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Human Umbilical Cord Mesenchymal Stem Cells Alleviate Nasal Mucosa Radiation Damage in a Guinea Pig Model

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ABSTRACT

Nasal complications after radiotherapy severely affect the quality of life of nasopharyngeal carcinoma patients, and there is a compelling need to find novel therapies for nasal epithelial cell radiation damage. Therefore, we investigated the therapeutic effect of human umbilical cord mesenchymal stem cells (hUC-MSCs) in guinea pig model of nasal mucosa radiation damage and explored its therapeutic mechanism. Cultured hUC-MSCs were injected intravenously immediately after radiation in the nasal mucosa-radiation-damage guinea pig model. Migration of hUC-MSCs into the nasal mucosa and the potential for differentiation into nasal epithelial cells were evaluated by immunofluorescence. The therapeutic effects of hUC-MSCs were evaluated by mucus clearance time (MCT), degree of nasal mucosa edema, and the nasal mucosa cilia form and coverage ratio. Results indicate that the hUC-MSCs migrated to the nasal mucosa lamina propria and did not differentiate into nasal epithelial cells in this model. The MCT and degree of mucosal edema were improved at 1 week and 1 month after radiation, respectively, but no difference was found at 3 months and 6 months after radiation. The nasal mucosa cilia form and coverage ratio was not improved 6 months after radiation. Thus, hUC-MSCs can migrate to the nasal mucosa lamina propria and improve MCT and mucosa edema within a short time period, but these cells are unable to differentiate into nasal epithelial cells and improve nasal epithelial regeneration in the nasal mucosa radiation damage guinea pig model. J. Cell. Biochem. 116: 331–338, 2015.

KEY WORDS: MESENCHYMAL STEM CELLS; RADIATION DAMAGE; STEM CELL TRANSPLANTATION; NASOPHARYNGEAL CARCINOMA

Nasal complications after radiotherapy severely affect the quality of life of nasopharyngeal carcinoma patients; yet, no effective treatment methods have been identified. Current treatments, such as nasal irrigation and functional endoscopic sinus surgery, only partly alleviate symptoms and do not affect the structure and function of nasal epithelial cells [Hu et al., 2008; Liang et al., 2008]. With the development of diagnosis and treatment methods in recent years, the survival rates and survival period of nasopharyngeal carcinoma patients have improved. As a result, nasal complications after radiotherapy are becoming more prominent, and there is a compelling need to find novel therapies for nasal epithelial cell radiation damage.

Previous studies have shown that bone marrow mesenchymal stem cells (BMMSCs) transplantation achieved good results for the intestinal, parotid gland, and other mucosal tissues radiation damage models. For example, Kudo et al. transplanted BMMSCs from male nude mice into the intestinal walls of irradiated female nude mice. The survival rate, mean body weight, and histology of the BMMSCs-treated group were significantly improved compared with the control group [Kudo et al., 2010]. Lin et al. [2011] found that cell therapy with BMMSCs aids in the functional regeneration of salivary glands after irradiation damage. In addition, Phulpin et al. [2011] injected autologous BMMSCs into irradiated tibias in rats, and bone blood flow and bone metabolism were significantly

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increased during the first week after cell transplantation. Although BMMSCs have been studied extensively, use of these cells for treatment of human mucosa radiation damage is limited by their low numbers and their invasiveness acquisition.

Umbilical cord mesenchymal stem cells (hUC-MSCs) share the phenotypic characteristics of BMMSCs [Fong et al., 2007]. Similar to BMMSCs, hUC-MSCs have a multilineage differentiation capacity and also exhibit similar in vitro regeneration properties [Wu et al., 2007; Fan et al., 2011]. In addition, hUC-MSCs induced in vitro can differentiate into cells that display lineage-specific protein. Furthermore, these cells exhibited therapeutic efficacy in animal models of cerebral ischemia [Koh et al., 2008], spinal cord injury [Yang et al., 2008], Parkinson's disease [Weiss et al., 2006], type I diabetes [Chao et al., 2008], and cirrhosis [Tsai et al., 2009]. Safety of hUC-MSCs has also been verified, as large doses of hUC-MSCs inoculated into nude mice demonstrated no tumorigenicity [Gauthaman et al., 2012] and no immunogenicity [Wang et al., 2012]. Meanwhile, because hUC-MSCs are isolated from umbilical cords, which are typically discarded after birth, these cells have the advantage of availability, noninvasive acquisition, and no ethical limits.

