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ORIGINAL

ENHANCING BONE REPAIR AND FUNCTIONAL RECOVERY: THE ROLE OF THREE-DIMENSIONAL SPHEROID CULTURE AND HYPOXIC PRECONDITIONING IN MESENCHYMAL STEM CELL OSTEOGENIC DIFFERENTIATION UNDER LOW-OXYGEN CONDITIONS

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ABSTRACT

Background: Currently, bone marrow mesenchymal stem cells (BMSCs) are commonly utilized as seed cells for the treatment of bone defects or nonunion. However, their efficacy in bone regeneration is limited due to the severe weakening or cell death that occurs after transplantation into the body when cultured in vitro. This discrepancy is attributed to differences in the cellular living environments between in vitro and in vivo conditions. Thus, the objective of our study is to develop a proficient method for preparing BMSCs with robust survival and osteogenesis ability through a combination of hypoxic preconditioning and three-dimensional (3D) culture specifically for BMSC-based cell therapy. **Methods:** The primary BMSCs underwent varied hypoxic preconditioning treatments including sustained hypoxia, intermittent hypoxia, and sustained normoxia. The hanging drops technique was carried out to generate 3D microspheres. CCK8 assay, apoptosis determination assay, and live-dead staining were used to evaluate the proliferation and survival potential of BMSCs. Alkaline phosphatase, alizarin red staining, and osteogenic-related

proteins were performed to assess the osteogenic differentiation abilities of BMSCs. **Results:** BMSCs showed stronger proliferation and survival abilities after intermittent hypoxic preconditioning and 3D culture. Moreover, cells cultured in the intermittent hypoxic preconditioning and 3D culture exhibited less apoptosis and higher osteogenic differentiation abilities, alkaline phosphatase activity, cell calcium content, and expression of osteogenic-related molecules than those cultured under conventional condition. **Conclusions:** The application potential of seed cells in bone regeneration can be significantly improved by subjecting BMSCs to intermittent hypoxic preconditioning and 3D spheroid culture at specific stages. These treatments promote the proliferation, survival, and osteogenic differentiation potential of BMSCs, which enhances their ability to support bone growth and regeneration.

KEYWORDS: BMSCs, Hypoxic Preconditioning, Spheroids Culture

1. INTRODUCTION

Bone defects and nonunion are significant clinical challenges in orthopedic and sports medicine, often resulting from trauma, fractures, or degenerative conditions(Liu et al., 2016; Yuan, Cui, Zhang, Liu, & Cao, 2007). These conditions not only impair mobility and physical performance but also significantly affect the quality of life and functional independence of patients(Chu et al., 2018; J. Li et al., 2021). Bone marrow mesenchymal stem cells (BMSCs) have emerged as a promising tool in regenerative medicine due to their ability to differentiate into osteoblasts, promote bone regeneration, and modulate the local microenvironment (Fannon, Bithell, Whalley, & Delivopoulos, 2021; Han et al., 2014). However, their therapeutic efficacy is often limited by poor survival, reduced functionality, and insufficient osteogenic potential following transplantation. This discrepancy primarily arises from the difference in environmental conditions between in vitro culture and in vivo implantation, particularly the low-oxygen (hypoxic) environment characteristic of damaged or regenerating tissues(Jiang et al., 2017; Potier et al., 2007). The physiological niche of BMSCs in the bone marrow is inherently hypoxic, with oxygen levels ranging between 1% and 7%. This low-oxygen environment plays a critical role in maintaining the stemness, proliferation, and differentiation capacity of BMSCs. In contrast, conventional in vitro culture conditions are typically normoxic (approximately 21% oxygen), leading to altered cellular behavior and reduced viability when transplanted into hypoxic in vivo environments. Hypoxic preconditioning, which involves exposing cells to low oxygen levels before transplantation, has been proposed as a strategy to improve the adaptation, survival, and functionality of BMSCs in hypoxic tissues. Among the various hypoxic regimens, intermittent hypoxia has shown superior results in enhancing cell viability, proliferation, and differentiation by mimicking the dynamic oxygen fluctuations of the in vivo microenvironment. In addition to hypoxic preconditioning, the three-dimensional (3D) culture of BMSCs represents a

