapnut (charcoal)[3-5], in which watermelon frost and vietnamese sophora root as sovereign drug with the effect of clearing heat-toxin. Scutellaria, rhizoma coptidis, and cortex phellodendron as minister drug with the effect of clearing heat and dampness, purging internal heat and detoxifying. Rhubarb with the effect of relieving heat accumulation, dredge the internal organs and relieving the heat; Rhizoma belamcandae with the effect of clearing away heat and relieving sore throat, Bulbus fritillariae thunbergii with the effect of nourishing yin and dispelling heat, reducing phlegm and relieving sore throat; Menthol with the effect of dispersing wind heat; Borax, realgar, natural indigo and soapnut as assistant drug with the effect of removing toxin for detumescence; Borneol as envoy drug with the effect of arousing consciousness and relieving pains. The synergy of whole prescription has the effect of clearing away heat and toxic materials, relieving swelling and relieving pain. Clinical application include swelling and pain in throat, sore tongue and aptha, swelling and aching of gum or bleeding, moth's sore mouth, goose's sore mouth in children, slight scald and traumatic hemorrhage; Acute and chronic laryngitis, tonsillitis, stomatitis, oral ulcer and other diseases. The purpose of this study is to explore the possibility of applying this drug in Pulp revascularization (PR) by observing the effect of watermelon frost on stem cells from human exfoliated deciduous teeth (SHED), so as to enrich the existing antiseptic drugs for revascularization and expand the clinical application of traditional Chinese medicine in China.

2. METHODS

Main experimental reagents and materials

α-MEM medium (Gibco, USA);Trypsin (Invitrogen, USA);FBS (Sijiqing, Hangzhou, China); Collagenase, Dispase enzyme (Gibco, USA);Anti-CD34-PE, CD45-PE (Biolegend, USA); Anti-CD146-PE (Abcam USA);Anti-CD34, DSP, NESTIN (Abcam, USA); Phosphate buffer (PBS) (Gibco, USA);Paraformaldehyde (Cellchip bio, China);Dexamethasone (Sigma, USA);β-Sodium glycerophosphate (Sigma, USA); Dimethyl sulfoxide (DMSO) (Xi 'an chemical reagent factory, China); Indomethacin (Sigma, USA); Toluidine blue (Beyotime Bio, China); MTT (Sigma, USA) Trizol Reagent (Invitrogen, USA);DEPC water (Beyotime Bio, China); RT-PCR kit (TOYOBO, Japan); RT-PCR primer (Shanghai bio, China); SYBRPremixExaq TMII (TaKaRa, Japan).

Isolated culture of SHED

The clinical patient's deciduous teeth close to the replacement were collected and placed in a culture medium centrifuge tube. In the ultra-clean table, the tooth was taken out and placed in a petri dish. After repeatedly rinsed with PBS, the tooth was moved to another petri dish to remove the pulp tissue. Pulp tissue was rinsed repeatedly with PBS until the surface blood was rinsed clean. In petri dishes, pulp tissue was cut up and placed in digestive juices contains type I collagenase and Dispase in dark conditions, then placed at 37 ℃ hatch for digestion of 40 min - 1 h, rocked gently during every 5 to 10 min, until no visible dental pulp tissue could be observed.

Subculture of SHED

When the cells in the 6-well plate grew to 80 - 85 %, the culture medium was discarded and washed with PBS for 2 - 3 times. After digestion with 2.5 % trypsin at 37 ℃ for 40s, the cells were observed under an inverted microscope until they were reduced to a round shape. Then an equal amount of serum-containing medium was added to stop digestion and the cells were blown to obtain a single cell suspension. After centrifugation at 800 r/min for 5 min, the supernatant was discarded and culture medium was added to re-suspend the cells, and the cells were inoculated and passed at a

density of 7×10^5 / cm^2.

SHED phenotype identification by flow cytometry

The P1 SHED in the logarithmic growth phase was selected, routinely digested and centrifuged. The supernatant was discarded, and the cells were re-suspended with PBS. The cells were divided into 1.5 mL EP tubes with 200 μL, and the number of cells per tube was no less than 1×10^6. 2 μL anti-human monoclonal fluorescent labeled antibodies CD34-PE, CD45-FITC, CD146-PE were added to each centrifuge tube except for the blank tubes. The cells were incubated in darkness at 4 ℃ for 2 h, then washed with PBS for 3 times, centrifuged at 800 r/min for 5min, and then re-suspended with 300 L PBS. The positive expression rate of cell surface antigen was measured by flow cytometry, expressed in percentage (%).

