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Engineering bone with mesenchymal stem cells: challenges and obstacles

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Abstract: Repair and reconstruction of large bone defects remain a significant challenge. Cell construct, containing mesenchymal stem cells (MSCs) and scaffold, is a promising strategy for addressing and treating major orthopaedic clinical conditions. However, the design of an ideal cell construct for engineering bone faces two critical challenges (i) matching the scaffold degradation rate to that of new bone formation and (ii) preventing the massive cell death post-implantation (caused by disruption of oxygen and nutrient supply). We will hereby primarily focus on the challenge of survival of MSCs post-implantation. Increasing evidence indicates that metabolic regulation plays a critical role in cell fate and functions. In cell metabolism, glucose is considered the major metabolic substrate to produce ATP via glycolysis when the availability of oxygen is limited. In this paper, we delineate the essential roles of glucose on MSC survival. We aim to provide a different perspective which highlights the importance of considering glucose in the development of tissue engineering strategies in order to improve the efficiency of MSC-based cell constructs in the repair of large bone defects.

Introduction:

The repair of large bone defects still represents a significant challenge for orthopaedic, reconstructive and maxillofacial surgeon^{1,2}. Current surgical techniques for the repair of bone defects utilize autogenous, allogeneic, and prosthetic materials to promote bone tissue regeneration. Autologous bone graft is the gold standard treatment; however, it is limited in the amount of bone tissue which can be harvested, posing a problem when the defect is large³. In addition, the required additional surgical procedure for bone graft harvest gives rise to a wide range of donor site co-morbidities, including infection, hematoma, fracture, nerve and vascular injuries, and chronic donor site pain^{4,5}. Therefore, new approaches to bone regeneration that would maximize the benefit and reduce risk of complications are needed. In this context, cell constructs have a promising role in addressing the aforementioned current limitations due to the technical advances in the field of biomaterials and stem cell biology^{6,7}.

Mesenchymal stem cells (MSCs), also referred to as multipotent stromal cells, are a small non-hematopoietic subpopulation of bone marrow derived cells. They are characterized by their expression of surface markers CD73, CD90, and CD105, and their lack of expression of surface markers CD34, CD45, HLA-DR, or CD11b, and CD19^{8,9}. MSCs were first studied for their role in the proliferation of hematopoietic stem cells¹⁰, yet now they are recognized for their therapeutic potential in tissue engineering applications. MSCs possess the ability to differentiate into adipocytes, chondrocytes and osteoblasts, as well as to induce a regenerative niche by secreting biologically active molecules that inhibit tissue scarring, apoptosis and inflammation; promote

angiogenesis; and stimulate mitosis of tissue-intrinsic stem or progenitor cells¹¹.

During the bone repair processes, scaffolds act as a temporary supporting structure, which (a) ensures that the defect space is suitable for bone tissue growth, maturation and remodeling; (b) provides mechanical functionality for proper transfer of loads acting on scaffolds to the adjacent host tissues while the tissue regenerates; and (c) facilitates in-growth of tissue regeneration¹². Previous studies have shown that MSCs loaded into calcium-based scaffolds actively contribute to the bone repair process in small animal models (for review)^{13,14}. The clinical applicability of such cell constructs was further evaluated in critical-size bone defects in large animal (dog and sheep) models; promising results were obtained in the repair of long, segmental bone defects in load-bearing situations using MSC-loaded ceramics (such as hydroxyapatite (HA)-tricalcium phosphate¹⁵, porous HA¹⁶, coralline HA¹⁷ and calcium carbonate¹⁸⁻²⁰). However, in these studies, the degradation of the material was a limiting factor, hindering new bone tissue from occupying the entire defect space and complete remodeling of the defect site. Temporal scaffold degradation is, thus, a key factor in bone tissue regeneration. It remains a challenge to optimize the degradation rate of ideal biological scaffolds to match the new bone formation rate while still providing mechanical support²¹.