significant advancement in cell therapy. Unlike traditional two-dimensional (2D) monolayer cultures, 3D spheroid culture better replicates the natural architecture and cell-cell interactions of the in vivo microenvironment(Haider & Ashraf, 2010). This approach enhances cell survival, promotes differentiation, and improves the secretion of bioactive factors that support tissue repair and regeneration(Kolar, Gaber, Perka, Duda, & Buttgereit, 2011; Yu et al., 2019). When combined with hypoxic preconditioning, 3D culture may further amplify the regenerative potential of BMSCs by synergistically enhancing their osteogenic differentiation and adaptation to low-oxygen environments. In the context of sports and rehabilitation medicine, optimizing the therapeutic potential of BMSCs is particularly critical for addressing skeletal injuries and promoting functional recovery. Athletes and physically active individuals frequently experience bone fractures or defects that require effective and timely interventions to restore performance and prevent long-term complications. Enhancing the regenerative capacity of BMSCs through intermittent hypoxic preconditioning and 3D culture could provide a robust strategy for improving outcomes in bone repair and regeneration(Fehrer et al., 2007). This study investigates the combined effects of intermittent hypoxic preconditioning and 3D spheroid culture on the proliferation, survival, and osteogenic differentiation of BMSCs under low-oxygen conditions(Chambers, Mosaad, Russell, Clements, & Doran, 2014; Zhang et al., 2018). By exploring this novel approach, we aim to bridge the gap between laboratory findings and clinical applications, offering insights into optimizing BMSC-based therapies for bone regeneration. Furthermore, the study emphasizes the relevance of these techniques in the field of sports medicine, where accelerated recovery and enhanced functional outcomes are essential for enabling individuals to return to active lifestyles. This study explores a rapid and effective method for obtaining BMSCs with stronger proliferation, osteogenesis, and survivability in harsh environments through the use of hypoxic preconditioning and the formation of BMSC spheroids. By addressing cell survival and functional maintenance, this approach may provide a solution for the transformation of BMSC cell therapy into clinical use.

2 Materials and Methods

2.1 Isolation and Culture of BMSCs

BMSCs were extracted from the femur and tibia bone marrow of 4-week-old SD rats and considered as passage 0 (P0). The P0 BMSCs were divided into three groups (Table 1): sustained hypoxia, intermittent hypoxia, and sustained normoxia.

The culture medium was replaced every two days, and cell passaging was done at 90% growth density. The cell morphology and growth were closely observed, and cells were cultured up to passage 3 (P3) for initial analysis of proliferation and osteogenic differentiation activity.

Table 1: Group and culture condition of P0-P3

	SUSTAINED HYPOXIA	INTERMITTENT HYPOXIA	SUSTAINED NORMOXIA
P0 0-48 HOURS	1%O_2	1%O_2	21%O_2
P0 48 HOURS-P3	1%O_2	21%O_2	21%O_2

Cells from the P3 intermittent hypoxia group were selected for future experiments in 2D monolayer or 3D spheroid culture. The cells were divided into four groups (Table 2). The experimental group (3D hypoxia-normoxia) underwent hypoxic preconditioning again for 48 hours with 3D microspheres. The other groups cultured in 2D monolayer or without re-hypoxic preconditioning were used as controls. After 96 hours of preconditioning, all cells were placed in a low-oxygen environment for analysis of survival and osteogenic differentiation capacity *in vitro*.

Table 2 Group and culture condition of intermittent hypoxia after P3

	2D NORMOXIA	2D HYPOXIA-NORMOXIA	3D NORMOXIA	3D HYPOXIA-NORMOXIA
0-48 H	21% O_2 Matrigel	21% O_2 Matrigel	21% O_2 Hanging-drop culture plate	21% O_2 Hanging-drop culture plate
48-96 H	21% O_2 Matrigel	1% O_2 Matrigel	21% O_2 Low-adherence culture plate with matrigel	1% O_2 Low-adherence culture plate with matrigel
96 H-21 D	1% O_2 Osteogenic induction	1% O_2 Osteogenic induction	1% O_2 Osteogenic induction	1% O_2 Osteogenic induction