We have isolated and cultured hUC-MSCs and analyzed the surface markers. We have further investigated their ability to differentiate into adipocytes, osteoblasts, and chondrocytes, thus meeting the definitive criteria for MSCs [Dominici and Krause, 2006]. We hypothesized that the transplanted hUC-MSCs will migrate to the nasal mucosa in the guinea pig model of radiation damage via chemotaxis in order to repair the nasal mucosa damage. In order to test this hypothesis, we intravenously injected hUC-MSCs into this guinea pig model to evaluate the therapeutic effects on mucus clearance time (MCT), nasal mucosa edema degree, and nasal mucosa cilia form and coverage ratio. Migration of hUC-MSCs to the nasal mucosa and the ability to differentiate into nasal epithelial cells were evaluated by immunofluorescence. The results of this research provide new insights for addressing radiation-induced mucosa injury and other radiation complications.

MATERIALS AND METHODS

ANIMALS

Guinea pigs (male, 300–450 g body weight) were purchased from Shanghai Laboratory Animal Center (Shanghai, China; http://slac.bioon.com.cn/) and bred in a specific pathogen-free animal facility. The animal study protocol was approved by the ethics committee of the Affiliated Eye and Ear, Nose and Throat Hospital of Fudan University. All the animals were handled in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

NASAL MUCOSA RADIATION DAMAGE GUINEA PIG MODEL

Guinea pigs were anesthetized and irradiated in a prone position using X-rays (5 Gy) once per week for three weeks. The size of the exposure field was 10×10 cm. The frontal boundary was the anterior naris, and the posterior boundary was the connection line of the bilateral front edge of the auricle. Guinea pigs with radiation-induced pathological changes in the nasal mucosa were identified for further study.

ISOLATION AND CULTURE OF hUC-MSCs

Ethical approval was obtained from the Fudan University, and signed informed consent forms were obtained from all donors. The umbilical cords from consenting full-term caesarian section patients were provided immediately upon delivery in a previously supplied vessel containing Hanks balanced salt solution, penicillin (100 U/ml), and streptomycin (100 μg/ml). To isolate stem cells, umbilical cord samples were rinsed in 75% ethanol for 30 s and then cut open in parallel to umbilical cord vessels in order to expose the vessels fully. The gelatinous tissue surrounding the vessels was excised and minced into very fine pieces of 0.5–1 mm^2 and then placed in a sterile 24-well cell culture plate (Corning, Ewloe, UK). Samples were incubated for 30 min at room temperature to facilitate tissue attachment. The minced tissue was carefully covered with 5 ml growth medium, which consisted of low-glucose Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) solution. The umbilical cord samples were incubated at 37°C in a humidified CO_2 incubator for 7–10 days before the monolayer of adherent cells reached confluence. Cells were transferred to 25 cm^2 flasks (Corning) at a density of 5×10^3 cells/cm^2 and cultured until reaching 70–80% confluence before data collection and cryopreservation. The third passage of hUC-MSCs was used for experiments.

IMMUNOPHENOTYPIC ANALYSIS

Flow cytometric analysis was used to characterize the phenotypes of the hUC-MSCs. At least 1×10^6 cells (in 100 μl phosphate buffered saline (PBS) with 0.5% bovine serum albumin) were incubated with fluorescein-labeled monoclonal antibodies against mouse CD45, CD90, CD105, and CD146 or with their respective isotype control antibodies (R&D Systems, Minneapolis, MN). After washing, the labeled cells were analyzed by flow cytometry with fluorescence-activated cell sorting (FACS) using a Calibur flow cytometer and Cell Quest Pro software (BD Biosciences, San Diego, CA).

MULTILINEAGE DIFFERENTIATION OF hUC-MSCs

Adipogenic differentiation was induced by culturing hUC-MSCs for 20 days in adipogenic medium. The protocol includes culturing in Adipogenic medium A (1 mg/ml glutamine, 2 μg/ml insulin, 1 μg/ml isobutylmethylxanthine, 1 μg/ml rosiglitazone, 1 μg/ml dexamethasone, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS in DMEM) for 3 days and then in Adipogenic medium B (1 mg/ml glutamine, 2 μg/ml insulin, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS in DMEM) for 1 day, and then the cycle was repeated five times. After induction, the cells were assessed using an Oil Red O stain as an indicator of intracellular lipid accumulation.