Furthermore, the overall therapeutic effectiveness of the MSC-containing ceramic scaffolds do not match the osteogenic capability and clinical success of the gold standard, autologous bone grafts¹⁹. The underlying reasons for the limited osteogenic potential of cell constructs are not yet fully understood but *in vivo*

massive death of transplanted cells after engraftment into cell constructs is a major, and serious, problem. Specifically, massive cell death was observed *in vivo* when MSCs, loaded into scaffolds, were implanted subcutaneously²²⁻²⁴, in mouse calvarial defects²⁵ and in femoral segmental bone defects²⁴. This massive cell death post-implantation is a general phenomenon in tissue engineering and as such represents a bottleneck in the use of MSCs in regenerative medicine. Thus, post-implantation massive cell death was also observed when using a single cell suspension of MSCs for the treatment of either cardiac ischemia in mice (reviewed in)²⁶ or acute kidney injury in rats²⁷.

Metabolic regulation plays a critical role in cell fate and functions²⁸⁻³⁰ and it is particularly important post-implantation as MSCs experience ischemia (lack of oxygen and nutrients), and oxidative stress at the delivery site^{22,31,32}. This adverse microenvironment affects, in all likelihood, their metabolism. In fact, the primary metabolic consequences of ischemia include the disruption of mitochondrial function and aerobic ATP production and the activation of anaerobic glycolysis and lactate production^{33,34}. In this scenario, glucose is considered as the major metabolic fuel for ATP production via glycolysis. Since vital cell processes are fueled through the metabolism of energy substrates supplied by the environment^{35,36}, the severely compromised MSC metabolism at transplantation sites may lead to cell death.

Both oxygen and glucose are needed to allow realignment of metabolic activity and engagement in regenerative activity of human MSCs (hMSCs), specifically survival, proliferation and differentiation. The critical roles of oxygen in stem cell biology have been reviewed comprehensively in the past³⁷. This paper aims to delineate the essential roles of glucose on MSCs survival. We hope this paper can provide a different perspective on recognizing the importance of glucose in the development and optimization of MSC-based constructs that aim to repair and regenerate large bone defects.

Metabolism of glucose in MSCs:

Carbohydrates, organic molecules composed of carbon, hydrogen, and oxygen atoms, are central to many essential metabolic pathways. The family of carbohydrates includes both simple and complex sugars. Glucose, a simple sugar with the molecular formula C₆H₁₂O₆, is the most important carbohydrate with respect to cell metabolism. In fact, glucose is metabolized by nearly all known organisms and releases energy in the form of the high-energy molecules adenosine triphosphate (ATP) for later use in cellular processes. MSCs metabolize glucose and produce ATP by two main pathways: glycolysis and oxidative phosphorylation (OXPHOS). In glycolysis, a glucose molecule is broken down into two molecules of pyruvate and ATP; then pyruvate is either converted into lactate or, in the presence of sufficient oxygen, further oxidized in mitochondria to acetyl-CoA for the tricarboxylic acid (TCA)³⁶. In OXPHOS, electrons of the TCA cycle are transferred through the electron transport chain (ETC) within the inner mitochondrial membrane and produce ATP molecules^{35,38}. Although, OXPHOS produces a significantly higher amount of ATP than glycolysis (specifically, 34-36 vs 2 ATP molecules per glucose molecule), MSC survival is not dependent on OXPHOS but rather on glycolysis. Thus, rat MSCs cultured with 2,4-dinitrophenol (DNP), a mitochondrial uncoupler that inhibits OXPHOS, displayed no increase in cell death up to 72

hours post-DNP treatment, whereas addition of 2-deoxyglucose, an analog of glucose that inhibits glycolysis, induced a significant decrease in cell viability as early as 24 hours after treatment under normoxic conditions³⁹. Moreover, in near-anoxia (0.1% pO₂), hMSCs treated with Antimycin A or Malonate (TCA cycle inhibitors) does not further impair the MSC metabolism nor does it significantly increase cell death⁴⁰. Altogether, these findings indicate that metabolism of glucose via glycolysis is crucial to MSC survival.

