High glucose concentration in cell culture medium does not acutely affect human mesenchymal stem cell growth factor production or proliferation

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Submitted 29 October 2008; accepted in final form 21 April 2009

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High glucose concentration in cell culture medium does not acutely affect human mesenchymal stem cell growth factor production or proliferation. Am J Physiol Regul Integr Comp Physiol 296: R1735–R1743, 2009. First published April 22, 2009; doi:10.1152/ajpregu.90876.2008.—Optimizing the function and proliferative capacity of stem cells is essential to maximize their therapeutic benefits. High glucose concentrations are known to have detrimental effects on many cell types. We hypothesized that human mesenchymal stem cells (hMSCs) cultured in high-glucose-containing media would exhibit diminished proliferation and attenuated production of VEGF, hepatocyte growth factor (HGF), and FGF2 in response to treatment with TNF-α, LPS, or hypoxia. hMSCs were plated in medium containing low (5.5 mM) and high (20 mM or 30 mM) glucose concentrations and treated with TNF-α, LPS, or hypoxia. Supernatants were collected at 24 and 48 h and assayed via ELISA for VEGF, HGF, and FGF2. In addition, hMSCs were cultured on 96-well plates at the above glucose concentrations, and proliferation at 48 h was determined via bromo-2′-deoxy-uridine (BrdU) incorporation. At 24 and 48 h, TNF-α, LPS, and hypoxia-treated hMSCs produced significantly higher VEGF, HGF, and FGF2 compared with control. Hypoxia-induced VEGF production by hMSCs was the most pronounced change over baseline. At both 24 and 48 h, glucose concentration did not affect production of VEGF, HGF, or FGF2 by untreated hMSCs and those treated with TNF-α, LPS, or hypoxia. Proliferation of hMSCs as determined via BrdU incorporation was unaffected by glucose concentration of the media. Contrary to what has been observed with other cells, hMSCs may be resistant to the short-term effects of high glucose. Ongoing efforts to characterize and optimize ex vivo and in vivo conditions are critical if the therapeutic benefits of MSCs are to be maximized.

STEM CELL THERAPY HAS SHOWN great promise as a treatment modality for a wide variety of ischemia and inflammatory-mediated diseases (28, 31). More than a decade has now passed since Ferrari et al. (12) first reported that bone marrow stem cells transplanted into injured muscle could regenerate healthy muscle tissue. During this time, a significant amount of progress has been made in terms of characterizing the benefits, mechanisms, and limitations of stem cell-based therapies for human diseases, and several human trials have already been completed or are currently underway (3, 7, 34). It was initially proposed that the beneficial effects of stem cells would be owed to their ability to differentiate into a wide variety of cell types (i.e., pluripotency), which could then incorporate into diseased tissue, repopulate this area with healthy tissue, and thereby improve its function (2, 12, 29). Although this mechanism likely plays a role to some degree, evidence suggests that it is unlikely to provide a full explanation for the beneficial effects that can be attributed to stem cells. Specifically, the beneficial effects of stem cells have been consistently observed to occur acutely without sufficient time for growth, division, and production of new tissue; stem cells engraft at much lower rates than would be expected if they were able to differentiate and give rise to enough new tissue to account for the observed functional improvement; and, perhaps most importantly, many of the beneficial effects of stem cells can be replicated with cell-free conditioned media alone (14, 40, 43).

As such, we and others have hypothesized that stem cells may exert their beneficial effects at least in part via paracrine mechanisms (6, 33, 38, 43). Indeed we have demonstrated that human mesenchymal stem cells (hMSCs) are capable of producing large quantities of angiogenic, proliferative, anti-inflammatory, and anti-apoptotic factors in response to stressors such as TNF-α, LPS, and hypoxia (9, 39). Substances such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (FGF2), for example, are potent angiogenic and tissue-growth promoting factors secreted by stem cells that may play key roles in mediating the beneficial properties of stem cells. Our group recently provided further evidence for the critical role that VEGF plays in terms of stem cell function by demonstrating that MSCs in which VEGF has been ablated by siRNA, compared with untreated MSCs, offer significantly less cardioprotection in the rat hearts subjected to ischemia/reperfusion injury via the Langendorff isolated heart preparation (25). Clearly the paracrine functions of stem cells are a key component to their therapeutic effects.

