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Reduced serum and hypoxic culture conditions enhance the osteogenic potential of human mesenchymal stem cells

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Abstract

Current protocols for inducing osteogenic differentiation in mesenchymal stem/stromal cells (MSCs) in culture for tissue engineering applications depend on the use of biochemical supplements. However, standard *in vitro* culture conditions expose cells to ambient oxygen concentrations and high levels of serum (21% O_2, 10% FBS) that do not accurately recapitulate the physiological milieu. While we and others have examined MSC behavior under hypoxia, the synergistic effect of low serum levels, such as those present in ischemic injury sites, on osteogenic differentiation has not been clearly examined. We hypothesized that a concomitant reduction of serum and O_2 would enhance *in vitro* osteogenic differentiation of MSCs by more accurately mimicking the fracture microenvironment. We show that serum deprivation, in conjunction with hypoxia, potentiates osteogenic differentiation in MSCs. These data demonstrate the role of serum levels in regulating osteogenesis and its importance in optimizing MSC differentiation strategies.

Keywords

Mesenchymal stem cell; Osteogenic differentiation; Serum deprivation; Hypoxia

1. Introduction

Bone marrow-derived mesenchymal stem/stromal cells (MSCs) have been intensely investigated for orthopaedic tissue engineering applications for over two decades due to their availability, proliferative capacity, and multi-lineage differentiation potential (1–5). These cells are especially well-adapted for treatments seeking to resolve nonunion bone defects because endogenous MSCs undergo osteogenic differentiation to participate directly in fracture repair and bone regeneration (6). To this end, *in vitro* biochemical supplements such as ascorbic acid, β-glycerophosphate, and dexamethasone are typically used to differentiate MSCs down the osteoblastic lineage (7, 8) and enhance the efficacy of subsequent *in vivo* delivery (9, 10). However, critical-sized defects and nonunion fractures

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Disclosures

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are poorly vascularized, resulting in an ischemic environment that is nutrient-deficient with oxygen levels as low as 0–3% (11, 12). Similarly, the MSC bone marrow niche is hypoxic, with an oxygen tension of less than 5% O_2 (13). Therefore, standard culture conditions utilizing media supplemented with 10% fetal bovine serum (FBS) and ambient air (21% O_2) (14) do not recapitulate the *in vivo* microenvironment, and phenotypic responses elicited under these conditions may deviate greatly from cellular behavior *in situ*.

We and others have shown that hypoxic culture has a significant effect on *in vitro* MSC behavior and osteogenic differentiation. For example, hypoxia-inducible factor 1-α (HIF-1α) is a transcription factor that is stabilized in the absence of oxygen and promotes cell survival and upregulates secretion of pro-angiogenic cytokines such as vascular endothelial growth factor (VEGF) (15, 16). Low oxygen tensions alone also stimulate proliferation and reduce senescence (17) while inhibiting osteogenesis in stromal cell populations (17–20). However, these studies are incomplete because they only take into account a single component of ischemia. Protocols targeting chondrogenic differentiation and maintenance of MSCs often call for the reduction or removal of FBS in order to mimic avascular cartilage (4, 14, 19), and induction media is typically supplemented with growth factors like transforming growth factor-β (TGF-β). However, the avascular cartilage niche contains levels of oxygen and nutrients that are lower than that of bone marrow (13) and may not be optimal for osteogenesis. The effects of simultaneous serum deprivation and hypoxia on MSC osteogenic differentiation have been poorly characterized and merit further study to develop improved protocols for *in vitro* differentiation prior to transplantation.

We hypothesized that combining serum reduction with hypoxia would more accurately simulate an ischemic defect site and enhance osteogenic differentiation of human MSCs compared to standard culture conditions. We investigated this hypothesis by incubating MSCs at varying oxygen tensions in media containing three different FBS concentrations, in the presence or absence of standard osteogenic supplements. We assessed osteogenic differentiation over time by measuring intracellular alkaline phosphatase (ALP) activity in cells exposed to each combination of stimuli. We also measured mineralization and calcium secretion of osteogenically-induced MSCs in serum-reduced and hypoxic conditions *in vitro* over the course of 3 weeks.

