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Microgravity-Modified ADSC-Exosomes; A Novel Approach to Amplifying Osteoprogenitor Cell Differentiation: A Hypothesis with Methodological Design

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ABSTRACT

Introduction: His hypothesis paper aims to evaluate the feasibility of utilizing microgravity (MG) as a bioreactor and microgravity-modified adipose mesenchymal stem cell-derived exosomes (ADSC-exos) as a novel approach in the field of bone regeneration.

Materials and Methods: The methodological procedure includes 5 essential steps; tissue harvesting and preparation, cell culture to obtain osteoprogenitor cells and ADSC-exosomes, microgravity application in groups I and III, final cell culture on scaffold-3D culture, flow cytometry analysis, and statistical analysis. SPSS software is employed for statistical analysis. The suggested statistical analyses are the Bonferroni or LSD post hoc test and the Kolmogorov-Smirnov test for normal distributed data.

Results: According to several previous studies, in which MG was utilized as a bioreactor factor compared to the control group, an enhanced osteoprogenitor cell differentiation and proliferation is comprehensible. An analogous orientation of advancement in group II (concerning the impact of ADSC-exos through paracrine factors) is expected compared to that in the control group; nevertheless, the significance of the increase in group II in comparison with groups I and III remains unpredictable.

Conclusion: Subject to the successful results of this hypothesis, the use of microgravity-modified ADSC-exosomes will be valuable as a novel approach for amplifying regeneration in osseous tissue.

Keywords: Microgravity; Regeneration; Bioreactors; Osteoprogenitor; Adipose Tissue-Derived Mesenchymal Stem Cells; Exosome

Abbreviations: ADSC-Exos: Adipose Mesenchymal Stem Cell-Derived Exosomes; MG: Microgravity; EPCs: Endothelial Progenitor Cells; MSCs: Multipotent Mesenchymal Stem/Stromal Cells; ECs: Endothelial Cells; IL-8: Interleukin 8; VEGF: Vascular Endothelial Growth Factor; 3D: Three-Dimensional; BFP: Buccal Fat Pad; BMSCs: Bone Marrow Stromal Cells; PBS: Phosphate-Buffered Saline; CM: Complete Medium; HEPES: 4-(2-Hydroxyethyl)-1-Piperazine Ethane Sulfonic Acid; FBS: Fetal Bovine Serum; GF2: Growth Factor-2; ADSCs: Adipose-Derived Stem Cells; FCM: Flow Cytometry; EDTA: Ethylenediaminetetraacetic Acid; RANKL: Receptor Activator of Nf-Kb Ligand; RANK: Receptor Activator of Nf-Kb; SPARC: Secreted Protein Acidic and Rich in Cysteine; BMI: Body Mass Index; HLA: Human Leukocyte Antigens; 2D: Two-Dimensional

Introduction

The subject of gravity, its altered states (microgravity and hypergravity), and its integration with the medical field (through medical Physics and tissue engineering) have garnered significant attention in recent decades. The potential future of life in outer space and the undeniable impact of microgravity on bodily organs and biological processes merits further exploration. The reconstruction of bony structures represents a significant clinical challenge, whereas environmental factors such as MG can potentially impact the quality and quantity of the final novel bone. Numerous prior studies have presented MG as a risk factor for and an impediment to the regular functionality of the cardiovascular system, Endothelial Progenitor Cells (EPCs), and endothelial cells. Conversely, some studies have shown that MG may promote cell differentiation and proliferation. According to a study by Ramaswamy et al (2016), prolonged exposure to MG (>12 h) is expected to rehabilitate angiogenesis in porcine blood-derived vascular stem cells. Other studies have suggested that culture in NG after exposure to MG, could enhance paracrine attributes and rates [1]. Building upon a study by Grigoryan EN and Radugina EA, the MG advanced the proliferation of stem-like cells in a broad range of up to 2-fold. Compared with those in the control group, the larger and more advanced regeneration in the treatment group was caused by accelerated cell dedifferentiation and sequential differentiation in the retina, lens, and limb. Notably, MG is capable of promoting lasting effects even when applied before operation [2].