Despite the observed benefits to their use in both in vitro and in vivo models of human disease, stem cells in many cases have offered only modest and short-lived benefit when tested in human clinical trials (30). Much of the underwhelming performance of stem cells observed to this point may be secondary to the fact that large numbers of transplanted cells fail to survive and proliferate in numbers substantial enough to exert an appreciable effect. It is not fully understood why stem cell survival is so poor following transplantation, but may be due in part to the oftentimes harsh conditions characterized by hypoxia and inflammation that stem cells must be forced to combat when used for human disease states (10, 27). Thus, a key component of stem cell research involves determining how these cells can best be expanded in culture, preconditioned, and modified to enhance both their survival and therapeutic capacity following transplantation.

Hyperglycemia is frequently encountered as part of many pathologic conditions including diabetes mellitus (DM), postinjury or postsurgical stress response, sepsis and the sys-
temic inflammatory response syndrome, and secondary to iatrogenically administered substances, such as total parenteral nutrition and corticosteroids. Chronic conditions characterized by hyperglycemia such as DM are often associated with progressively worsening micro- and macrovascular disease. Interestingly, investigators have reported that numbers and mobilization of endothelial progenitor cells (EPCs), minimally differentiated cells that play an important role in vasculogenesis, are diminished in diabetics, offering a potential mechanism by which vascular disease may develop in DM (13, 20, 23). Given its effect on EPCs, it is possible that the hyperglycemia associated with DM could affect the proliferation and function of hMSCs, as these cells share similar characteristics with one another. Studies have also confirmed that high glucose cell media induces senescence, decreases proliferation rates, and increases apoptosis in rat MSCs (37). Recent evidence also suggests that high glucose may suppress growth factor production by rat multipotent adult progenitor cells (rMAPCs), which are closely related to MSCs (22). The effect that high glucose concentrations may have on the proliferation and growth factor production of hMSCs, however, is unknown. We hypothesize that elevated glucose concentrations will attenuate hMSC proliferation and diminish their production of VEGF, HGF, and FGF2 in response to treatment with TNF-α, LPS, and hypoxia.

MATERIALS AND METHODS

hMSC. Poietics hMSC were purchased from Cambrex BioScience (Walkersville, MD). hMSCs are harvested and cultured from normal human bone marrow. Cells are tested by the manufacturer for purity by flow cytometry and for their ability to differentiate into osteogenic, chondrogenic, and adipogenic lineages. Cells are positive for CD105, CD166, CD29, and CD44; and negative for CD14, CD34, and CD45. Thawing of cells and initiation of culture process were performed according to the manufacturer’s instructions. hMSCs were plated in T-225 tissue culture flasks from Corning (Corning, NY) and cultured with mesenchymal stem cell basal medium purchased from Lonza (Basel, Switzerland) containing mesenchymal cell growth supplement (Lonza) and 1% penicillin-streptomycin at 37°C, 5% CO2, and 90% humidity. Per the manufacturer, glucose concentration of the mesenchymal stem cell basal medium is 5.5 mM. Medium was changed every 2–3 days.

Experimental groups. hMSCs were obtained between passages 6 and 7 at 80% confluence and plated in 12-well plates (Corning) at 0.1 × 10⁶ cells per well in 1 ml of mesenchymal stem cell basal medium containing either 5.5 mM glucose, 20 mM glucose, or 30 mM glucose. Cells were plated in triplicate at each of the preceding glucose concentrations among four groups: 1) control (untreated), 2) TNF-α treated (50 ng/ml), 3) LPS treated (100 ng/ml), and 4) hypoxia treated. All cells except for the hypoxia group were then incubated at 37°C, 5% CO2, and 90% humidity for 24 or 48 h. The hypoxia-treated cells were incubated in a Ruskinn Invivo2 300 hypoxia chamber (Pencoed, UK) at 37°C, 5% CO2, and 1% O2; for 24 or 48 h. Cell supernatants were collected from all plates for ELISA. Cell counts were performed in duplicate for each well so that concentrations of growth factors in supernatants as determined by ELISA could be normalized to the number of cells in each well. After trypsinization, a 20-ml aliquot of cell solution was mixed with an equivalent amount of trypan blue. Viable cells, determined by the ability to exclude trypan blue, were then counted via the aid of an automated cell counter (Nexcelom Bioscience, Lawrence, MA).