2.2 Cell proliferation and Cell Counting Kit-8 (CCK8) assay

To evaluate the cell proliferation ability, BMSCs were seeded from P1 at a density of 3×10^3 cells/cm^2 in T25 cell culture flasks (Corning, USA). Cell counting was conducted daily using Image J software until P4, and the cell population doubling times during the exponential growth phase were calculated using the following formula, where t_x is the time interval considered, N_t is the cell count at the beginning of the time interval, and N_0 is the cell count at the end of the time interval. All experiments were performed in triplicate.

$$T_D = \frac{t_x \times \log 2}{\log \frac{N_t}{N_0}}$$

The cell viability was monitored with CCK8 (Dojindo, Japan). P3 BMSCs

were uniformly seeded into 96-well plates at a density of 5×10^3 cells per well in a hypoxic environment, and their proliferation capacity was evaluated at five time points (day 0, 1, 2, 3, 4). Briefly, the medium in each well was discarded, then 10 μL CCK-8 reaction solution and 100 μL medium mixture were added to each well under dark conditions and incubated for 2 hours. A microplate reader was used to record the OD value at 450 nm. The mean value was calculated and the cell proliferation curve was drawn.

2.3 Live-Dead Staining

The Live-Dead Cell Viability Test Kits (KeyGEN, China) were used to determine the survival of BMSCs under hypoxic conditions at 24 hours and 48 hours. P3 BMSCs in Table 1 were evenly seeded into 96-well plates at a density of 5×10^4/mL and transferred to hypoxic culture. At 24 hours and 48 hours, the culture medium in the well plate was discarded, and the plates were rinsed thrice using phosphate buffered saline (PBS). The live-dead solution was then added to each well, and the cells were stained at room temperature for 45 min. Percentages of live and dead cells were calculated based on fluorescence readings at 530 nm and 645 nm.

2.4 Generation of BMSCs spheroids via the Insphero hanging drops technique

The hanging drop technique was used to generate BMSCs spheroids based on Insphero (InSphero AG, Schlieren, Switzerland) manufacturer's instructions. P3 BMSCs, preconditioned with intermittent hypoxia, were finally selected for constructing 3D BMSCs microspheres. It was divided into three groups based on seeding densities of 2.4×10^4, 4.8×10^4 and 9.6×10^4 per well to construct BMSCs microspheres. Live-dead cell staining and apoptotic determination assay were used to determine the optimal cell densities for the construction of the microspheres.

2.5 Apoptosis determination assay

The apoptosis activity of caspase was determined in two parts. Caspase-glo reagent (100 ul, Promega，USA) was added to each well on one 96-well plate. The samples were incubated at room temperature for 3 hours, and the fluorescence value of each sample was measured using a fluorescence luminescence instrument.

Nucleic acid quantitative analysis reagent (100 ul, Sigma, USA) was added into each well on another 96-well plate and allowed to react for 5 minutes. The DNA content of the sample was determined using a fluorescence luminescence instrument. Apoptotic activity was compared by calculating the ratio of the two data sets.

2.6 Alkaline phosphatase (ALP) and alizarin red staining (ARS)

P3 BMSCs were cultured with osteogenic induction medium to induce osteogenic differentiation. The BMSCs were evenly seeded into 6-well plates at a density of 2×10^5 cells per well. On day3, 5, 7 of osteogenic induction, the alkaline phosphatase dye (Beyotime, China) was added to each well for 30 minute. An inverted microscope was used to visualize the stained cells. Cell lysates were collected after seven days of osteogenesis induction. Alkaline phosphatase content was assessed using an alkaline phosphatase assay kit (Beyotime, China). The absorbance was then measured at 405 nm. On day7, 14, 21 of osteogenic induction, to fix the cells, 4% paraformaldehyde was used for 15 minutes. The residual paraformaldehyde solution was removed by washing thrice using PBS buffer. Alizarin red staining was performed and the positive area of alizarin red staining were evaluated using Image J software.

2.7 Cell Calcium Content

The cells were collected on the day 7, 14, 21 during osteogenesis induction, and the cells were washed twice with PBS. The matrix glue containing cells was collected and divided into two groups. The cells were added into two 96-well plates and centrifuged in a low-speed centrifuge, and the supernatant was collected for subsequent analysis. The pre-prepared Calcium Chromogenic Working Solution (Leagene, China) was added to samples in a dry test tube, mixed well, then allowed to stand at room temperature for 5 minutes. The absorbance was detected at 575 nm. Nucleic Acid Quantitative Analysis Reagent (100 ul, Sigma, USA) was added to each well of another 96-well plate, and allowed to react for 5 minutes. The DNA content of the sample was determined using a fluorescence luminescence instrument. The relative calcium content was compared by calculating the ratio of the two data.