Effects of glucose on MSC survival under near-anoxia

In order to better understand the effect of post-implantation ischemia and design a “niche” that reduces MSC sensitivity to ischemia, Deschepper *and al.* established an *in vitro* model and investigated the respective roles of glucose deprivation and continuous near anoxia (the two major insults associated with ischemia) on MSC viability⁴¹. In this model, MSCs were exposed to severe and continuous near-anoxia (pO₂ ≤ 0.2%) with or

without glucose shortage. Without glucose, no viable cells were observed at day 3 of culture, whereas, with glucose, viable cells were observed up to day 12 of culture⁴¹. These findings challenge the current paradigm that the massive cell death observed upon implantation is ascribed to near-anoxia *per se* and provide clear evidence that MSCs can withstand exposure to severe, continuous near anoxia provided that a glucose supply is available. From a tissue engineering perspective, these results suggest that MSCs loaded into glucose-enriched material scaffolds can overcome an episode of long-term, severe near-anoxia and may remain functional with respect to new tissue formation. These results, which have been initially established with sheep MSCs, are now confirmed and published with hMSCs over a period of up to 21 days *in vitro*^{40,42}. Most importantly, in an ectopic mice model, transplantation of hMSCs in constructs enriched with glucose resulted in a four-to-five-fold enhancement of hMSC survival when compared to the transplantation of hMSCs in constructs without glucose at 14 days post-implantation⁴². Taken together, these data establish that glucose is an essential and missing piece to the puzzle of MSC survival in cell constructs post-implantation.

The pro-survival effect of glucose on hMSCs was further reinforced by studying the potential benefits of alternative energy substrates (specifically, glutamine, serine and pyruvate) on hMSC survival. Moya *et al.* confirmed experimentally that hMSCs cultured under 0.1% pO₂ were unable to use exogenous glutamine and serine to produce ATP, and exhibited the similar survival rate to hMSCs cultured in the absence of glucose. In contrast, hMSCs are able to produce ATP by metabolizing pyruvate into lactate, but while viability of hMSCs remained high at more than 60% at day 7, it sharply decreased at day 14⁴⁰. Most importantly, the analyses of gene expression profiles of metabolic enzymes revealed, for the first time, that glycolysis (not pentose phosphate, TCA, or glycogenolysis) is the only metabolic pathway involved in hMSC survival under near-anoxia. These results were further confirmed by addition of inhibitors of glycolysis (either 2DG or NaOx) which induced a drastic decrease in cell viability. In fact, there were no viable cells after 3 days of culture, suggesting a pivotal role of glycolysis for ensuring hMSC survival in near-anoxia⁴⁰. Altogether, these findings indicate that glucose plays a paramount role on MSC survival by supplying ATP via glycolysis.

It is noteworthy that, apart from ATP depletion, inhibition of glycolysis as a result of glucose deprivation also induces the loss of crucial anti-apoptotic signals such as Akt^{36,43,44}. Moreover, decreased glucose metabolism due to glucose deprivation represses the generation of many biosynthetic precursor molecules, including nucleic acids, fatty acids, and acetyl-CoA for acetylation⁴⁵; as well as inhibits proper glycosylation and protein folding in the endoplasmic reticulum⁴⁶. There is also evidence that glucose deprivation as a metabolic stress activates the cellular process autophagy as a protective mechanism to mitigate damage and provide nutrients for short-term survival^{47,48}.

Conclusion:

Undoubtedly, MSCs have great therapeutic potential in bone tissue engineering applications. However, massive MSC death upon transplantation is a major impediment that results in a low engraftment rate and subsequently therapeutic shortcomings and/or failure. Metabolic activity of MSCs, which is highly sensitive to environmental cues, plays critical roles in cell fate and functions. Recent studies have focused on the importance of glucose to the survival rate of transplanted MSCs by sustaining bio-energetic levels so that these cells better adjust to the hostile and injured microenvironment. In such cases, glucose-enriched scaffold is a promising approach to ensure the metabolic demands of transplanted MSC are met, thus improving their survival and their functions pertinent to bone tissue regeneration.

