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Review Article

Hypergravity as A Possible Way of Bone Tissue Engineering in Osteoblastic Differentiation From Mesenchymal Stem Cell: A Systematic Review

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ABSTRACT

Background: Tissue engineering development has become a highlight in recent decades. One of the key areas of focus is producing mature bone tissue to overcome orthopedic problems, such as bone defects. Various cultures have been implemented on stem cells; some induce osteoblastic differentiation markers, while others have the opposite effect. Microgravity has been proven in several studies to inhibit the expression of osteogenic differentiation markers. Conversely, hypergravity is expected to have the opposite impact, supporting stem cells in the osteogenesis pathway.
Methods: A literature search was conducted using online databases including Sciencedirect, PubMed, and Proquest, covering the period from 2008 to 2022. This search considered only experimental studies published in English. The keywords used in this research were "hypergravity" and "mesenchymal stem cell." All acquired data were processed and analyzed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (2020).
Results: Initially, 190 studies were collected from online databases based on relevant keywords. After screening, 5 studies were included in the final analysis, focusing on hypergravity treatment and its effects on mesenchymal stem cells (MSCs).
Conclusion: Hypergravity shows a significant and strong impact on osteoblastic differentiation. This study revealed that a gravity force of 30G and a culture duration of 7 to 14 days are the most optimal combination for inducing osteoblastic differentiation in MSCs.

Keywords: Bone; Human and medicine; Hypergravity; Mesenchymal stem cell; Osteoblast; Osteogenic differentiation

INTRODUCTION

Tissue engineering development has become a highlight in recent decades. One of its key aims is producing mature bone tissue to overcome orthopedic problems, such as bone defects. When bone defects occur, advanced tissue engineering solutions, like three-dimensional bone products that are safe and free of carcinogenic characteristics, can be developed to replace the damaged tissue and serve as new bone complements. In this context, there is a growing interest in how physical stimuli influence stem cells, prompting them to differentiate into other cells or grow three-dimensionally. This interest arose when scientists began to observe the impact of microgravity at a cellular level, coinciding with humanity's achievements in space exploration and access to real microgravity environments. In con-

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junction with microgravity research, paradoxical physical stimuli like hypergravity are also being investigated. Some studies show that both real and simulated microgravity inhibit the osteogenic differentiation of mesenchymal stem cells (MSCs).[1–9] These studies also investigated the counterpart stimuli, examining whether hypergravity affects osteogenic differentiation. Our knowledge is still limited, as research is ongoing. However, some current results support the hypothesis that hypergravity has a positive effect on osteogenic differentiation in MSCs.

Mesenchymal stem cells have the potential to differentiate into a variety of specialized cell types. To guide their transformation into specific desired cells, appropriate tissue engineering techniques must be employed. For instance, osteoblasts can be directly differentiated from MSCs through intramembranous ossification, a process of bone formation.[10–12] Certain markers can detect the differentiation of MSCs into osteoblasts, but not into other specific cell types. Research has identified several such markers, including Core Binding Factor α1 (Cbfa1) also known as Runt-related transcription factor 2 (RUNX2), Alkaline Phosphatase (ALP), Bone Sialoprotein (BSP), Osteopontin (OPN), Osteocalcin (OCN), β-Catenin, type 1 collagen (COL1A1), and Osterix (OSX). These markers may induce or support osteogenic differentiation, and increased expression of these markers can indicate that the osteogenic differentiation process is occurring.[8–11] The osteogenic differentiation process is also accompanied by the presence of calcium deposits, as evidenced by increased levels of calcium in the extracellular matrix.[13]

Osteoblastogenesis has some stages, from MSC becoming a mature osteoblast to finally becoming an osteocyte. Each stage may show different markers presence or different power of expression in the same markers. Huang et al. explained that ALP, BSP, and COL1A1 are early osteogenic differentiation markers, while Pentatricopeptide repeat (PPR) and OCN are late in presence.[14] Zhang showed increased expression levels of Type 1 collagen and ALP during the transition from biopotential cells to pre-osteoblasts, with RUNX2 consistently expressed throughout these two stages. Miron and Zhang also demonstrated significant expression of Type 1 collagen, ALP, RUNX2, and Osterix in the pre-osteoblast stage.[14–16] This study aims to determine the extent to which hypergravity affects osteogenesis, specifically the culture duration and gravity force needed to achieve optimal results. Varying marker expression patterns also lead to a hypothesis about the ideal time and gravity force to combine with an osteogenic inducer for method optimization.