2. Materials and Methods

2.1 Cell culture

Human bone marrow-derived MSCs (Lonza, Walkersville, MD) were expanded without further characterization in growth medium (GM) composed of minimum essential alpha medium (α-MEM, Invitrogen, Carlsbad, CA), 10% FBS (JR Scientific, Woodland, CA) and 1% penicillin-streptomycin (P/S, Mediatech, Manassas, VA). Cells were expanded under standard culture conditions in a humidified incubator and used at passages 4–6. When described, osteogenic differentiation was induced by culturing cells in osteogenic medium (OM: GM supplemented with 10 mM β-glycerophosphate, 50 mg/mL ascorbate-2-phosphate, and 100 nM dexamethasone, Sigma-Aldrich, St. Louis, MO). Medium was replaced every 3 days.

To assess the effect of varying serum and oxygen levels on MSCs, cells were seeded in GM on 12-well tissue culture plates at 30,000 cells/cm^2 and allowed to attach overnight. After 24h, medium was refreshed with GM or OM containing 1, 5, or 10% FBS and incubated in Heracell 150i tri-gas incubators (Thermo Scientific, Waltham, MA) at 1, 5, or 21% oxygen (n = 4 for every combination).

2.2 Biochemical characterization of osteogenic differentiation

Intracellular alkaline phosphatase (ALP) activity was quantified in MSCs at 7, 14, and 21 days as previously described (21). Briefly, cells were rinsed in phosphate buffered saline (PBS, Invitrogen) and collected in passive lysis buffer (Promega, Madison, WI). ALP activity was measured using a *p*-nitrophenyl phosphate (PNPP) assay and normalized to DNA quantified by a Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen).

After rinsing cells with PBS, calcium was collected from the bottom of each well in 0.9N H_2SO_4 using a cell scraper and incubated overnight at 37°C. Secreted calcium was quantified by reacting with *o*-cresolphthalein complexone as previously described (22) and assessed qualitatively using an Alizarin red stain (18).

2.3 Statistical analysis

Data are presented as mean ± standard error unless otherwise stated. Statistical analysis was performed using paired Student's *t*-tests and one-way ANOVA with Tukey's Multiple Comparison *post*-test where applicable. *P* values less than 0.05 were considered statistically significant.

3. Results

3.1 Serum reduction increases intracellular ALP activity in undifferentiated MSCs

At 7 days, MSCs cultured in GM containing 5% FBS exhibit trends for higher ALP activity compared to cells grown in 10% serum in both 1% and 5% O_2 (Fig. 1A–B). In 21% O_2, MSCs exposed to 5% serum have significantly higher ALP levels (Fig. 1C) and maintain the highest activity through 21 days of culture, although statistical significance was not achieved compared to 10% FBS at 3 weeks (Fig. 1F, I). Similarly, undifferentiated MSCs in 5% O_2 have significantly higher ALP activity when supplemented with 5% FBS for 2 weeks compared to cells in full serum (Fig. 1E). After 21 days, cells in 5% serum have ALP activity that is greater than or equal to cells in standard media with 10% FBS at all oxygen tensions (Fig. 1G–I). MSCs in 1% FBS GM had the highest ALP levels compared to both other serum concentrations in 5% O_2 at all time points (Fig. 1B, E, H).

3.2 Serum deprivation potentiates the effect of osteogenic supplements on MSCs

As expected, MSCs cultured in the presence of standard osteogenic supplements had significantly higher ALP activity compared to cells in GM under every condition (Fig. 2). After 7 days, MSCs in 1% and 5% FBS had higher ALP levels than those in 10% serum under all oxygen tensions except 1% O_2, where the 5% FBS group did not achieve statistical significance (Fig. 2A–C). Cells in reduced serum also expressed more ALP after 14 days in 5% O_2 (Fig. 2E). After 3 weeks, MSCs in 5% O_2 and OM containing 1% and 5% serum

again had higher intracellular ALP compared to those cultured in 10% FBS (Fig. 2H). Similarly, cells in ambient air had significantly elevated ALP levels when supplemented with 5% FBS (Fig. 2I). Over the course of 21 days, MSCs cultured in 1% O_2 and OM with 1% or 5% serum experienced significant cell loss and lack of proliferation *(data not shown)* and exhibited significantly lower osteogenic response (Fig. 2D, G). To avoid confounding results due to apoptosis and drastically lower cell number, 1% O_2 conditions were not investigated in subsequent experiments.