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Disclosure Statement

The authors declare no competing financial interests

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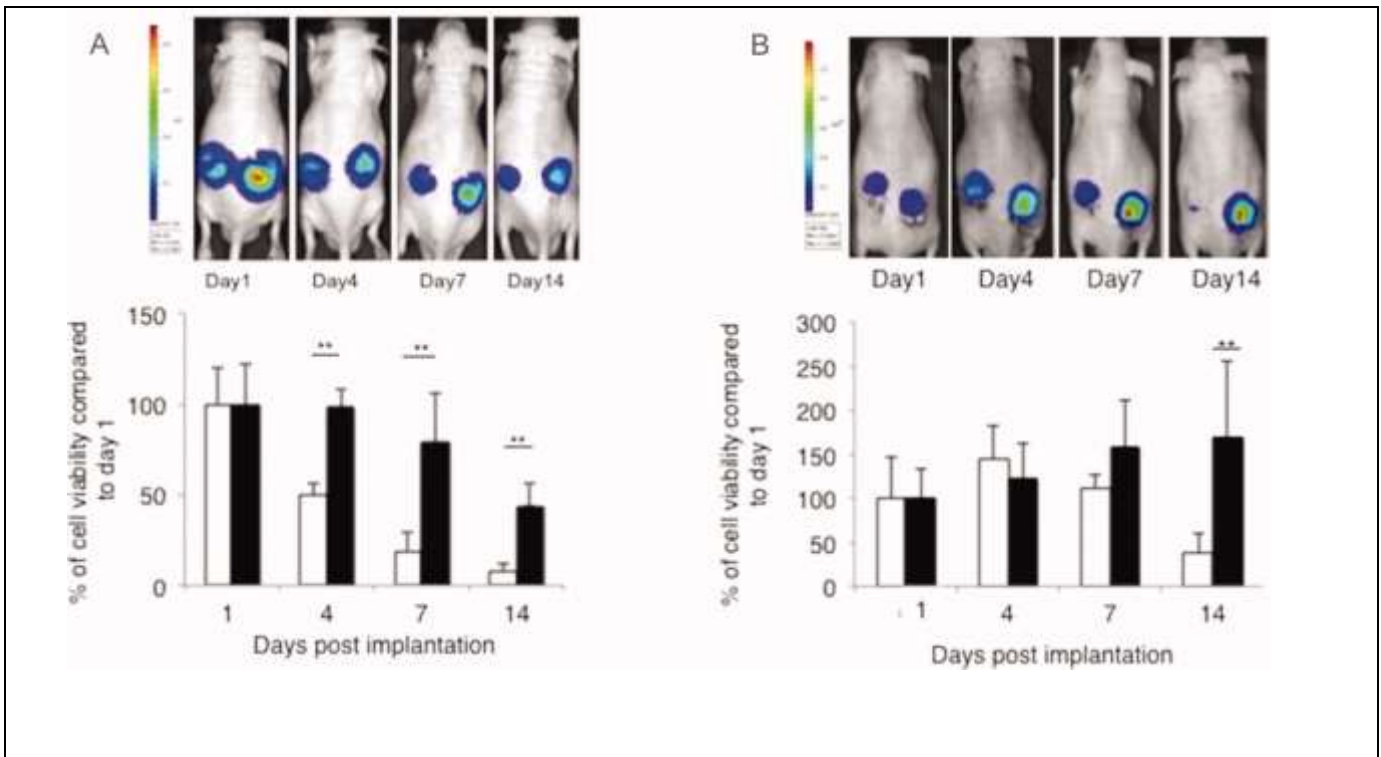


Figure 1: Glucose enhances in vivo cell survival in a 3D construct. (A): Imaging and viability quantification of hMSCs seeded in PASM scaffolds filled with fibrin gel (loaded [black bars] or not [white bars] with glucose). (B): Imaging and viability quantification of hMSC seeded in PASM scaffolds filled with hyaluronic acid (2%) loaded (black bars) or not (white bars) with glucose. A striking increase of cell viability was observed in cell constructs loaded with glucose independent of the type of hydrogel (i.e., hyaluronic acid or fibrin gel) used. At day 14, a four- to fivefold increase in cell numbers obtained for cell-containing constructs loaded with glucose when compared to the respective control (i.e., cell-containing constructs without glucose). Data are represented as mean \pm SD. *, $p < .05$; **, $p < .001$. This figure is reprinted with the permission from Deschepper *et al.*⁴². Copyright John Wiley & Sons, 2013.