2.8 Real-time PCR analysis

On day 21 of osteogenic induction, the cells were collected and total RNA was isolated using TRIzol (TaKaRa, China). The quantity of total RNA was determined using a spectrophotometer (Implen, Germany) by measuring its absorbance at 260 nm. The A260: A280 ratio of RNA samples was between 1.8 and 2.0 to ensure high purity. A total of 1 μg total RNA sample was used as a template for conversion into cDNA using a SuperScript First-Strand Synthesis System kit (Thermo Fisher Scientific, USA). A real-time PCR system (Thermo Fisher Scientific, USA) was used to carry out qPCR, with the reaction system composed of cDNA, primers, and SYBR Premix Ex Taq™ (Takara Bio, China). The specificity of single-target amplification was confirmed through post-PCR melting curves. Additionally, the fold change of the target gene relative to GAPDH was determined. All the reactions were performed in triplicate to ensure

consistency. Quantification of mRNA was performed with the comparative threshold cycle method (ΔΔCt) with GAPDH as the internal reference, and relative gene expression was reported as $2^{-\Delta\Delta CT}$. The following primer sequences were used for qPCR.

2.9 Western blot analysis

The contents of Runx2 and Col1a1 were detected using western blot after 21 days of osteogenesis induction. Precooled cell lysate (800 μL, KeyGEN, China) was added to each well of the plate. Total cell protein was extracted using a total protein extraction kit (KeyGEN, China). Protein concentration was measured using a BCA protein assay Kit (KeyGEN, China). Aliquots of 20 μg of each sample were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, USA), followed by blocking with 5% (w/v) nonfat milk for 1 hour. The membranes after blocking were incubated overnight with the primary antibodies at 4°C. Then, membranes were washed with Tris-buffered saline with Tween (TBST: 20mM Tris-HCl [pH 7.6], 1% Tween 20, 137mM NaCl), incubated with horseradish peroxidase(HRP)-conjugated secondary antibody in blocking buffer for 1h at room temperature, detected immunoreactive products using ECL plus western blotting detection reagent, and photographed by Quantity One software (Bio-Rad, CA, USA).

2.10 Statistical Analysis

Data are expressed as mean ± standard deviation. Data analysis was performed using a one-way analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test using GraphPad Prism 9.0 software. $P < 0.05$ was considered a significant level.

3. Results

3.1 Intermittent hypoxic preconditioning improves BMSCs survival and proliferation

The morphology of P0 and P3 passages of BMSCs were observed to evaluate the effects of different hypoxia pretreatment conditions on the survival and proliferation capacity of non-physiological monolayers BMSCs *in vitro*. We observed no remarkable morphological differences among cells with different hypoxic preconditioning. Moreover, we analyzed the population doubling time under different hypoxic pretreatments. The population doubling time was significantly shorter in the intermittent hypoxia group than that in the sustained hypoxia and sustained normoxia groups ($P<0.0001$, $n=6$), indicating a shorter passage time interval of BMSCs after intermittent hypoxia preconditioning and higher culture efficiency. After preconditioning, P3 BMSCs were cultured in a hypoxic environment to plot the cell proliferation curve. The proliferation ability

of cells in the sustained hypoxia group gradually increased with culture duration. Conversely, the proliferation ability of cells in the intermittent hypoxia and sustained normoxia groups decreased initially before increasing after exposure to hypoxic conditions, with the lowest point being observed at 48 hours. Moreover, the proliferation ability of cells in the intermittent hypoxia group reduced after exposure to the hypoxia environment compared to that of cells in the sustained hypoxia group. However, the proliferation ability of the cells in the intermittent hypoxia group recovered faster after 48 hours than that of cells in the sustained normoxia group, which exceeded that of cells in the sustained hypoxia group on the 3rd day. These findings indicate that sustained hypoxic culture and intermittent hypoxic culture can improve the proliferation ability of P0 BMSCs in a hypoxic environment. Cell survival was detected by live-dead cell staining and counting. Massive BMSCs in the intermittent hypoxia and sustained normoxia groups died upon exposure to hypoxia. However, cell survival was higher in the intermittent hypoxia group than that in the sustained normoxia group. This indicates that BMSCs in the intermittent hypoxia group had a stronger survival ability than that in the sustained normoxia group under a hypoxic environment.