Proliferation. Proliferation was assessed by the use of the 5-Bromo-2’-deoxy-uridine Labeling and Detection Kit III (BrdU; Roche Applied Science, Indianapolis). Cells were plated at 10,000 cells/well on a 96-well culture plate (Corning) in 100 ml of mesenchymal stem cell basal medium containing either 5.5 mM glucose, 20 mM glucose, or 30 mM glucose (12 wells per group). Cells were then incubated at 37°C, 5% CO2, and 90% humidity for 48 h. The cells were then assayed for their ability to incorporate BrdU according to the manufacturer’s instructions. We also cultured LLC-PK1 renal tubular epithelial cells (RTECs), whose proliferation rates are known to be acutely reduced by high glucose concentrations, under identical conditions to serve as a positive control (1). In addition to being cultured at each of the three glucose concentrations, RTECs were also cultured in medium containing equimolar concentrations of mannitol to control for the effects of osmolarity.

ELISA for VEGF, HGF, and FGF2. Production of VEGF, HGF, and FGF2 by hMSCs was determined by ELISA of cell culture supernatants using commercially available kits (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. All samples were plated in duplicate. Concentrations were obtained in picograms per milliliter and subsequently expressed as pg/10⁶ cells, based on previously obtained cell counts.

Protein isolation and Western blot analysis. The JAK/STAT and p38 mitogen activated kinase (MAPK) signaling pathways may be involved in mediating growth factor production and proliferation of hMSCs (39, 41). Additionally, the expression of signaling molecules in these pathways in other cell types has been found to be affected by high glucose concentrations (20, 22). Thus, the STAT3 and p38 MAPK signaling molecules were analyzed by Western blot to determine whether or not their activity was altered by glucose concentration. hMSCs from all groups were collected in cold buffer containing 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na3VO4, 1 g/ml leupeptin, and 1 mM PMSF and centrifuged at 12,000 rpm for 10 min. Protein concentrations of each sample were determined via Bradford protocol using a biophotometer. Equal amounts of protein extracts were subjected to electrophoresis on a 12% Tris·HCl gel (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane. The membranes were incubated in 5% dry milk for 1 h and then incubated with the following primary antibodies: 1) STAT3, 2) p38 MAPK, 3) phosphorylated STAT3 (Tyr 705), and 4) phosphorylated p38 MAPK (Thr180/Tyr182) (all antibodies were obtained from Cell Signaling Technology, Beverly, MA). Membranes were subsequently incubated with secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and detected using SuperSignal West Pico stable peroxide solution (Pierce, Rockford, IL).

Presentation of data and statistical analysis. Values are expressed as means ± SE (n = 3 for ELISA, n = 12 for BrdU, and n = 2–3 for Western blots). Data were compared using a one-way ANOVA with Tukey’s post hoc test where appropriate. P < 0.05 was considered statistically significant.

RESULTS

Effects of TNF-α, LPS, and hypoxia on VEGF, HGF, and FGF2 production by hMSCs. At 24 h TNF-α, LPS, and hypoxia-treated hMSCs produced significantly higher VEGF, HGF, and FGF2 compared with control, except in the case of HGF production by hypoxia-treated hMSCs, which was less than control. Similar results were obtained for hMSCs treated for 48 h (Fig. 1). Hypoxia-induced VEGF production by hMSCs was the most pronounced change over baseline among all groups, with hypoxia-treated hMSCs producing approximately seven to eight times the amount of VEGF than untreated hMSCs.