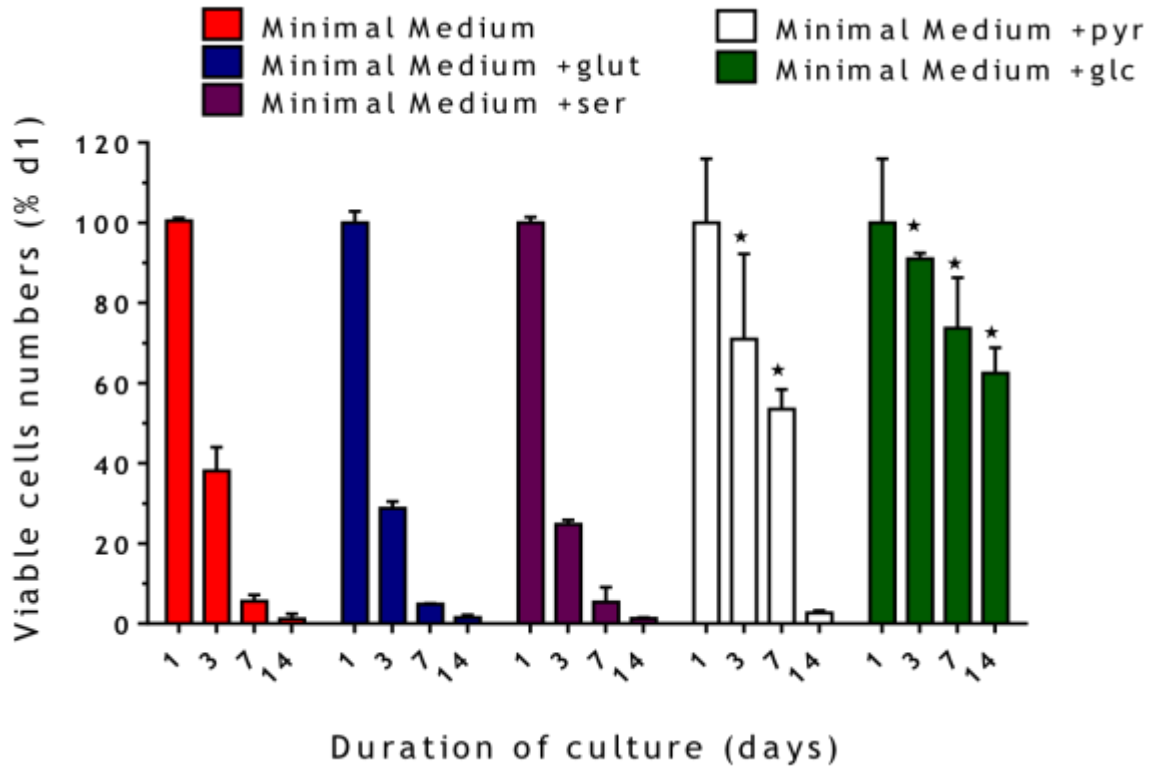


Figure 2: Extracellular glucose (glc) is necessary to ensure long-term survival of human mesenchymal stem cells (hMSCs) exposed to near-anoxia. Viability of hMSCs cultured under near-anoxia (0.1% O₂) in a minimal medium supplemented or not with either glutamine (glut), serine (ser), pyruvate (pyr), or glc at a final concentration of 5 g/l for up to 14 days ($n = 3$). Results are expressed as means \pm SD. *, $p \leq .05$ versus the minimal medium cell group (two-way analysis of variance followed by Bonferroni's post hoc test). This figure is reprinted with the permission from Moya *et al.*⁴⁰. Copyright John Wiley & Sons, 2018.