Another study confirming the probable positive effect of MG on cell proliferation, by Ratushnyy A et al. suggested that MG could evoke Multipotent Mesenchymal Stem/Stromal Cells (MSCs) to Support Endothelial Cells (ECs) functions (proliferation and migration through the production of paracrine angiogenesis factors such as interleukin 8 (IL-8) and Vascular Endothelial Growth Factor (VEGF)) [3]. Recognizing the limitations inherent in the bone regeneration process and the significant impact of the quality and quantity of newly formed bone on patient quality of life, this study aims to present a novel approach for enhancing the quality and predictability of treatments in the field of osseous tissue regeneration.

Materials and Methods

This hypothesis has been designed to evaluate the feasibility of utilizing microgravity as a bioreactor and microgravity-modified ADSC-exos as an innovative approach to improve osteoprogenitor cell differentiation and proliferation during regeneration in osseous tissue.

There are three major questions:

i. Can MG modification amplify osteoprogenitor cell differentiation during the bone regeneration process?

ii. Does MG modification enhance the regenerative effects of ADSC-exos on osteoprogenitor cell differentiation?

iii. Is it feasible to minimize or prevent postsurgical complications related to bone repair deficiency with the use of MG as a bioreactor?

Structurally, four study Groups were considered to Assess the Defined Study Questions

Control: Osteoprogenitor cells + Three-dimensional (3D) culture

Group I: Osteoprogenitor cells + MG modification + 3D culture

Group II: Osteoprogenitor cells + ADSC-exos + 3D culture

Group III: Osteoprogenitor cells + ADSC-exos + MG modification + 3D culture

Fundamentally, the desired case selection needs to rely on the necessity of both the surgical procedure in the maxilla (implant placement, wisdom tooth extraction, or osteotomy) and buccal fat pad (BFP) removal surgery in the same patient. According to the method of human tissue usage in this study, case selection to obtain the desired tissue for yielded ADSC-exos and osteoprogenitor cells could be complicated. There are other available depots for both osteoprogenitor cells and adipose tissue in the human body. Consequently, maxillary osteotomy-harvested bone particles or BFP could be replaced with other depots such as the thigh (for ADSC-exos) and hip (for bone marrow). Regarding any unpredictable obstacle to providing the desired human tissue, animal substitutes such as harvested tissues from female Sprague-Dawley rats could be considered. Additionally, subject to the agreement of related organizations, a licensed research tissue bank could be an alternative for obtaining essential tissue sources. Based on the method of human tissue usage in this study, any legal or ethical consent must be obtained. Presuming that animal tissues should be used as replacements, all animal experimental protocols need to be reviewed and approved by related organizations.

Osteoprogenitor Cell Culture

Since the oral cavity is likely an available easy depot for a small amount of bone marrow, osteotomy-harvested bone particles from the maxilla provide bone marrow stromal cells (BMSCs) and yield osteoprogenitor cells. These bony particles are generally accessed during several surgical procedures in the oral area such as drilling for implant placement, wisdom tooth extraction, or osteotomy.

I. The samples are diluted with Phosphate-Buffered Saline (PBS) (ratio of 1:3). A density gradient in the solution causes nucleated cell isolation. The complete medium (CM) consists of 4.5 mg/mL d-glucose, 0.29 mg/mL l-glutamine, 1 mmol/L sodium pyruvate, 100 mmol/L 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid (HEPES) buffer, 100 U/mL penicillin, 100 µg/mL streptomycin, 0.1 mmol/L nonessential amino acids, and alpha-MEM supplemented with 10% fetal bovine serum (FBS) [4].

II. The plate phase is performed at a density of 100000 cells/

cm2 in the CM supplemented with 5 ng/mL fibroblast growth factor-2 (GF2) and 10 nmol/L dexamethasone [4].