Effect of glucose concentration on production of VEGF, HGF, and FGF2 by hMSCs. At both 24 and 48 h, glucose concentration did not affect production of VEGF, HGF, or FGF2 by MSCs in control, TNF-α, LPS, or hypoxia-treated groups (Figs. 2–5). No statistically significant differences in growth factor production in response to treatments were noted.
between the 5.5 mM, 20 mM, and 30 mM glucose concentrations. In most cases, an increasing trend in levels of growth factors occurred in the 48-h groups compared with 24-h; however, this did not reach statistical significance at any point.

Effect of glucose concentration on proliferation of hMSCs. Proliferation of hMSCs as determined via BrdU assay at 48 h was unaffected by glucose concentration in the media (Fig. 6A). Proliferation of RTECs, however, was significantly reduced at the 20 mM and 30 mM glucose concentrations (Fig. 6B). This same effect was not observed with equimolar concentrations of mannitol.

JAK/STAT and p38 MAPK signaling in hMSCs treated with high glucose. Stimulation of hMSCs with TNF-α and hypoxia induced STAT3 phosphorylation at 24 h (Fig. 7A). LPS-induced STAT3 phosphorylation was less pronounced. Phosphorylation of STAT3 at 48 h was attenuated in all stimuli groups (Fig. 7B). Stimulation of hMSCs with TNF-α and LPS induced p38 MAPK phosphorylation at 24 h (Fig. 8A). Hypoxia-induced p38 MAPK phosphorylation was less pronounced. Phosphorylation of p38 MAPK at 48 h was attenuated in the TNF-α and LPS-stimulated groups, but appeared to be enhanced in the hypoxia-stimulated group compared with 24 h (Fig. 8B). Glucose concentration had no effect on STAT3 or p38 MAPK activation.

DISCUSSION

Maximizing the beneficial properties of MSCs while overcoming barriers to their use are essential steps that must be taken to optimize the benefit of their use in the treatment of human disease. With this series of in vitro experiments we aimed to determine whether or not glucose concentration of the culture medium influenced the short-term ability of hMSCs to both proliferate and produce growth factors in response to a variety of stimuli. Contrary to our hypothesis, we determined that short-term exposure to high glucose does not affect the proliferative capacity of MSCs nor their ability to produce paracrine growth factors.
An abundance of evidence has been gathered to suggest that transplanted stem cells mediate many of their benefits to injured tissues within just minutes to hours (8). Many of these short-term protective effects may be secondary to these cells’ ability to secrete paracrine factors such as VEGF, HGF, and FGF2 among others. Each of these growth factors possesses potentially beneficial and protective properties for at-risk or injured tissue. VEGF and FGF2 are important factors involved in neoangiogenesis, proliferation and recruitment of endothelial cells, and wound healing. Their release from stem cells mediates several of the beneficial effects of these cells including protection of the myocardium against ischemia/reperfusion injury, improved tissue perfusion and reduced tissue infarct following myocardial and peripheral tissue ischemia, and protection from spinal cord damage and infarction following injury (17, 19, 25, 44). VEGF and FGF2 may also act in an autocrine fashion to mediate the survival of stem cells following transplantation, thereby optimizing their effectiveness (15). Additionally, HGF is an important factor involved in tissue healing that may also be released by stem cells in response to injurious stimuli. HGF can acutely inhibit cellular apoptosis and may play a role in activating endogenous, resident tissue-specific stem cells (33, 35). Evidence has thus shown that stem cell-derived HGF plays several important roles following cellular therapy, including limiting infarct size and preserving tissue function following myocardial or peripheral ischemia and preserving liver function following damage or loss to hepatic parenchyma (4, 45, 47). Although HGF is produced by MSCs, it appears to be in lesser quantities than other growth factors as was the case with this set of experiments. Thus, maximizing HGF expression by MSCs is an area of ongoing research (46).