3.3 MSC calcium secretion and matrix mineralization is enhanced in low serum

Compared to cells induced in OM containing full serum under 5% O_2, MSCs cultured in 5% FBS under 5% O_2 deposited significantly more mineral at 14 days and comparable amounts after 21 days as visualized by Alizarin red staining (Fig. 3A). Chemical quantification of calcium secretion after 3 weeks confirms that OM supplemented with 5% FBS results in the highest amount of mineralization at 5% O_2 (Fig. 3B). Under atmospheric oxygen tension, calcium staining was slightly stronger in conventional OM at 14 days, but differences were not discernable by 21 days (Fig. 3C). In fact, quantification reveals that MSCs differentiated in 5% FBS produced twice as much calcium by the end of the study compared to those cultured in full serum (Fig. 3D).

4. Discussion

In vitro osteogenic differentiation protocols are commonly used in an effort to enhance the therapeutic effectiveness of MSCs delivered to nonunion and critical-sized bone defects. Osteogenic induction prior to implantation encourages MSCs to participate directly in bone repair by secreting and calcifying a collagen matrix (1, 3), thus improving functional *in vivo* outcomes (23). Standard osteogenic media supplements include ascorbic acid to promote extracellular matrix production, β-glycerophosphate as a source of phosphate ions for mineralization, and dexamethasone to stimulate transcription of osteoblast genes such as bone sialoprotein (7, 8, 14). However, these supplements are not found *in situ*, and MSCs dedifferentiate and regain their multipotency with the withdrawal of these cues in culture (24). More broadly, conventional cell culture conditions call for supplementation of basal media with 10% FBS and incubation in ambient 21% O_2 to promote proliferation. These parameters are not physiologically relevant, especially compared to ischemic fractures and defects with little nutrient supply and oxygen tension below 1% (11, 25).

In an effort to more accurately recapitulate *in vivo* conditions, many groups have investigated the effect of hypoxia on MSC behavior in both 2D and 3D. Compared to 21% O_2, hypoxic culture inhibits *in vitro* osteogenic differentiation of adipose-derived stromal cells (18), MIAMI cells (17), and MSCs (20, 26) while upregulating trophic factor production (18, 27). Nonetheless, the results of these studies may be misleading because they fail to consider the concomitant lack of serum that accompanies hypoxia in poorly vascularized tissue. Indeed, serum deprivation alone selects for bone marrow MSCs with a more pluripotent phenotype (28) and enhances their pro-angiogenic capacity (29). Due to the avascular nature of cartilage, serum-free and serum-reduced cultures have been investigated in the expansion and maintenance of chondrocytes (19, 30), but the collective effect of

hypoxia and serum deprivation on MSC osteogenic differentiation has not been clearly reported. Therefore, we specifically examined the effect of multiple degrees of serum removal (1,5, 10% FBS) and oxygen levels (1,5, 21% O_2) on biochemical markers of MSC osteogenesis over 3 weeks.

Even in the absence of inductive cues, MSCs in GM had elevated levels of intracellular ALP in low-serum cultures (Fig. 1). In 5% O_2, ALP was consistently highest at 1% and lowest at 10% FBS over the course of 21 days (Fig. 1B, E, H). At ambient oxygen tension, media with 5% serum resulted in the highest cellular production of ALP at all time points (Fig. 1C, F, I). With the addition of osteogenic supplements, MSC ALP activity increased by at least an order of magnitude compared to cells in GM (Fig. 2), consistent with our previous findings (21). Also in agreement with our earlier work, MSCs in full serum exhibited reduced ALP activity when cultured in hypoxia. However, cells induced with media containing 1% and 5% FBS exhibited equal or higher levels of ALP compared to standard media under every oxygen tension and at every time point. These data demonstrating osteogenic differentiation in both the presence and absence of osteogenic supplements highlight the significant, complex interplay between serum and oxygen levels in governing cellular response, a phenomenon that merits further investigation.