Cell immunofluorescence identification

The P2 SHED during the logarithmic growth phase was inoculated into a 24-well plate and cultured in conventional culture medium. After 8 h of culture, the cells were observed under the microscope. When the cells were completely attached to the wall, the culture medium was discarded, washed with PBS for 2 - 3 times, and fixed with a fixed solution for 30 min. Washed with PBS for 2 - 3 times, and treated with 1 % Triton for 25 - 30 min. Washed with PBS for 2 - 3 times. The sections were treated with goat's serum for 30 min at 37 ℃. Goat's serum was directly discarded without washing, and the first antibody was added and treated at 4 ℃ for 24 h. Reheated for 30min and washed with PBS for 2 - 3 times the second day. Fluorescent secondary antibodies were added under the dark condition and the section was treated in incubators at 37 ℃ for 3 h. Washed with PBS for 2-3 times, added Hochst for 15 min, washed with PBS for 2 - 3 times and dried it for observation with laser confocal microscope.

Detection of cell proliferation capacity

The SHED during logarithmic growth were inoculated into 96-well plates with the density of 1000 cells per well. After 24 h, the cells of the experimental group were treated with 1 % sodium hypochlorite and physiological saline, and detected on day 1, 2, 3, 4, 5 and 6, respectively. 20 μL MTT was added to each well. The plate was incubated at 37 ℃ for 4 h, then culture was terminated, and culture medium was discarded. 150 L DMSO/each hole was added and slightly shaken for 10min to fully dissolve. The absorbance of each hole was measured by enzyme-linked immunometric meter at the wavelength of 490 nm, and the mean value was calculated.

Cytotoxicity test

SHED during logarithmic growth were treated with sodium hypochlorite and physiological saline, and the culture medium containing calcium hydroxide, triple paste and watermelon frost was placed in the cell petri dish and cultured for 24 h, respectively. The SHED was prepared with physiological saline into a cell suspension of appropriate concentration for later use. 0.4 % trypan blue dye was absorbed with a dropper and added to the cell suspension at a ratio of 1:1. After wiping the counting plate with anhydrous ethanol or 95 % ethanol solution, wipe it off with a silk cloth, wipe off the cover slip and put the cover slip over the counting plate. Slowly drip from the edge of the counting plate to fill the space between the counting plate and the cover slip. After a while, place the counting plate under a low power lens(10 × 10) to observe the count. When counting, only the complete cells were counted. The cells in a group are counted as a cell. In a large square, if there are cells on the line, the down-line cells are counted while up-line cells not, and the left-line cells are counted while right-line cells not. The error of the secondary repeat count shall not exceed ± 5 %. Under the microscope observation, where refraction is strong and not coloring for living cells, with blue for dead cells.

Statistical analytical procedure

The results of this experiment were analyzed by SPSS 16.0 statistical software, and the t-test was used for comparison between groups. The level of inspection showed $\alpha = 0.05$ and $P < 0.05$, suggesting

significant difference.

3. RESULTS

Isolated culture and identification of SHED

The isolated SHED was similar in morphology to those of permanent dental pulp stem cells (DPSC), with slightly smaller cells, mostly spindle-shaped or long-spindle shaped (Fig. 1). According to previous literatures, CD146 was selected as a positive marker, and its expression rate was 35.89 %; CD34 as a negative marker with a positive expression rate of 1.07 %. The results indicated that the cells were derived from mesenchymal stem cells rather than hematopoietic stem cells. Cell immunofluorescence showed that NESTIN was positively expressed, and it could be seen that positive red fluorescence signals were expressed around the cell nucleus. Meanwhile, CD34 was expressed negatively (Fig. 2).

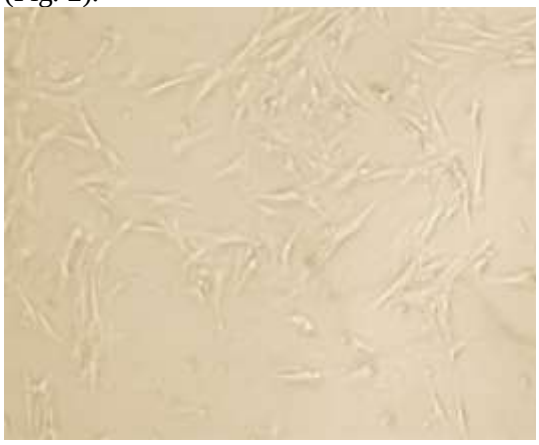

Fig. 1 The third generation of SHED (× 200).

Most of SHED were spindle-shaped and fibrocyte morphology, a few of which are polygonal-shaped or ovoid-shaped.