The paracrine functions of stem cells are critical to these cells’ ability to protect against cellular injury and repair damaged tissue. Thus, research focused on preserving and maximizing stem cells’ ability to secrete beneficial growth factors is essential. Examples of how MSCs have been manipulated to optimize their benefit include alterations of ex vivo culture conditions, pretreatment with stressors such as hypoxia, via genetic or epigenetic modifications to block or enhance production of certain proteins, and by varying the methods and conditions of transplantation (11, 16, 24, 25, 32). Based on previous observations with other cell types, glucose concentration could conceivably affect stem cell function and proliferation and is a variable that can easily be manipulated to optimize both ex vivo expansion of stem cells and in vivo function and survival of these cells. This is particularly pertinent given the presence of hyperglycemia as a commonly encountered comorbidity along with many of the diseases in which stem cell transplantation may provide benefit.

Glucose concentrations for this experiment were chosen to reflect what is generally considered a normal serum glucose level as well as those that may be near the higher end of what may be observed clinically. Specifically, a glucose concentration of 5.5 mM is equivalent to ~99 mg/dl in terms of conventionally reported serum glucose levels, whereas concentrations of 20 mM and 30 mM are approximately equal to levels of 360 and 540 mg/dl, respectively. Glucose concentrations exceeding these values generally occur only in extreme circumstances and often produce their own acute, critical pathologic manifestations (diabetic ketoacidosis and nonketotic hyperosmolar coma, for example). As glucose concentrations relate to cell culture medium, 5.5 mM is considered a low glucose media, while 30 mM is considered high glucose media. Furthermore, media containing 5.5 mM glucose is currently what is most often used and recommended for maintaining hMSCs in culture (36). As such, our choice of glucose concentrations reflect what is considered normal and what is considered high both in terms of cell culture and serum glucose levels.

Despite our hypothesis, our experiments demonstrate that hMSC proliferation and growth factor production in response to glucose

Fig. 3. In TNF-α-treated hMSCs, glucose concentration did not affect levels of growth factor production at 24 or 48 h.

Fig. 4. In LPS-treated hMSCs, glucose concentration did not affect levels of growth factor production at 24 or 48 h.
to the chosen stimuli are unaffected by glucose concentration of the culture medium. The hypothesis was formulated chiefly upon data gathered from studies involving MSCs, rMAPCs, and EPCs indicating that high glucose concentrations can have detrimental effects on these cell types even in the short term. A small amount of data from studies of rat MSCs and rMAPCs, for instance, indicate that elevated glucose concentrations induce senescence, increase apoptosis, and attenuate basal levels of VEGF secretion in these cell types (22, 37). Similarly, a growing abundance of data indicates that diabetes and high glucose levels lead to diminished numbers and function of EPCs. As EPCs play an important role in vasculogenesis and vascular repair, their impairment by high glucose levels is thought to be a mechanism by which vascular pathology occurs at an accelerated rate in diabetic individuals. To the contrary, higher glucose concentrations have been shown to enhance the proliferation of embryonic stem cells grown in culture (18).

Clearly the effects of glucose are heavily dependent on the cell-type in question. Although the mechanisms by which glucose exerts its effects on cells are not fully understood, recent evidence has shown that glucose concentration influences a variety of cellular pathways and functions. Liu et al. (22) found, for example, that high glucose concentrations attenuated basal VEGF production by rMAPCs and that this attenuation was associated with inhibition of JAK/STAT signaling. Similarly, Kuki et al. (20) demonstrated that hyperglycemia-induced EPC senescence is associated with activation of the p38 MAPK pathway. The JAK/STAT and p38 MAPK pathways are stimulated by a variety of stimuli including cytokines, hypoxia, and osmotic stress and regulate a variety of cellular functions, including growth factor production, proliferation, and apoptosis. Previous work has outlined a role for these signaling pathways in the production and secretion of growth factors by hMSCs (39, 41). We again confirmed that the JAK/STAT and p38 MAPK pathways, in most cases, are activated in response to injurious stimuli and appear to be correlated with increased growth factor production by MSCs. This was particularly noteworthy with TNF-α and hypoxia-induced STAT3 phosphorylation at 24 h and with TNF-α and LPS-induced p38 phosphorylation at 24 h. In situations where increased STAT3 and/or p38 phosphorylation were not clearly correlated with growth factor production, it is likely that other signaling pathways are concurrently involved in mediating growth factor production (9, 42).