III. The incubation is done under humidified conditions at 37°C and 5% CO2 [4].

ADSC-exos Culture

BFP removal is one of the most common plastic surgeries for providing adipose tissue. In this procedure, after local anesthesia the buccal mucosa (beneath the parotid duct) will be incised to observe the BFP, followed by grasping and moving through the incision with forceps. Based on several studies, due to the possibility of xenogeneic infection transmission and immunization, it is recommended that the typical 10% FBS be replaced with human platelet lysate. At present, collagenase digestion is the gold standard method for isolating adipose-derived stem cells (ADSCs) [5].

I. After decontamination of the specimens with povidone-iodine, rinsing with PBS, and dividing them into equal-weight samples are the next steps [6].

II. Samples are immersed in an appropriate medium in centrifuge tubes (Dulbecco's modified Eagle), including 10% FBS, 100 µL/mL streptomycin, 100 IU/mL penicillin, and collagenase NB [6].

III. The tube is shaken at 37 °C for 1 h and centrifuged at 400×g at 4 °C for 5 min, which is accompanied by a gentle shaking (30 s), and centrifuged again [6].

IV. The top layer is removed and the remaining solution is filtered through 100 and 40 µm filters. Then this step is completed by centrifuging samples at 1500×g at 4 °C for 5 min and aspirating the supernatant [6].

V. The Following steps involve resuspending the cells in the modified medium and seeding them into 10 cm cell culture dishes. Nonadherent cell removal will be performed after 24 h of culture [6].

VI. The next steps are culture medium replacement every 2–3 days and incubation in a 5% CO2 air-humidified atmosphere at 37 °C [6].

VII. Before continuing to the next stages, identity approval of cultured ADSCs is essential. Flow cytometry is an acceptable analysis for this importance. However, the use of definitive markers to impressively discriminate ADSCs is controversial, some of these markers such as CD44, CD105, and CD90 will be useful [5].

VIII. The cultured medium is collected and centrifuged at 13000×g for 30 min [7].

IX. Two milliliters of ExoQuick-TCTM exosome precipitation solution is utilized for exosome isolation [7].

X. The final steps are overnight incubation at 4 °C, centrifuging at 10000×g for 30 min at 4 °C, washing, and centrifuging the exosomes at 10000×g for 15 min at 4 °C. ADSC-exos will be suspended (in 100 of µL PBS) and stored at – 80 °C for later analyses [7].

XI. Similar to ADSCs identity approval, ADSC-exos must be characterized. CD63 and CD9 could be used as biomarkers for this step. In addition, the morphology of ADSC-exos is detectable through transmission electron microscopy [7].

MG Modification and 3D Culture

In groups, I and III, the ADSC-exos are exposed to MG (10-3) before being added to the 3D culture, where long-term exposure (>12 h) is suggested. Osteoprogenitor cells and ADSC-exos will be applied to the prepared 3D cultures. The scaffold-3D culture system is selected for use. The proposed medium for ultimate 3D culture is CM which is described above. In a study by Kong L et al., Gravite® (Space Bio-Laboratories Co., Ltd., Hiroshima, Japan) was employed to simulate MG. This machine creates a comparable environment to that of outer space by spinning the cells around two axes and integrating the gravity vector with the temporal axis. As a result, 10-3 g is generated and monitored through a gravity acceleration sensor. The culture flasks were placed in a 3D clinostat at 37 °C in a 5% CO2 chamber, where air bubbles in the flasks were fully evacuated to remove the influence of shear stress [6]. The same technology (the Gravite®) is used for MG modification in this study.

Data analysis

To assess the final results and compare the differentiation ratios, flow cytometry is an in-access and reliable analysis method.

Flow cytometry (FCM)

I. Sample Preparation, includes two steps:

a. Adherent cell removal with enzymatic solutions or calcium chelation reagents [8].

b. Washing the cells, suspending in buffer, centrifuging at 400×g for 5 min, replacement of buffer, and resuspending in FCM staining buffer (in a small volume of buffer) [8].

II. Blocking: Anti-Fc antibody dilution is applied to the washed cells, after that, the staining antibody dilution is added [8].