MATERIAL AND METHODS

This paper systematically assesses the role of hypergravity in the osteogenic differentiation of mesenchymal stem cells. Relevant studies were identified using three databases: PubMed, ScienceDirect, and Proquest. The keywords "hypergravity" and "mesenchymal stem cell" were used in the search. The literature search and study selection were performed according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines (2020).

Selection Criteria

This research included experimental studies published in English between 2008 and 2022, focusing on the effect of hypergravity on mesenchymal stem cells. Studies were required to contain the keywords "hypergravity" and "mesenchymal stem cell." Abstracts relevant to hypergravity treatment and MSCs were reviewed. Included studies examined osteogenic marker expression after hypergravity treatment compared to normal gravity. Studies were excluded if they: (1) did not use MSCs as a sample or used MSCs in combination with other cell types; (2) involved failed MSC cultures or experiments; (3) were not written in English or lacked an English translation; or (4) were published outside the research period of 2008 to 2022.

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Study Selection

The first step involved screening titles for relevance to the keywords and inclusion criteria. Articles that did not match all keywords were excluded. Next, abstracts of the selected titles were screened. Abstracts that did not correlate with the keywords and inclusion criteria were also excluded. Full texts were obtained for the remaining articles, and relevant results were extracted and used as data for this research.

RESULTS

The initial literature search yielded a total of 190 articles. After removing one duplicate article sourced from both Proquest and PubMed, and two duplicate titles from ScienceDirect and PubMed, 187 unique articles remained. Of these, 181 were excluded based on irrelevant titles, leaving six articles for further screening. These six articles contained relevant samples and treatments and were reviewed comprehensively. One study was excluded because it did not examine the osteogenic differentiation of MSCs, despite having relevant samples and treatments. Finally, five articles were included in this research. A summary of the study selection process can be seen in Figure 1.

Of the five studies (Table 1), three used MSCs derived from mice or rats, while the remaining two used human MSCs. All studies treated the MSCs with hypergravity, with magnitudes ranging from 2G to 40G and durations varying from 48 hours to 2 weeks. One study compared the effects of hypergravity with and without an osteogenic inducer and showed increased expression of osteogenic markers such as COL1A1, RUNX2 (Cbfa1), and ALPL in both conditions.