In order to characterize functional *in vitro* osteogenic differentiation, we qualitatively and quantitatively assessed calcium deposition by osteogenically induced MSCs. As expected, cells conditioned in OM with 10% FBS secreted a calcium-rich matrix by 3 weeks as evidenced by heavy Alizarin red staining. MSCs in 5% serum under 5% O_2 had noticeably stronger staining by 14 days and comparable staining by 21 days (Fig. 3A). Although MSCs in reduced serum did not mineralize their matrix as rapidly in ambient oxygen, the extent of calcium staining in 5% and 10% FBS was indistinguishable by 21 days (Fig. 3C). Biochemical quantification of calcium at 3 weeks confirmed that MSCs produce the most mineral in OM supplemented with 5% FBS regardless of oxygen tension. Notably, the 5% serum and 5% O_2 condition elicited the strongest osteogenic response from MSCs, surpassing calcium secretion by cells in 21% O_2.

These data clearly demonstrate that serum content and oxygen tension synergistically affect MSC differentiation and have significant implications for *in vitro* protocols for osteogenic induction. For example, many groups seek to increase the therapeutic potential of MSCs by preconditioning them under hypoxic environments prior to *in vivo* injection. This strategy upregulates pro-survival and angiogenic markers (31, 32), increases Wnt signaling (33), and improves *in vivo* osteogenesis (34). Given our findings, modulation of serum levels in these conditioning regimens could significantly enhance their efficacy and merits additional study.

Our results underscore the complexity of cellular response to environmental cues and the importance of considering multiple parameters when optimizing culture conditions. In this case, hypoxia alone is not sufficiently representative of an *in vivo* milieu – future studies seeking to reproduce a physiological environment *in vitro* must consider both FBS and oxygen content. From a practical standpoint, we have shown that a superior level of osteogenic differentiation can be achieved simply by halving the amount of costly FBS in

culture medium. This in turn will allow for more cost-effective expansion and conditioning of larger numbers of MSCs for use in large-scale therapeutic applications.

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Highlights

- Serum levels, in addition to hypoxia, have a significant effect on MSC osteogenic differentiation.
- Both naïve and osteogenically induced MSCs exhibit higher osteogenic markers in reduced serum.
- MSCs deposit the most calcium under 5% O_2 in osteogenic media supplemented with 5% FBS.
- Standard culture conditions (21% O_2, 10% FBS) may not be optimal for MSC osteogenic differentiation.