III. Antibody incubation: An optimal concentration of specific antibodies conjugated to bright fluorophores, is used for dilution in FCM assay buffer [8].

IV. Fixation and storage: Cell fixation will be done with paraformaldehyde in phosphate-buffered saline to store the cells overnight at 4°C. At least twice washing will be necessary before further usage [8].

V. Data acquisition [8].

Flow cytometry's essential materials and their suggested composition are available in Table 1.

Table 1: Flow cytometry essential materials and their suggested compositions [12].

Flow Cytometry Essential Materials	Suggested Composition and Suggested CD Markers
Cell sorting buffer	500 ml PBS, 100 µl 500 mM EDTA, pH 8.0, and 1% (w/v) bovine serum albumin.
FCM staining medium	500 ml Hank's buffered salt solution, 15 ml heat-inactivated fetal calf serum, 2 ml 500 mM EDTA, pH 8.0, and 1 ml 1 M HEPES, pH 7.0.
Osteoblast's CD markers	CD90/Thy-1, alkaline phosphatase.
Osteoclast's CD markers	RANKL/CD254 or RANK/CD265, CD68, CD115.
Osteocyte's CD markers	SPARC, podoplanin (GP38).

Note: EDTA: ethylenediaminetetraacetic acid, RANKL: receptor activator of NF-κB ligand, RANK: receptor activator of NF-κB, SPARC: secreted protein acidic and rich in cysteine.

Statistical Analysis

SPSS software is used for the statistical analysis. All the data are presented as the mean ± standard deviation. Regarding the number of groups (four) and the multivariate nature of the study, the suggested statistical analysis would be the Bonferroni or LSD post hoc test and the Kolmogorov-Smirnov test for normally distributed data [9].

Results

On the basis of several previous studies in this field, we anticipate that the results of groups I and III (where MG is utilized as a bioreactor factor) will demonstrate enhanced osteoprogenitor cell differentiation and proliferation compared to those of the control group. Furthermore, a similar trend toward an increase in group II (concerning the impact of ADSC-exos via paracrine factors) is expected compared to that in the control group; nevertheless, the significance of the increase in group II in comparison with groups I and III remains unpredictable.

Discussion

The reconstruction of bony structures represents a significant clinical challenge. Generally, the bone grafting procedure is associated with insufficient vascularization as an obstacle during the initial phase after implantation. Nutrient and gaseous exchange potentially affect the survival rate of cells within the integration phase of the harvested graft with host tissue, which is accomplished by blood microcirculation (waste product removal) and vasculature (calcium and phosphate delivery for mineralization) [10]. Environmental factors such as MG can potentially impact the quality and quantity of the final novel bone. Owing to the limited amount of autologous cancellous bone sources available for harvesting, reconstruction, and autografting treatment plans, innovative methods combining a bioreactor such as MG and tissue engineering could lead to advancements in this area. In general, within the context of regenerative treatment protocols, clinicians may encounter various complex medical conditions, such as systemic disorders, that could notably impact the ultimate outcomes of tissue regeneration. Moreover, challenges arise in situations such as traumatic bone injuries and tumor resections, where ensuring adequate resources for effective reconstruction of the defect becomes a substantial obstacle. Similarly, in fields such as dental implantology and occlusal rehabilitation, instances of bone graft demands are prevalent. In all these clinical procedures, clinicians could take advantage of tissue engineering approaches to enhance cellular proliferation and differentiation within osseous tissues.