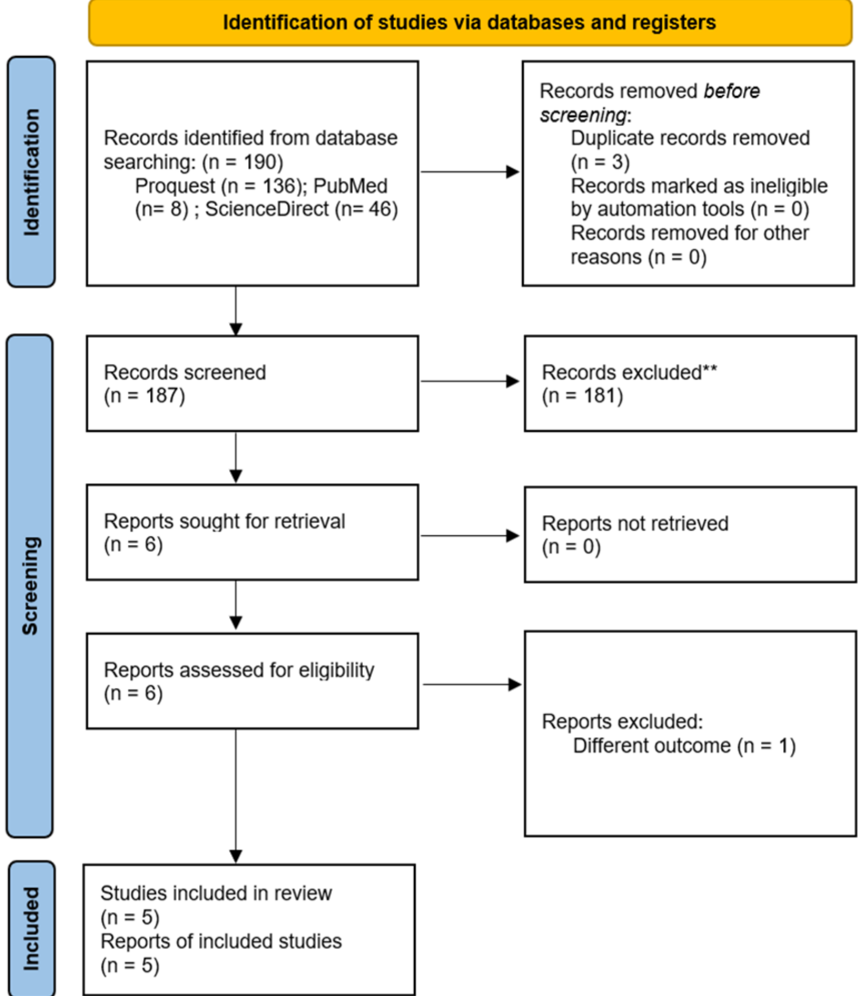

Figure 1. Flowchart for determining articles to be used from ScienceDirect, PubMed, and ProQuest, using the PRISMA 2020 guidelines.

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Table 1. Results and summary of MSCs cultured under hypergravity (from online databases)

No.	Author	Year	Sample	Power	Duration	Result
1	Rocca et al.[17]	2015	Rat MSC	20G	2 days	Gene transcription level 1. Significantly Upregulated RUNX2 (1.5-fold at 20G; 1.8-fold at 20G + BTNPs) and Ras homolog gene family member A (RHOA) (2.5-fold at 20G; 2.8-fold at 20G + BTNPs) 2. Significantly Enhanced COL1A1 (COL1A1 enhanced 1.5-fold only in the double-stimulation 20G + BTNPs, other treatments are decreasing the transcription) 3. Significantly Enhanced Alkaline phosphatase gene (ALPL) (about 1.2-fold at 20G; 1.6-fold at 20G + BTNPs) Protein expression level 1. Increased expression of COL1 (1.3-fold at 20G; 1.5-fold at 20G+BTNPs)
2	Huang et al.[4]	2009	Rat MSC	2G	1 - 7 days	1. Increase expression of ALP, COL1A1, Cbfa1 in both samples with and without osteogenic inducer, although sample with inducer shows higher expression 2. The highest expression of those 3 proteins were in day 7
3	Prodanov et al.[18]	2015	Rat MSC	10G	1 - 7 days	Hypergravity Only (7days) compared to ground control 1. β-Cat upregulated almost 3-fold 2. RUNX2 upreguated almost 4-fold 3. Osteocalcin upregulated almost 2-fold Hypergravity + Nanotextured Substrate (7 days) compared to ground control 1. β-Cat upregulated about 1.5-fold 2. RUNX2 upregulated almost 2.5-fold 3. Osteocalcin upregulated almost 3.5-fold *Day 1 have no significant difference compared to ground control
4	Nakaji-Hirabayashi et al.[19]	2022	Human MSC	5G	14 days	1. Increasing of ALP (significant upregulated ALP is in the day 14) 2. Increasing of Ribonucleic Acid (RNA) expression of Receptor Activator of Nuclear Factor-κB-ligand (RANKL) (about 3x higher) and OPG (about 4x higher) 3. Decreasing of MSC markers of CD44 and CD105 4. Increasing of Calcium deposition (almost 4 times higher than sample control)
5	Lingens et al.[20]	2022	Human MSC	10G until 40G	12 days	Hypergravity enhance Ca+ content : 10G increase 115% Ca+ content 20G increase 122% Ca+ content 30G increase 123% Ca+ content 40G increase 107% Ca+ content