Osteogenic differentiation was induced by culturing hUC-MSCs for 21 days in osteogenic medium (1 mg/ml glutamine, 0.1 μg/ml dexamethasone, 1 mg/ml β-glycerophosphate, 2 μg/ml ascorbic acid, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FBS

in DMEM). After induction, the cells were examined for extracellular matrix calcification using Alizarin Red S staining.

Chondrogenic differentiation was induced by culturing hUC-MSC pellets for 21 days in chondrogenic medium (0.1 μg/ml dexamethasone, 3 μg/ml ascorbic acid, 1 mg/ml cell culture additives contain insulin, transferrin, and selenium (ITS) + supplement, 1 μg/ml sodium pyruvate, 1 μg/ml proline, and 1 mg/ml TGF-β3). Pellets were formalin fixed and embedded in Optimal Cutting Temperature (OCT) compound for Alcian Blue staining. All differentiation mediums and staining solutions were purchased from Cyagen Biosciences, Inc. (Guangzhou, China).

HUC-MSCs INTRAVENOUS INJECTION

Guinea pigs (n = 20 in each group) were randomly assigned to each of the three groups: Group A: Normal control group. Group B: Radiation control group. Group C: MSCs injection group (Fig. 1).

hUC-MSCs were transfected with a recombinant replication-defective adenovirus carrying green fluorescent protein (1×10^{11} colony-forming unit/ml; SinoGenoMax, Beijing, China) for 24 h (Multiplicity of Infection [MOI] = 100). The cells were washed with PBS and amplified with culture medium containing 10% FBS. Then hUC-MSCs were harvested with trypsin, washed twice in PBS, and resuspended immediately before transplantation. Using an insulin syringe, approximately 1×10^6 hUC-MSCs labeled with GFP were injected into each animal into the superficial dorsal veins of the penis immediately after radiation. Each guinea pig was injected once per week for 3 weeks. The guinea pigs in the control group were injected with an equivalent volume of PBS.

ANALYSIS OF hUC-MSCs MIGRATION AND DIFFERENTIATION

Guinea pigs injected with GFP-labeled hUC-MSCs were sacrificed at 1 week, 1 month, 3 months, and 6 months after the final radiation. Nasal mucosa were frozen in liquid nitrogen and then cut into 10 μm sections. The two adjacent sections were used to detect GFP-positive cells via fluorescence confocal microscopy (Leica, Wetzlar, Germany) or were stained with an antibody against GFP (Santa Cruz Biotechnology, Santa Cruz, CA) and visualized using a fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Santa Cruz Biotechnology).

Immunohistochemical staining was used to identify the origin of the GFP-positive cells within the nasal mucosa. Antibodies to β-tubulin (1:200; Abcam, Cambridge, MA) were used to detect nasal epithelial cells. Corresponding FITC-conjugated secondary antibodies (1:200; Abcam) were used. Nuclei were labeled using DAPI (Sigma–Aldrich, St. Louis, MO). Images were obtained by confocal microscopy.

MEASUREMENT OF MUCUS CLEARANCE TIME

Nasal mucociliary clearance was evaluated using the charcoal-powder method. The guinea pigs were anesthetized, and charcoal powder mixed with PBS was dripped into the nose of guinea pigs. Next, a 2.7 mm endoscope (Carl Storz, Tuttlingen, Germany) was inserted to observe the laryngeal pharynx. The MCT (from the time of nose drip to observation of the charcoal powder in the laryngeal pharynx) was recorded (Figs. 2A and B).

HISTOLOGIC ANALYSIS OF NASAL MUCOSA EDEMA

The whole maxillary bone including the nasal septum and turbinate was removed, fixed with 10% neutral-buffered formalin for 7 days, and decalcified with 10% trichloroacetic acid for 14 days at room temperature. Samples were embedded in resin (Heraeus Kulzer, Wehrheim, Germany). Sections were stained with hematoxylin and eosin and assessed using light microscopy with a high-powered magnification. Mucosal edema degree was measured by two blinded observers.