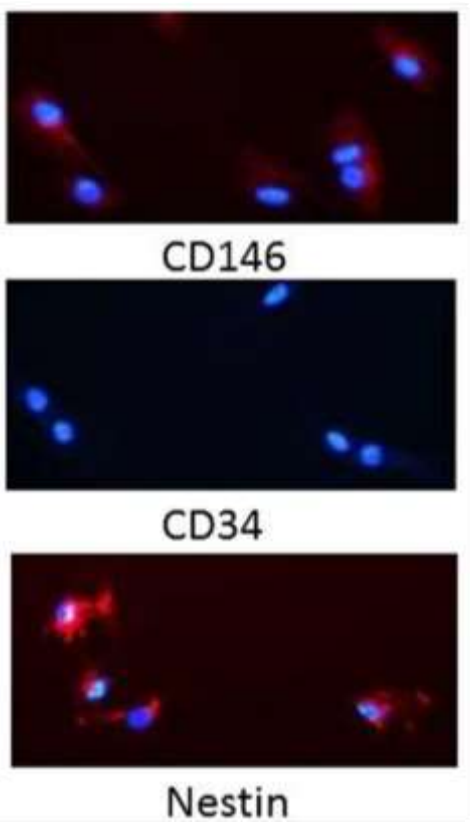

Fig. 2 SHED cell identification

According to the literature, CD146 was selected as the positive marker and CD34 as the negative marker, indicating that the positive expression of red fluorescence signal was observed around the nucleus.

Comparison of cell proliferation capacity

MTT showed that OD value of SHED was higher than that in permanent teeth from day 3, with no statistic difference between the two groups(Fig. 3) ($P < 0.05$).

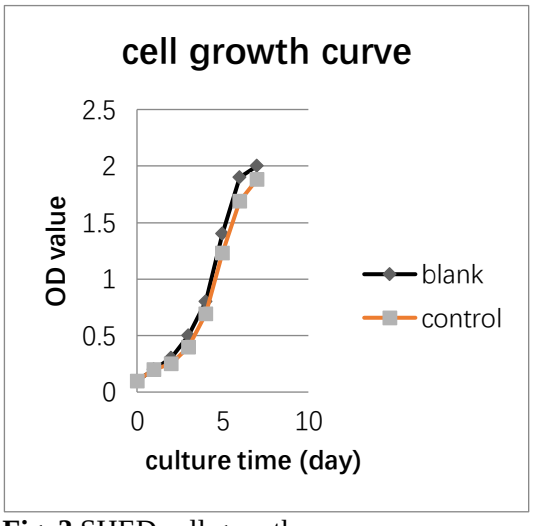

Fig. 3 SHED cell growth curve

The growth curve drawn with time as the abscissa and absorbance as the ordinate showed that the number of SHED increased with the extending of incubation time, which showed rapid growth on the 3rd day and entered the growth platform on the 6th day.

Comparison of Cytotoxicity

After sterilization for 24 h, observe survival situation of SHED, SHED (-) as the group only treated with sodium hypochlorite and physiological saline, namely the group A, while the calcium hydroxide treated as group B, triple antibiotic paste treated as group C and Guilin Watermelon Frost treated as group D. After group B to group D was treated with disinfectant, their cell number was lower than that of group A. The result was statistically different, and the conditions of cell proliferation of group B is better than that of group C, while group C is better than that of group D. There were statistical differences between the groups (Fig. 4)