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DISCUSSION

Five relevant articles were included in this research. These studies utilized human and rat mesenchymal stem cells (MSCs). All MSCs were subjected to hypergravity using centrifugation devices, with each study employing a different magnitude of gravity. One study compared the effects of hypergravity on MSCs with and without an osteogenic inducer. The outcomes were evaluated by assessing the expression of osteogenic differentiation markers.

The initial steps in bone tissue engineering involve harvesting MSCs from various tissues, such as bone marrow, umbilical cord, and endometrium. After harvesting, the MSCs are assessed for their potential to differentiate into osteoblasts by examining their expression of osteogenic differentiation markers.[21-24] Osteogenic differentiation is typically measured by assessing the expression of specific markers, such as RUNX2, ALP, BSP, OPN, OCN, β-catenin, COL1A1, and OSX.[5,14,21,22] The results demonstrated increased expression of osteogenic markers and calcium deposition. As noted by Graneli et al., RUNX2 is a key transcription factor that acts as a master regulator of osteogenic differentiation.[5,23,24] Other articles also said that there are early and late differentiation markers. Huang et al. said that ALP, BSP, and Type 1 collagen are early osteogenic differentiation markers, while PPR and OCN appear late.[14] Wang et al. identified ALP as a marker for the early stages of differentiation.[25] The study by Wan et al. showed that both ALP and OCN were strongly upregulated during the intermediate and late phases of differentiation.[26] Based on the articles reviewed, RUNX2 remains a major marker of osteogenic differentiation. ALP, strongly upregulated in early and intermediate differentiation, can