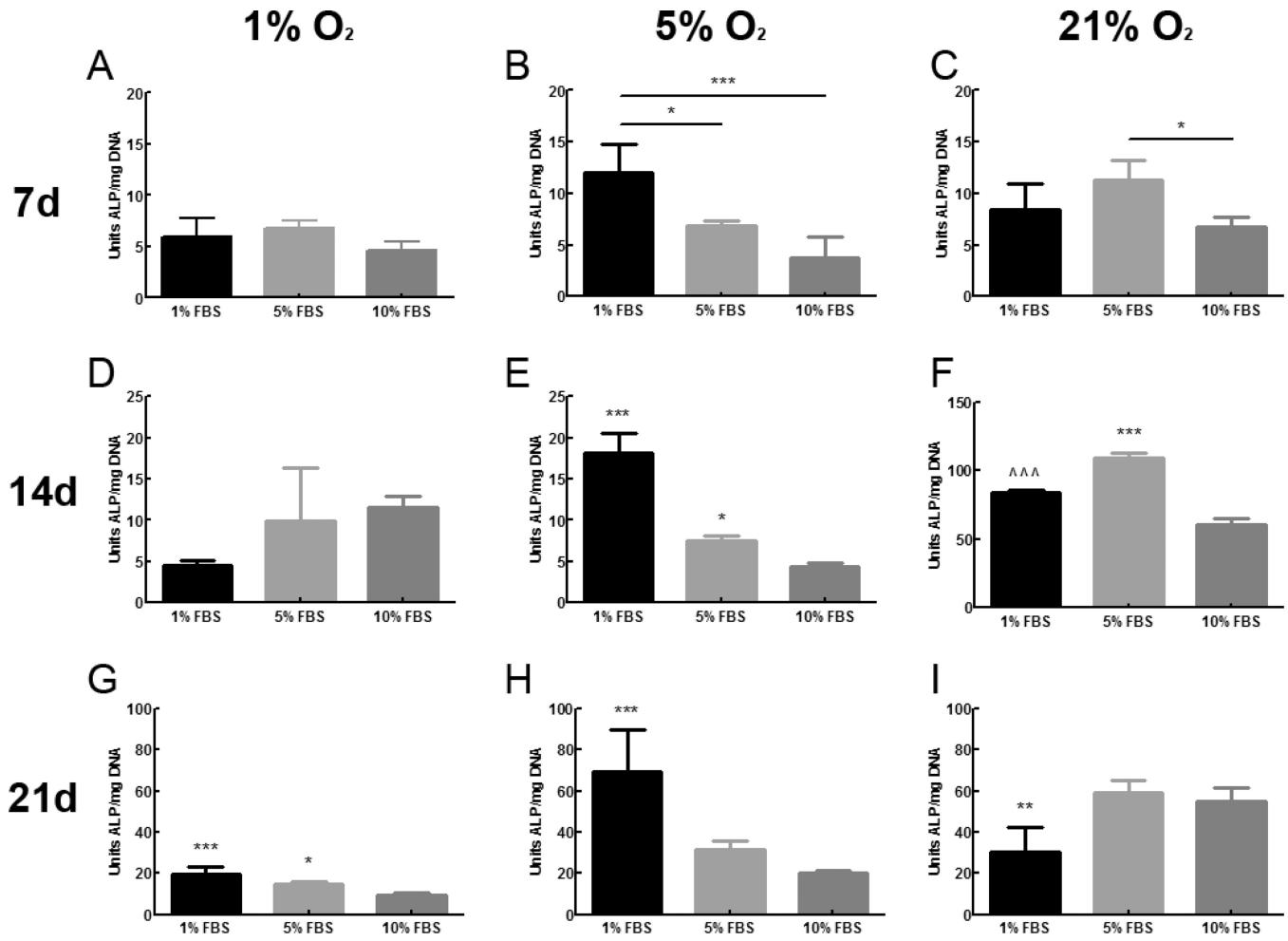

Fig. 1.

MSCs cultured in GM express higher levels of intracellular ALP in medium containing reduced serum levels compared to media with 10% FBS. (*A–C*) At 7 days, (*B*) MSCs in 5% O_2 have significantly higher ALP expression in GM supplemented with 1% FBS, while (*C*) cells in ambient oxygen have the highest ALP in 5% serum. Similarly, (*D–F*) at 14 days, GM containing either 1% or 5% serum produced significantly more ALP than cells in 10% FBS at both (*E*) 5% O_2 and (*F*) 21% O_2 tension. (*G–I*) After 3 weeks, MSCs in (*G*) 1% and (*H*) 5% O_2 had highest ALP content in medium containing 1% FBS, with trends for higher ALP in 5% FBS at (*H*) 5% O_2 and (*I*) ambient air. *p<0.05 vs 10% FBS, ***p<0.001 vs 10% FBS, ^^^p<0.001 vs 5% and 10% FBS (n = 4).