HISTOLOGIC ANALYSIS OF NASAL MUCOSA CILIA

Nasal mucosa samples were removed and preserved in formaldehyde solution. In order to remove formaldehyde residue and to dehydrate the samples for scanning electron microscopy (SEM) analysis, the samples were washed in mixtures of distilled water and ethanol and

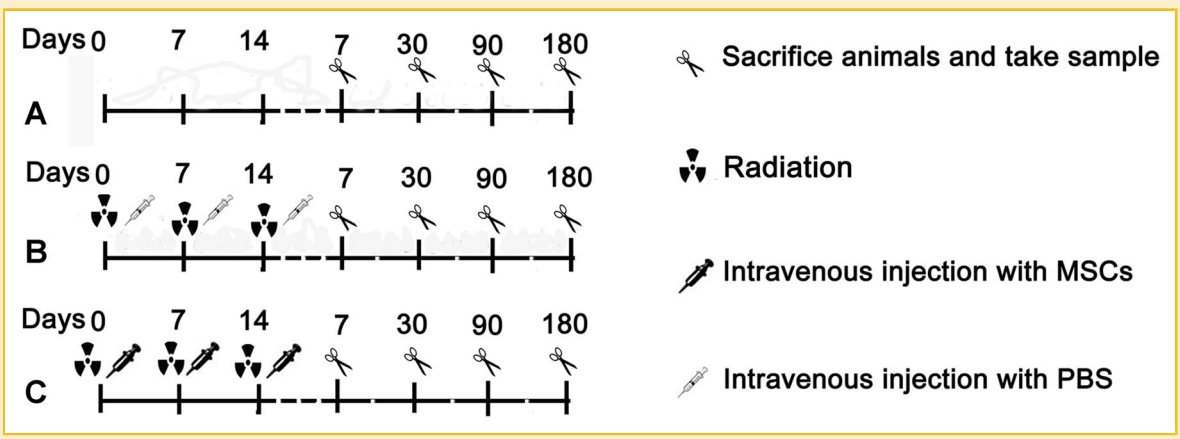

Fig. 1. Experimental protocol. Group A: Normal control group, guinea pigs without radiation or hUC-MSCs injection. Group B: Radiation control group, guinea pigs were irradiated once a week for 3 weeks and injected with PBS daily. Group C: MSCs injection group, guinea pigs were irradiated once a week for 3 weeks and injected with hUC-MSCs daily. All guinea pigs were examined for MCT, and samples were obtained 1 week, 1 month, 3 months, and 6 months after the last radiation treatment.