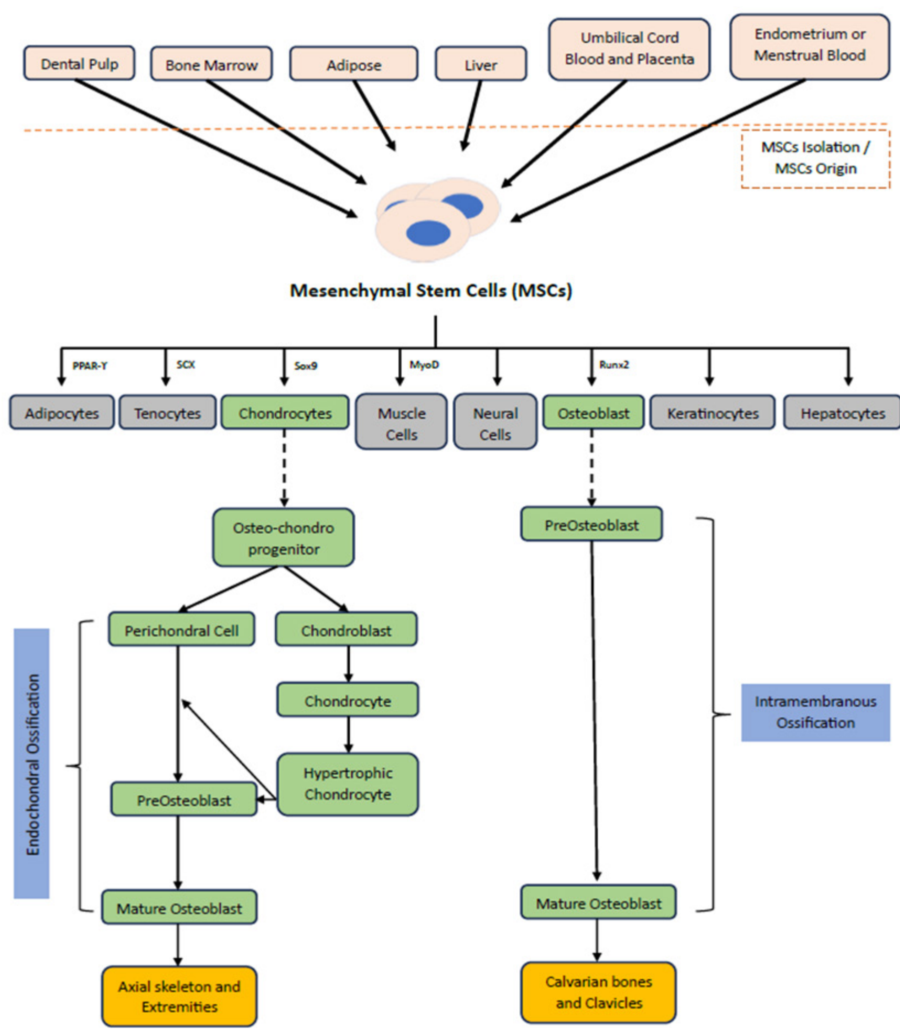

Figure 2. Flowchart of osteogenic differentiation.

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be a marker for short-duration cultures due to its prominent presence in these stages. Conversely, OCN, consistently expressed in late differentiation, may be a more suitable marker for long-duration cultures. Although different markers were measured in almost all samples, each osteogenic marker showed positive expression.

Figure 2 illustrates two pathways of osteogenic differentiation from MSCs: (1) intramembranous differentiation, where neural crest-derived mesenchymal cells directly differentiate into osteoblasts, and (2) endochondral ossification, where mesenchymal cells first differentiate into cartilage, which is later replaced by bone.[27,28,32] The ability to directly create bone cells with controllable shape and structure is a primary goal of bone tissue engineering, aiming to address various orthopedic problems.

The differentiation of MSCs into mature osteoblasts occurs in three stages, a process that takes at least 21 days. In the first stage, pre-osteoblasts proliferate and express fibronectin, collagen, Transforming Growth Factor-β (TGFβ) receptor 1, and OPN. The second stage involves the initiation of cell differentiation and extracellular matrix maturation, marked by the presence of ALP and collagen. The final stage, matrix mineralization, occurs when OCN enriches the organic scaffold.[5,33,34] Hypergravity enhanced both the expression of osteogenic markers and proliferation in stage 1 of osteoblast differentiation.[35] Enhanced proliferation in the initial stage of osteoblast differentiation may result in faster and more efficient bone tissue engineering.

Studies have shown that between day 0 and day 7, there is paradoxical activity between proliferation (stage 1) and matrix deposition (stage 2), followed by the beginning of matrix mineralization (stage 3) as proliferation ends. A similar paradoxical relationship exists between stage 2 and stage 3, with matrix deposition ending and matrix mineralization optimizing by day 21. This demonstrates that the differentiation process does not proceed in a strictly linear fashion. In contrast, human pluripotent stem cells (hPSCs) treated with hypergravity, a newer method, differentiate from a mesenchymal-like cell to an osteocyte. Osteocalcin is highly expressed late in differentiation, while type I collagen is highly expressed in the pre-osteoblast stage.[33,34,36] The results demonstrate positive expression of osteogenic markers. However, the magnitude of marker expression can be influenced by several factors, including culture duration, gravity, the use of inducers, pre-culture conditions, and marker detection methods. While some factors, such as osteogenic inducers and potentially gravity, may significantly affect marker expression, one experiment showed significant expression of osteogenic markers like RUNX2, type 1 collagen, and ALP even without the use of an inducer.