In this paper, the investigation relied on the properties of osteoprogenitor cells, ADSC-Exos, and MG effects. This study aims to examine the novel effect of microgravity-modified ADSC-exos as a potent agent for enhancing the differentiation rate of osteoprogenitor cells. Why osteoprogenitor cells? Osteoprogenitor cells (osteogenic cells) which settle in the bone marrow and endosteum (the cellular layer of the periosteum), are known as stem cells. The infant mesenchymal origin and differentiation process of these cells into osteocytes and osteoblasts, make them a substantial cell resource for bone regeneration studies [4]. Why Adipose Mesenchymal Stem Cell-Derived Exosomes (ADSC-exos)? Adipose-Derived Stem Cells (ADSCs) can preserve self-renewal and augment multidifferentiation potential through paracrine factors release and extracellular vesicles. This allows them to be authorized for tissue and damaged organ repair [5]. The efficiency and attributes of ADSCs (such as gene expression patterns and the tendency to differentiate into a specific germ layer) depend on variable criteria, for instance: the harvesting method, adipose tissue depot, patient medical comorbidities, metabolic index, body mass index (BMI), gender, and age [5]. Available evidences reveal that the thigh could be a more appropriate depot for harvesting ADSCs [5]. In addition to the thigh, the BFP is another precious adipose tissue with a rich blood supply, easy harvesting, and a low patient complication rate [11]. Aging, high BMI (>30 kg/m2), diabetes mellitus, radiotherapy exposure, and endocrine therapy are suspected to negatively affect ADSC properties [5].

The general ADSC harvesting methods incorporate direct excision, liposuction, and aspiration (the most common method involves slight negative pressure applied via a syringe). According to prior studies, mesodermal and ectodermal differentiation are more prone following direct excision while liposuction is likely to promote differentiation to the endoderm [5]. About BFP, direct excision is the preferred harvesting method.

Substantially, secreted exosomes from stem cells are responsible for impressive effects on tissue repair and regeneration. Exosomes are extracellular vesicles with a lipid bilayer membrane bounded at a diameter ranging from 30 to 150 nm, that carry bioactive proteins,

RNAs, and lipids. After being endocytosed by target cells, they can regulate their function. In contrast to the stem cell therapy, exosomes do not contain DNA and Human Leukocyte Antigens (HLA) antigens, so this method prevents tumorigenicity and immunogenicity risk [12]. Why 3D cultures? In early in vitro studies, a two-dimensional (2D) culture system (the spreading of cells on a flat monolayer surface) was used, which indicated unnatural proliferation as a drawback. Due to advancements, it is now feasible to create 3D models (a fibrous network) similar to an extracellular matrix or an ECM (a natural hydrogel) by the use of well-defined biopolymers (collagen and fibrin) and other synthetic polymers. This structure facilitates cell growth, spreading, and the movement of metabolic chemicals through large holes (millimeters in size).

These properties make hydrogel-based 3D systems a common choice for tissue engineering in ground-based microgravity research [13]. As noted in several studies, in addition to the viability and proliferation of MSCs, even osteogenic differentiation could be enhanced through 3D culture in comparison with conventional 2D culture [14]. Why MG Modification? MG disposed the expression levels of microRNAs and mRNAs in cardiovascular progenitor cells which control cell migration, mechanotransduction signals, osteogenic differentiation, and angiogenesis. Contingent upon the successful results of this hypothesis in the future, the application of microgravity-modified ADSC-exosomes (through appropriate infusion techniques), could be a reasonable novel approach to upgrade the osteoprogenitor cell differentiation. It is essential to note that for future clinical usage, any available adipose depot in a patient's body could be considered a potential source for isolating and culturing ADSC-exos. Limitations and strengths: Considering that the quantitative values outlined in the steps of the method have relied on previous studies, the specific quantitative values may be altered based on future preferences.

Conclusion

This hypothesis attempts to evaluate the feasibility of utilizing microgravity as a bioreactor and microgravity-modified ADSC-exos as a viable prospective methodology in the field of bone regeneration. On the basis of the successful results of this study, it will be a valuable novel approach to amplify regeneration in osseous tissue.

Declarations

Ethics Approval and Consent to Participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

Not applicable.

Competing Interests

The authors have no relevant financial or non-financial interests to disclose.

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Authors Contributions

MH contributed to the study conception and study design, wrote the main manuscript text, and substantively reviewed the study. SOK contributed to the study conception and revised the paper. All the authors reviewed and approved the final manuscript.

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Artificial Intelligence

The authors declare that they have not used artificial intelligence in this study.

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