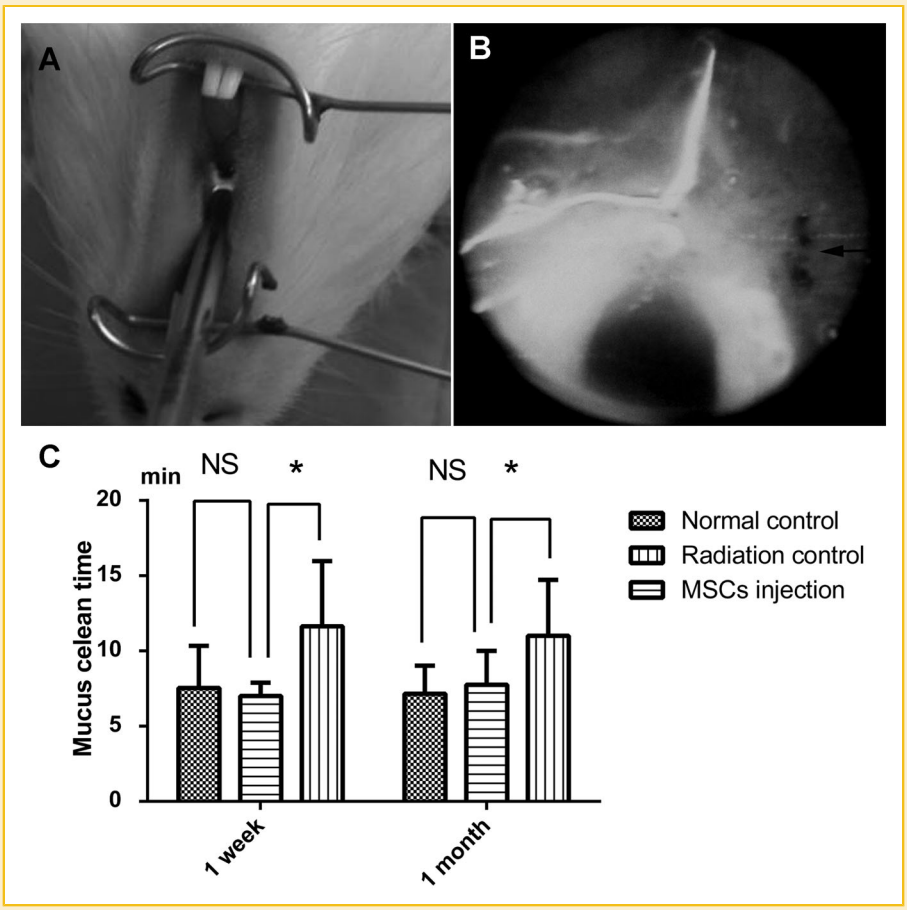

Fig. 2. Mucus clearance time change after hUC-MSCs transplantation. Charcoal powder mixed with water was dripped into the noses of guinea pigs (A). Measurement of the MCT occurred when the charcoal powder was observed in the laryngeal pharynx using an endoscope (B). At 1 week and 1 month after radiotherapy, MCT of the animals in the MSC injection group was significantly shorter than that in the animals in the radiation control group but was not different than that in the animals of the normal control group (*$P > 0.05$; C).

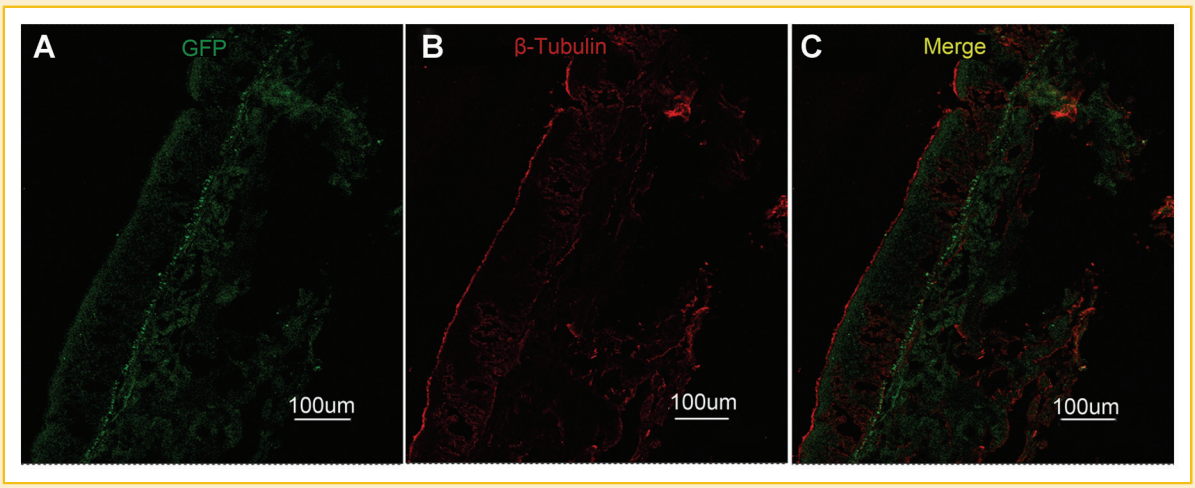

Fig. 3. Immunofluorescence of injected hUC-MSCs from the nasal mucosa in radiated guinea pigs. Immunofluorescence staining revealed GFP-positive cells (Stained green) distributed in the lamina propria of the nasal mucosa (A). The nasal epithelial cells expressed specific protein (β-tubulin, Stained red), which is located in the surface of nasal mucosa (B). The lack of overlap between these two cells indicates that the GFP-positive MSCs did not differentiate into nasal epithelial cells (C).

absolute ethanol and isoamylacetate. Finally, the samples were separated from the isoamylacetate, placed in a Petri dish, covered with porous cellulose paper, and dried for 24 h. The dried samples were sharply cut and coated with gold, and then the specimen was placed on a carbon substrate for inspection with a thermal field emission electron microscope (HITACHI su8010, Tokyo, Japan). The samples were analyzed to two blinded, scanning electron microscope experts. The morphological features and the coverage of the epithelial cilia were recorded.

STATISTICAL ANALYSIS

Data are presented as mean ± SEM. Statistical significance was assessed by an unpaired *t*-test using the SPSS software package version 18.0 (SPSS Inc., Chicago, IL). A *P*-value < 0.05 was considered significant.