3.2 Intermittent hypoxic preconditioning stimulates BMSCs osteogenesis differentiation

Alkaline phosphatase staining was performed following osteogenic induction of the P3 BMSCs. There was a gradual increase in the alkaline phosphatase activity of BMSCs in the intermittent hypoxia and sustained normoxia groups. In contrast, the alkaline phosphatase activity of the sustained hypoxia group slightly decreased. Moreover, after 7 days of osteogenic induction, the alkaline phosphatase activity of BMSCs in the intermittent hypoxia group was significantly higher than that in the sustained hypoxia and sustained normoxia groups. Alizarin red staining was also performed and evaluated after osteogenic induction of the P3 BMSCs in each group. Mineralized nodules gradually increased after osteogenic induction in the intermittent hypoxia and sustained normoxia groups. Few nodules were mineralized after osteogenic induction in the sustained hypoxia group. The number of mineralized nodules was significantly higher in the intermittent hypoxia and sustained normoxia groups than that in the sustained hypoxia group after 14 and 21 days of osteogenesis induction, particularly in the intermittent hypoxia group. These findings indicate that sustained hypoxic culture can inhibit the osteogenic activity, whereas intermittent hypoxia preconditioning can enhance the osteogenic activity of BMSCs.

3.3 Comparison of the viability of BMSCs spheroids with different diameters under hypoxic conditions

P3 BMSCs of the intermittent hypoxia group were selected for

subsequent experiments in 2D or 3D culture (Table 2). After being transferred to hypoxic culture for 48 hours, the apoptotic activity was measured to compare the survival ability of BMSCs microspheres with different cell densities. Microspheres with 4.8×10^4 and 9.6×10^4 cell densities had greater apoptosis than that with 2.4×10^4 cell density ($P<0.0001$, $n=12$); this indicates that the BMSCs microspheres with 2.4×10^4 cell density had stronger viability than that with 4.8×10^4 and 9.6×10^4 cell densities. After exposure to hypoxic culture for 24 hours and 48 hours, the dead cells were primarily concentrated at the core of the spheres. We also compared the staining results of live-dead cells and the percentage of dead cells cultured in a hypoxic environment for 24 hours and 48 hours in each group. Cell death was significantly lower in the 2.4×10^4 group than that in the 4.8×10^4 and 9.6×10^4 groups after hypoxic culture for 24 hours and 48 hours. This suggests a significantly higher survival rate in the 2.4×10^4 group than that in the 4.8×10^4 and 9.6×10^4 groups ($P<0.0001$, $n=12$).

3.4 The survival and osteogenic activity of BMSCs microspheres increase under hypoxic conditions after intermittent hypoxic preconditioning

Previous sections (3.1-3.2) revealed that the proliferation, survival, and osteogenic ability of BMSCs were significantly enhanced after intermittent hypoxia preconditioning *in vitro*. Section 3.3 shows the optimal seeding density (2.4×10^4) for generating 3D BMSCs microspheres. P3 BMSCs preconditioned with intermittent hypoxia were divided into four groups (Table 2) for subsequent experiments. The caspase activity was determined. The apoptosis curves showed that the 2D monolayer cultured BMSCs had higher apoptotic activity than the 3D BMSCs microspheres, indicating that BMSCs microspheres harbored stronger survival activity than the 2D monolayer or normoxic cultured BMSCs ($P<0.0001$, $n = 6$). Alkaline phosphatase assay was performed after osteogenic induction of BMSCs based on the preconditions shown in Table 2. The ALP levels observed in each group were time-dependent, with the activity of ALP gradually increasing in correlation with the duration of osteogenic induction. ALP content was not significantly different among all groups on days 0-3. However, ALP content was significantly different on days 5-7 ($P<0.0001$, $n = 6$). We also determined the Intracellular calcium concentration in each group . The calcium content was time-dependent, as indicated by a gradual increase in correlation with the duration of osteogenic induction. Notably, calcium content was observed to differ among all groups on day 7. Furthermore, the calcium content exhibited a significant increase at 7-14 days and 14-21 days. It was observed that the osteogenic calcium contents were highest in the 3D hypoxia-normoxia group. Real-time PCR analysis was performed to detect the expression of osteogenesis-related genes in BMSCs on the 14th day of osteogenic induction. The results demonstrate that the expression of osteoblast-specific markers, Runx2, Opn, Bsp and Col1a1, was upregulated in BMSCs following the intermittent hypoxia preconditioning or three-dimensional culture. Notably, the expression of osteogenesis-related markers was