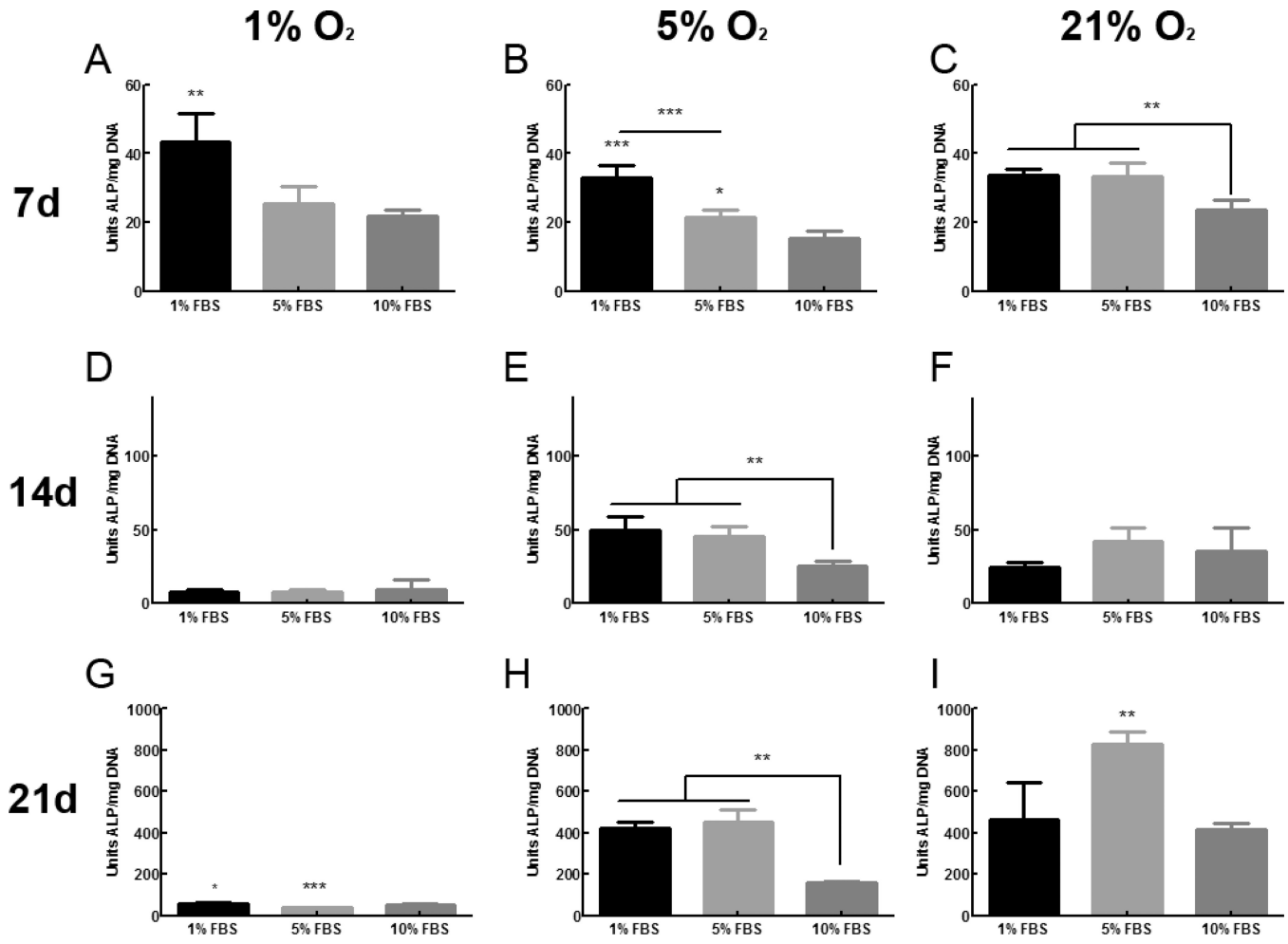

Fig. 2.

MSCs cultured in OM express equal or higher levels of intracellular ALP in medium containing lower serum levels compared to cells in 10% FBS at all oxygen tensions. (*A–C*) At 7 days, (*A*) MSCs in 1% O_2 have highest ALP expression in OM supplemented with 1% FBS. (*B*) Cells in 5% and (*C*) ambient oxygen have higher ALP in both 1% and 5% serum compared to 10%. Similarly, (*D–F*) at 14 days, (*E*) OM containing either 1% or 5% serum produced significantly more ALP than cells in 10% FBS at 5% O_2. (*G–I*) After 3 weeks, MSCs in (*G*) 1% had highest ALP content in medium containing 1% FBS, with the highest ALP in 5% FBS at (*H*) 5% O_2 and (*I*) ambient air. *p<0.05 vs 10% FBS, **p<0.01 vs 10% FBS, ***p<0.001 vs 10% FBS (n = 4).