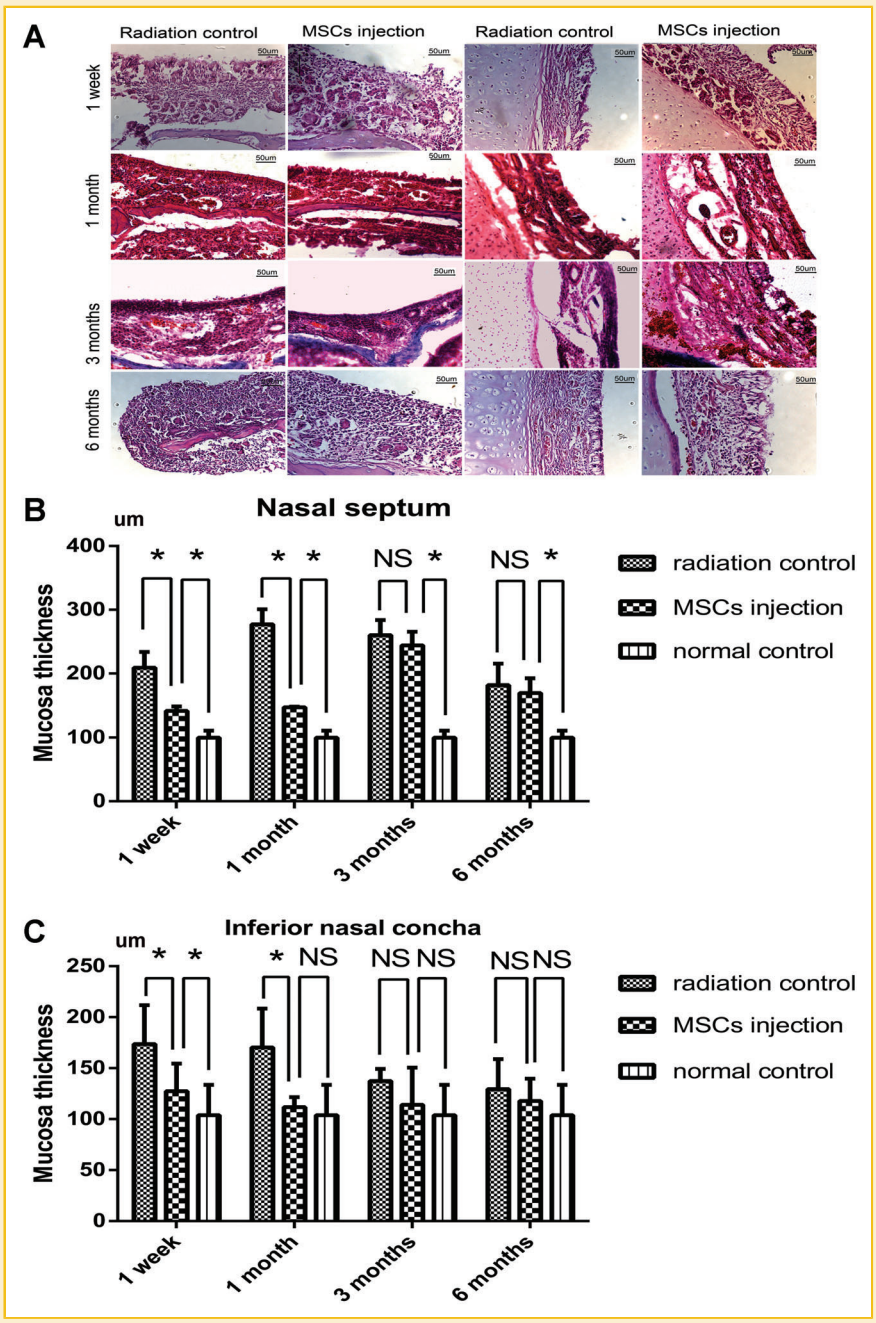

Fig. 4. Histology change after hUC-MSCs transplantation. (A) Histology of inferior turbinate and septum nasal mucosa. The thickness of the nasal mucosa was changed according to different intervention methods and times. Sections were stained with hematoxylin and eosin (magnification 200×). Results are representative of five independent experiments. (B) One week and one month after radiation, the inferior turbinate mucosa thickness in the animals of the MSC injection group was less than that in the radiation control group (*$P < 0.05$). But three and six months after radiation, there was no significant difference (NS, non-significant difference, $P > 0.05$). (C) One week and one month after radiation, nasal septum thickness was less in the MSC injection group than in the radiation control group(*$P < 0.05$). But three and six months after radiation, there was no significant difference (NS, non-significant difference, $P > 0.05$).

RESULTS

IMMUNOPHENOTYPE OF hUC-MSCs

hUC-MSC cultures were analyzed for expression of cell-surface markers. hUC-MSC cultures were negative for CD45, but expressed the surface proteins CD90, CD105, and CD146. The percentage of $CD90^+$, $CD105^+$, and $CD146^+$ cells in hUC-MSCs was 96.7%, 34.9%, and 92.4%, respectively.