significantly increased in BMSC microspheres pretreated with intermittent hypoxia. Western blot analysis was used to measure the content of osteogenic-related proteins on day 21 of osteogenic induction. It was found that the expressions of Runx2 and OPN were highest in the 3D hypoxia-normoxia group, followed by the 3D normoxia group, 2D hypoxia-normoxia group, and lowest in the 2D normoxia group. These results were consistent with the quantitative determination of calcium and alkaline phosphatase activities. Taken together, these observations suggest that BMSCs preconditioned with intermittent hypoxia exhibit greater osteogenic potential in a hypoxic environment.

4. Discussion

Proper preconditioning of BMSCs before in vivo transplantation improves their proliferation, survival, and osteogenic activity and is thus required for the further clinical application of BMSCs (Shi et al., 2018; Xu et al., 2020). This study provides an in vitro preconditioning method for BMSCs (combination of intermittent hypoxia preconditioning and three-dimensional culture) for efficient transplantation in vivo. The cell behaviors change during the in vitro culture is attributed to the significant changes in the growth environment (Quarta et al., 2021; Van Hemelryk et al., 2021). Following the results of this study, after long-term culture under normoxic conditions in vivo, the proliferation ability of cells will be inhibited within a certain time after being unexpectedly transferred to a hypoxic environment. The cells in the intermittent hypoxia and sustained normoxia groups showed poor potential to enter the hypoxic environment in the first two days. During the first two days, the intermittent hypoxia and sustained hypoxia groups exhibited growth arrest. These cells underwent a substantial time period in normoxia and required an adaptation period upon initial exposure to the hypoxic environment. Indeed, a certain adaptation time is crucial for cells to regain their proliferative potential when exposed to a challenging growth environment. However, by the 4^{th} day, the proliferation rate of the intermittent hypoxia group was the highest. This indicates that the intermittent hypoxia group not only had a strong adaptive ability to a low oxygen environment in vitro but also eventually acquired a strong proliferative ability. Only a limited number of primary MSCs can be directly extracted from the body. However, an adequate number of cells can be acquired by generating 3D culture microspheres after a specific period of *in vitro* culture (Ballester-Beltrán et al., 2017; Bhang, Lee, Shin, Lee, & Kim, 2012). Nonetheless, the adverse effects of hypoxia on cells were not ignored. The results revealed that sustained hypoxia had a certain inhibitory effect on the osteogenic differentiation of BMSCs. Therefore, we analyzed the effects of different hypoxic pretreatment methods on cells, optimized the traditional hypoxic culture method, and finally selected the intermittent hypoxic culture method for further analysis. Under the intermittent hypoxic culture approach, BMSCs could proliferate and survive in a hypoxic environment while maintaining osteogenic differentiation to a maximum extent. Moreover, all