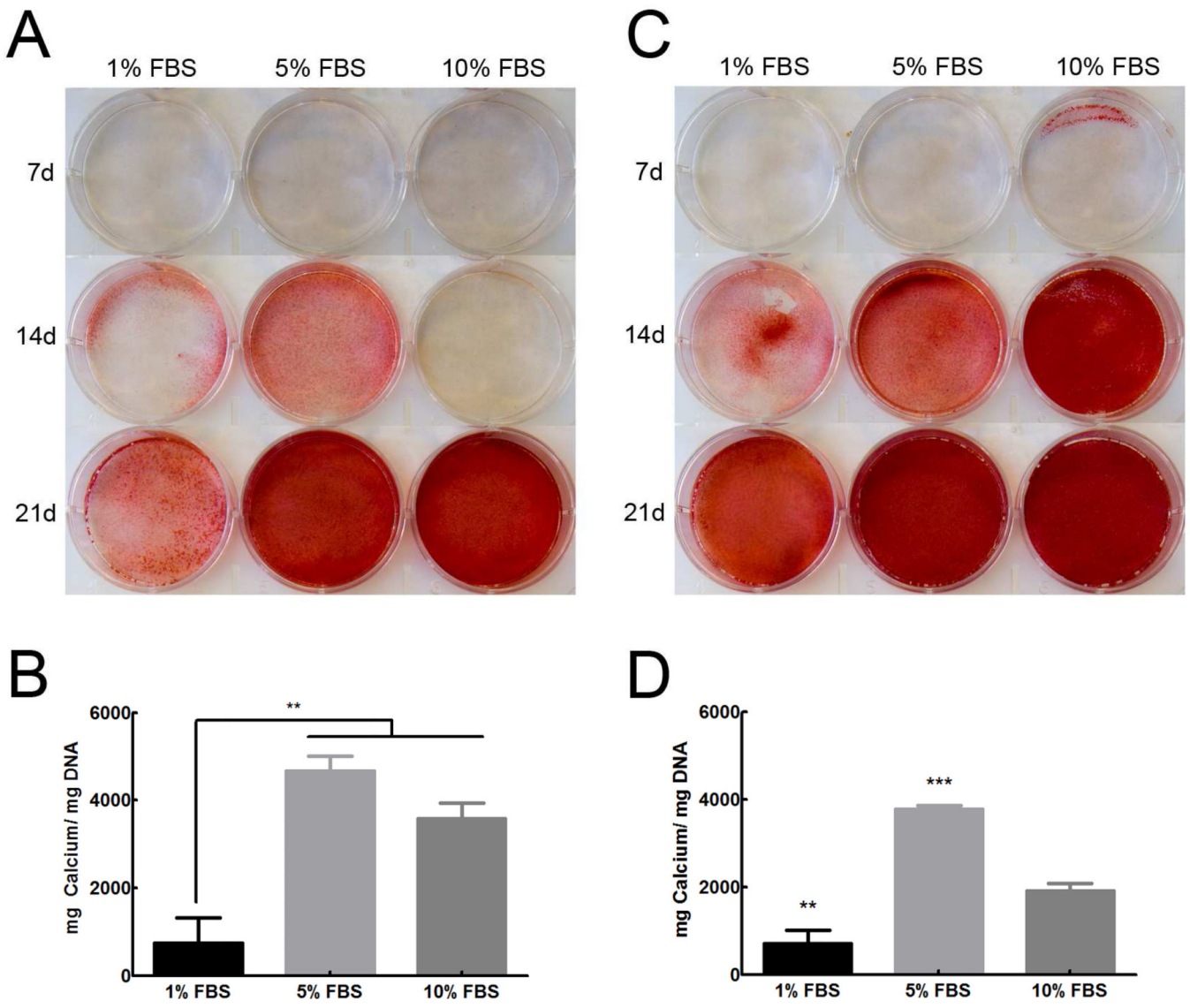

Fig. 3.

OM-induced MSCs secrete more calcium under serum- and oxygen-reduced conditions compared to standard culture conditions. (*A*) Alizarin red staining reveals that MSCs cultured at 5% O_2 and 5% FBS deposit the most total calcium at 14 and 21 days. These visual assessments are confirmed by (*B*) chemical quantification of samples at 21 days. (*C*) In 21% O_2, MSCs deposit the most calcium in OM supplemented with 10% FBS at 14 days, but cells grown in 5% serum deposit a higher amount by 21 days. (*D*) Calcium quantification reveals that cells grown in 5% FBS secrete twice as much calcium at 21 days. $^{**}p<0.01$ vs 10% FBS, $^{***}p<0.001$ vs. 1% and 10% FBS ($n = 4$).