DIFFERENTIATION CHARACTERIZATION OF hUC-MSCs

To examine the multilineage capacity of hUC-MSCs, cells were differentiated toward the adipogenic, osteogenic, and chondrogenic lineages using lineage-specific induction factors in culture for 3 weeks. A significant fraction of the cells cultured in adipogenic media contained multiple, intracellular lipid-filled droplets that stained with Oil Red O. Consistent with osteogenesis, several regions stained with Alizarin Red S staining, indicative of calcified extracellular matrix, were observed. Consistent with chondrogenic differentiation, regions that stained with Alcian Blue, which indicates mucopolysaccharide expressed by the cells, were observed.

DETECTION OF hUC-MSCs IN NASAL MUCOSA

To determine whether injected hUC-MSCs migrated into the nasal mucosa in irradiated guinea pigs, five nasal mucosa sections from each animal were examined with fluorescence microscopy. Purified stem cells (2×10^6) were injected intravenously following the last radiation of the guinea pigs. Fluorescence microscopic examination showed that green GFP-positive hUC-MSCs were present in the subepithelial layer of the nasal mucosa; however, these cells were not detected in the unirradiated guinea pigs. Furthermore, immunofluorescence staining examination revealed that the GFP-positive cells in the lamina propria of the nasal mucosa did not express the nasal epithelial cells-specific protein β-tubulin, indicating that these cells did not differentiate into nasal epithelial cells (Fig. 3).

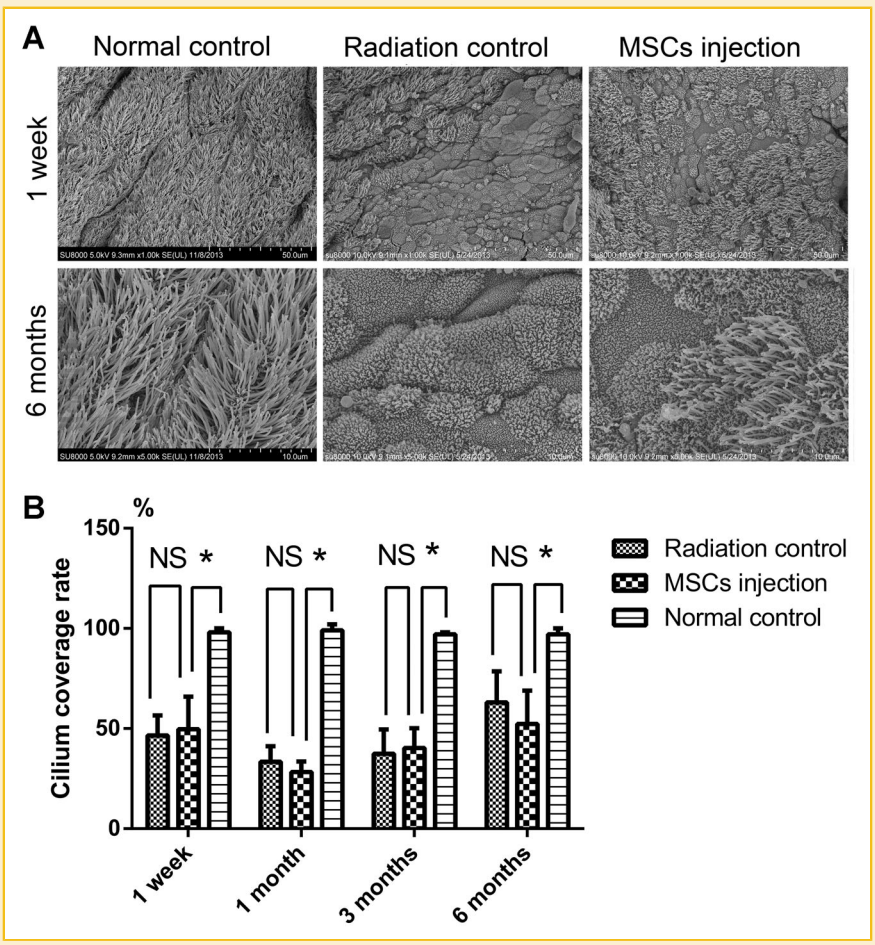

Fig. 5. Cilium coverage rate change after hUC-MSCs transplantation. (A) One week after radiation, the cilia of normal control group flourished, but the cilia of irradiated group were curly and deciduous. After 6 months, the cilia of the radiation control group and the MSC-injection group exhibited limited improvement (magnification 5000×). (B) After 1 week, 1 month, 3 months, and 6 months, the cilia coverage rates of the radiation control group and the MSC-injection group were low, and no significant differences were observed (NS, non-significant difference). At all time points, the MSC-injection group and the unirradiated control group exhibited significantly different cilia coverage rates (*$P < 0.05$).