aspects of the biological properties of cells were balanced, making it ideal for subsequent application. The 3D cell culture technology can simulate the physiological environment of cells in organisms and maintain the contact between cells, hence its more suitable for maintaining the biological behavior of isolated cells. Currently, 3D cell culture techniques are grouped into two main categories: those with and without scaffolds (Bhang, Lee, Lee, et al., 2012; N. Li et al., 2014). The 3D cell culture techniques with scaffolds, such as hydrogel technology, primarily rely on the support and guidance of natural or artificial scaffolds to promote cell growth on scaffolds and are widely used at present (W. Li et al., 2016). The 3D cell culture techniques without scaffolds mainly include suspension drop technology, suspension technology, and ultra-low adhesion surface. Stent-free-3D cell culture technology primarily uses a specific culture plate to suspend and aggregate cells into a 3D sphere (Bijonowski, Fu, et al., 2020; Bijonowski, Yuan, Jeske, Li, & Grant, 2020). Unlike the 3D cell culture technology with scaffolds, 3D cell culture without scaffolds can yield a single cell sphere without dealing with cell scaffolds. Moreover, the volume and number of cell spheres can easily be controlled when using 3D cell culture without scaffolds. Herein, the cell suspension was added above the hole of the automatic suspension plate (Gentile et al., 2012). The plate geometry guides the cells and culture medium through the hole to form a stable suspension (Knight & Przyborski, 2015). Each well generates a droplet, which contains a sphere whose size can be determined by the density of the seeded cells. The InSphero automatic hanging drop plate can be sterilized and reused, thereby significantly saving costs. As a result, this stent-free 3D cell suspension culture technique was used in the *in vitro* cell experiments (Strem et al., 2005). It is true that 3D cultures, such as microspheres, can replicate the 3D growth environment of cells *in vivo*. However, the impact of large microspheres on cell apoptosis cannot be overlooked. The diameter of microspheres can influence plasma osmotic pressure, oxygen content, and nutrient supply in cell cultures within these microspheres. Therefore, it is imperative to consider the size of microspheres to optimize cell survival and minimize apoptosis in 3D cultures (Barros et al., 2019). When the diameter of BMSCs microspheres is extremely large, cells at the center rapidly undergo apoptosis within a short period. Although increasing the seeding density can produce microspheres with a greater volume, it does not necessarily mean that a higher number of cells can maintain a high survival rate. As a result, to enhance the effectiveness and efficiency of cell application, BMSCs microspheres should maintain a specific range of diameter. Additionally, minimizing resource waste can lead to practical applications with good potential for clinical use of seed cells.

5. Conclusion

This study demonstrates that the combination of intermittent hypoxic preconditioning and three-dimensional (3D) spheroid culture significantly enhances the proliferation, survival, and osteogenic differentiation of bone

marrow mesenchymal stem cells (BMSCs) in a low-oxygen environment. By mimicking the physiological niche of BMSCs, this approach addresses critical challenges associated with in vitro culture, including poor cell survival and functionality after transplantation. The findings highlight the potential of these advanced culture techniques to optimize the therapeutic efficacy of BMSCs in bone regeneration and repair. The synergistic effects of intermittent hypoxia and 3D culture enhance cellular adaptation to hypoxic environments, reduce apoptosis, and promote the expression of osteogenic markers, such as alkaline phosphatase and calcium deposition. These improvements in cell functionality not only support better integration and performance of BMSCs in vivo but also provide a foundation for developing more effective regenerative therapies. This is particularly relevant in the context of sports and rehabilitation medicine, where accelerated bone healing and recovery are crucial for restoring physical performance and preventing long-term complications. The practical implications of these findings extend to the treatment of bone defects, nonunion, and skeletal injuries in physically active individuals. By improving the regenerative capacity of BMSCs, this approach offers a pathway to faster recovery, enhanced functional outcomes, and better quality of life for patients. Furthermore, the integration of these techniques into clinical protocols can pave the way for more personalized and effective therapies, ensuring optimal outcomes for individuals with diverse needs. Future research should focus on validating these findings in vivo and exploring their long-term impact on bone repair and functional recovery. Additionally, studies investigating the combination of this approach with other advanced therapies, such as biomaterials or growth factors, could further enhance its therapeutic potential. In conclusion, the combined use of intermittent hypoxic preconditioning and 3D spheroid culture represents a promising strategy for advancing BMSC-based therapies. This innovative approach has the potential to revolutionize bone regeneration practices, aligning with the goals of sports medicine and rehabilitation to support faster recovery and improved physical resilience in patients with skeletal injuries or conditions.

Ethics Approval and Consent to Participate

This research was approved by the Ethics Committee of West China College of Stomatology, Sichuan University, China (WCHSIRB-D-2016-206).

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Authors' Contributions

The specific contributions of the authors are as follows. (1) XQ, ZF and WJ contributed to the conception and design of the study; (2) GL, RM, XT, JT

and BY acquired, analyzed and interpreted the data; (3) XQ and ZF were the main writers of the manuscript, contributed equally to this work and should be considered co-first authors; (4) XQ, ZF and WJ were responsible for the critical revision of the manuscript. All authors have read and approved the final manuscript.

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