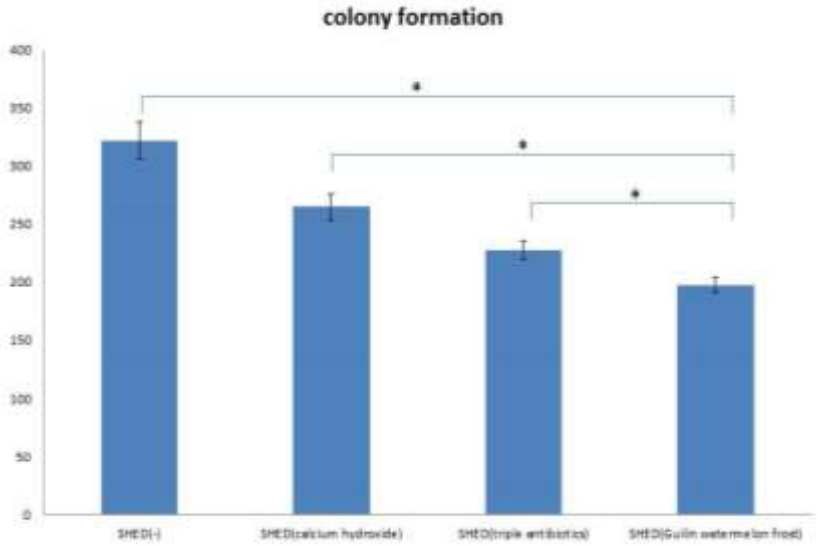

Fig. 4 Cytotoxicity of disinfectants

4. DISCUSSION

Odontogenic stem cells are undifferentiated cells with stem cell characteristics existing in tooth related tissues, which can be divided into embryonic odontogenic stem cells and adult odontogenic stem cells according to the developmental stage. The stem cells in the pulp of deciduous teeth have the properties of early embryonic mesenchymal stem cells. Studies have shown that they can not only express the surface specific markers of STRO-1 and CD146 of mesenchymal stem cells, but also express the molecular markers Oct4 and Nanog of embryonic stem cells. Although SHED is found in exfoliated deciduous teeth, its essence is stem cells from human deciduous teeth, which have the biological behavior of neural crest-derived cells. Many studies have confirmed that SHED has a high capacity for proliferation and extensive multidirectional differentiation, and some animal model experiments have also confirmed that SHED can repair spinal cord nerve injury, Parkinson's disease and stroke [6-8]. Although SHED cells are considered as adult odontogenic stem cells, they are essentially embryonic odontogenic stem cells. In addition, as the only deciduous organ that can be replaced in the process of human growth and development, SHED cells, like cord blood stem cells, are easy to obtain and minimally invasive, with a wider range of sources, while fully meeting the ethical requirements. Therefore, it is becoming the most concerned seed cell for stem cell therapy and tissue engineering.

As a new treatment option for immature

permanent teeth with infected or necrotic root apical holes, pulp revascularization (PR)is based on the traditional apexification. By controlling periapical tissue infection, it uses K/ H files to stimulate the remaining dental pulp and periapical tissues causing the bleeding, forming blood clots in the root canal and closing pulp cavity with mineral trioxide aggregate (MTA), which will induce the undifferentiated mesenchymal cells in the blood-borne and root zone to proliferate and differentiate, making pulp revascularization and root development. In endodontic revascularization, blood enters the root canal by puncturing the periapical tissue. In addition to clotting, platelets in the blood also secrete a variety of growth factors, including platelet-derived growth factor (PDGF), TGF-β factor, insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF). These growth factors can bind to relevant receptors on the surface of the undifferentiated mesenchymal cell membrane, initiate endogenous signaling pathway [1-9], promote cell proliferation and matrix secretion, and induce differentiation [10].

In addition, after the endodontic dentin tissues were disinfected and treated, fresh presenting could be exposed, which contained a variety of dentin matrix proteins (such as collagen, DMP-1, DSP, DPP, BSP, OPN, OCN, etc.) and cytokines (including TGF- β1, BMPs, IGF, PDGF, EGF, VEGF, FGFs, MMP, etc.). Many previous in vitro studies have confirmed that dentin tablets not only have pore size and porosity that match dentin cells, but also can provide a variety of important factors necessary for tooth development and regeneration, which can induce the adhesion, proliferation and differentiation of seed cells (such as SCAP, SHED and DPSC) to form the dentin-pulp complex. So, the root canal cavity has unique micro-environmental conditions for induction[11].

To sum up, the research purpose of this paper is to explore the possibility of applying traditional Chinese medicine in SHED cells, and sorting dental pulp regeneration disinfectant. During revascularization, the remaining living pulp and apical papilla should be preserved as far as possible. No or minimal mechanical preparation of the root canal is performed, because even in the case of infection, viable cells that may remain in the root canal will play a role in the recovery of pulp vitality. Root canal disinfection reagents and chemical flushing were used as the principal methods to remove the infected substances in root canal. Commonly used rinse solutions include 1.5 – 3 % sodium hypochlorite solution and 17 % EDTA solution with the purpose of reducing root canal infection and promote the release of cytokines in dentin tubule[12].After chemical preparation, the root canal was sealed with triple antibiotic paste consisting of ciprofloxacin, metronidazole and minocycline (or cefaclor, clindamycin, etc.) or calcium hydroxide paste. The crown was temporarily sealed with glass ionomer, and the patient was arranged a return visit after 3 - 4 weeks. If the symptoms are not completely relieved or the root tip exudates inflammatory during the return visit, repeat this procedure until the infection is successfully controlled and the root canal is kept sterile. The triple antibiotic paste has stronger antibacterial power than calcium hydroxide, but has stronger stem cell toxicity as well[13].Therefore, the stronger the antibacterial force of root canal antibiotics, the lower the risk of inflammation recurrence, but the greater the impact of antibiotics on root tip stem cells. A balance needs to be struck between antimicrobial strength and toxicity. In the present experiment, the survival rate of the cells treated with watermelon frost solution was lower than that treated with calcium hydroxide and triple antibiotics, and the reasons need to be further analyzed. Under the existing research conditions, the effect of single recipe contained in watermelon frost could be tested for further observation.

5. COMPETING INTERESTS

This work was mainly performed in Department of pediatric dentistry, Stomatological Hospital, Southern Medical University. The authors declare that they have no conflicts of interest.

6. ACKNOWLEDGMENTS

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