MEASUREMENT OF MUCUS CLEARANCE TIME

One week after radiotherapy, MCT of guinea pigs in the MSC injection group (7.0 ± 0.89 min) was significantly shorter than that in the animals of the radiation control group (11.64 ± 4.34 min) but was not different than that in the animals of the normal control group (7.54 ± 2.80 min). One month after radiotherapy, MCT of the guinea pigs in the MSC injection group (7.75 ± 2.25 min) was significantly shorter than that in the animals in the radiation control group (11.0 ± 3.73 min) but was not different than that in animals in the normal control group (7.15 ± 1.87 min; Fig. 2C). Except for the normal control group, MCT of the other groups after 3 months and 6 months was not detected in 30 min, so these values were not collected.

HISTOLOGICAL ANALYSIS OF NASAL MUCOSA EDEMA

To evaluate the histological changes in the nasal mucosa after hUC-MSC injection, the degree of edema of the nasal mucosa was measured. One week and one month after radiation, the inferior turbinate thickness of the guinea pigs in the MSC injection group was significantly less than that of the animals in the radiation control group ($P < 0.05$); however, this thickness was not different in the animals in the normal control group ($P > 0.05$). After 3 months and 6 months after radiation, however, this difference was not statistically significant (Fig. 4).

HISTOLOGICAL ANALYSIS OF NASAL MUCOSA CILIA

To evaluate histological changes in the nasal mucosa after injection of hUC-MSCs, the cilia morphology and coverage rate were measured. Six months after radiation, the morphology of the animals injected with MSCs was improved, but the cilia coverage rate in the MSC-injection group was not different than that in the radiation control group ($P > 0.05$; Fig. 5).

DISCUSSION

Nasal mucus cilia clearance system damage plays a key role in nasal complications after radiotherapy for nasopharyngeal carcinoma. MSCs have been reported to have a regeneration function, as transplanted MSCs can migrate to the damaged tissue and repair injured mucosa by cell replacement or other paracrine mechanisms [Kang et al., 2012; Bongso and Fong, 2013]. Hence, MSCs transplantation may have therapeutic effects for nasal mucosa radiation damage. Although bone marrow has been the predominant source for isolation of multipotent MSCs, the umbilical cord is alternative source offering larger quantities of MSCs [Bongso and Fong, 2013].

In guinea pigs, radiation injury results in prolonged MCT, nasal mucosa edema, thickness increase, decreased cilia coverage rate, and cilia morphology variation. Therefore, guinea pigs in the radiation control group exhibit altered MCT, mucosal thickness. The improved parameters of the MSC-injection group indicate that intravenous injection of hUC-MSCs induces repair of nasal mucosa radiation damage.

Furthermore, we demonstrated that hUC-MSCs administered via intravenous injection migrated into the nasal mucosa in the radiated guinea pig model, but these cells were not detected either in other organs of the radiated guinea pigs or in the nasal mucosa of unirradiated guinea pigs. These results suggest that an inflammatory response to radiation may improve the hUC-MSC recruitment environment at the radiation site.

Generally, stem cells are thought to repair tissue damage by alternative or paracrine mechanisms. In the present study, although transplanted hUC-MSCs migrated to the nasal mucosa, these cells were limited to the lamina propria, failed to reach the epithelium, and did not express the epithelial specific protein β-tubulin. In addition, the cilia coverage and morphology of MSC-injection group did not improved. Then, the hUC-MSCs do not repair the damage mucosa by a cell-alternative mechanisms. Therefore, these hUC-MSCs may repair the damaged mucosa via a paracrine mechanism, however, the underlying molecular mechanisms require further research.

In addition, due to the promising prospect and abundant supply of hUC-MSCs, the human MSCs rather than autologous and allogeneic MSCs were used in guinea pig model in this research. Although many research have reported hUC-MSCs got promising result in disease model of rat, toxicity study also showed hUC-MSCs transplantation did not affect the general health of cynomolgus monkeys, the accurate influence of xenogeneic MSCs transplantation on cells migration and repairing function need further research [Koh et al., 2008; Fan et al., 2011; Wang et al., 2012].

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