The optimal gravity level for osteogenic differentiation remains unclear. This study compared two samples exposed to the same gravity level with another sample exposed to a different level to determine the most effective magnitude for osteogenic differentiation. Regarding calcium deposition, a gravity level between 20G and 30G appears most promising. A comparison of 5G, 10G, 20G, and 40G on osteoblast cultures for post-translational collagen production revealed that 20G and 40G, after 72 hours of culture, showed significant differences compared to stationary conditions. Specifically, 20G and 40G induced higher collagen accumulation, increased ALP expression, and promoted proliferation compared to the stationary control. However, 40G showed greater effects for all three parameters compared to 20G.[37] Further research has demonstrated that short-duration (3 hours) hypergravity culture at 20G significantly upregulates Ras homolog gene family member A (RHOA), a marker of proliferating cells.[17] Research on adipose-derived stem cells treated with hypergravity (10G, 20G, 40G, and 60G) revealed that cell proliferation peaked at 40G (1.44 times higher than the ground control), fol-

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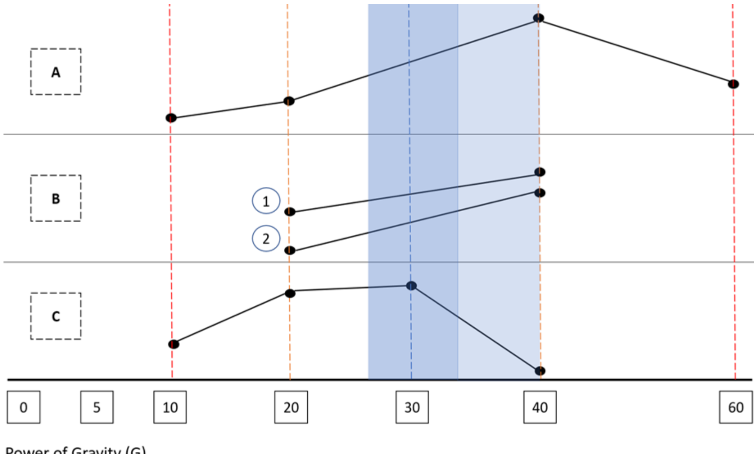

Figure 3. Impact of gravity on osteogenic differentiation. (A) Effect of hypergravity on cell proliferation in adipose-derived stem cells (ADSCs). (B) Effect of hypergravity on (1) cell proliferation and (2) ALP expression in osteoblasts. (C) Effect of hypergravity on calcium deposition.

lowed by 60G (1.40 times higher), 20G (1.26 times higher), and lastly 10G (1.13 times higher).[35]

Figure 3 combines data from three studies investigating the impact of varying gravity levels on osteogenic differentiation markers. Two of the studies indicate that marker expression increases most at 40G. However, calcium deposition was lowest at 40G compared to other gravity levels, with 30G showing the highest deposition. While other gravity levels also tend to increase marker expression, the effect is not as pronounced as with 40G.[20,35,37]

While hypergravity culture durations vary across studies, research suggests that specific durations may optimize osteogenic differentiation. For example, seven days of hypergravity culture at 2G and 10G significantly increased or optimized the expression of osteogenic markers (RUNX2, β-catenin, COL1A1, ALP, and OCN).[4,18] Another study demonstrated that ALP expression reached its peak on day 14 of culture under 5G gravity.[19] Based on these data, it appears that the optimal culture duration may be between 7 and 14 days. Two samples, cultured under different gravity levels (2G and 10G), showed optimal marker expression on day 7. This suggests that optimal expression may occur at a similar culture duration, regardless of the gravity level applied. However, further research is needed to confirm this hypothesis.

CONCLUSION

Mesenchymal stem cells possess the remarkable ability to differentiate into various cell types, depending on the treatment applied. Hypergravity has a significant impact on osteogenic differentiation. While the precise parameters for optimizing this process remain to be fully elucidated, the results presented here suggest that a gravity level of 30G and a culture duration of 7 to 14 days may be optimal, with shorter durations being preferable. Optimizing gravity and culture duration could potentially reduce or eliminate the need for osteogenic inducers. Indeed, hypergravity culture without inducers has been shown to enhance the expression of certain osteogenic differentiation markers.

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