In contrast to what has been observed with other progenitor cell types, we determined from these experiments that JAK/STAT and p38 MAPK signaling in hMSCs are unaffected by glucose concentration in the short term. Thus, we reason that stem cell proliferation and growth factor production are unaffected by glucose concentration because of these cells’ ability to maintain functionality of the JAK/STAT and p38 MAPK signaling pathways even in high glucose medium. How exactly this is accomplished in hMSCs when it is not the case in other cell types remains unclear. It is likely, however, that certain phenotypic and metabolic characteristics of hMSCs may offer a possible explanation. The number and types of sodium-dependent or independent glucose transporters (SGLTs or GLUTs, respectively), for example, are known to be differentially expressed among various cell types and may play an important role in cells’ ability to utilize and respond to glucose in their environments. Emerging evidence suggests that MSCs

![Fig. 6. Glucose concentration had no effect on proliferation at 48 h as measured by 5-bromo-2′-deoxy-uridine (BrdU) incorporation (A). Conversely, renal tubular epithelial cell (RTEC) proliferation at 48 h was significantly diminished at the 20 mM and 30 mM glucose concentrations (B). Medium containing equimolar concentrations of mannitol had no effect on RTEC proliferation. Absorbance is measured at a wavelength of 405 nm with a reference wavelength of 490 nm. *P < 0.05 vs. 5.5 mM group.](http://ajpregu.physiology.org)
may be able to alter glucose transporters expression depending upon the environment, even so far as to adopt a beta cell-like fate (5). Downstream pathways such as the thioredoxin reductase (TrxR)/thioredoxin (Trx) system, a system of cellular components that plays a role in several functions including gluconeogenesis, glycolysis, apoptosis, and neutralization of reactive oxygen species, may also respond to glucose concentrations and may play a role in the short-term maintenance of hMSC functions when exposed to high glucose concentrations (21, 26).

One potential criticism of our study could center on the time periods chosen for investigation (i.e., 24 and 48 h). Indeed we have not characterized the long-term effects of high glucose concentration with this set of experiments. Interestingly, Li

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Fig. 7. STAT3 expression in cellular lysates of hMSCs treated with no stimulus (control), TNF-α, LPS, or hypoxia for 24 h (A) or 48 h (B). The concentration of glucose in the cell culture medium was 5.5, 20, and 30 mM. Western blots are representative of typical results. Corresponding control bands and experimental bands are from the same blots.
et al. (21) examined the effects of high glucose on proliferation and apoptosis of hMSCs in culture at either 3–4 days or 4 wk and found either no effect or even enhanced proliferation in response to high glucose at the time periods chosen. Our objectives with this study, however, were slightly different. Namely, we aimed to determine whether or not high glucose concentration would affect the secretion of paracrine growth factors and proliferative capacity of MSCs as these may relate...
to the ability of these cells to be transplanted as a therapy for acute conditions characterized by tissue injury and dysfunction. From a technical standpoint, it would be difficult to precisely quantify cellular growth factor production by measuring levels in cell culture supernatant beyond 24–48 h secondary to the importance of changing the cell culture media of these cells in culture every 2–3 days. Regardless of any technical considerations, however, our objective with these experiments is important to consider, particularly given that, in vivo, many acute conditions where stem cells may be employed therapeutically are characterized by hyperglycemia secondary to co-morbid diabetes or acute stress. Thus, whether or not this hyperglycemia might affect the acute therapeutic potential of stem cells is potentially very pertinent when considering the use of these cells as a therapeutic modality.

**Perspectives and Significance**

Stem cell therapy is becoming a reality for a wide variety of conditions including myocardial ischemic disease, peripheral vascular disease, neurologic injury, and several others. Many of the short-term benefits to stem cell therapy can be attributed to the paracrine actions of these cells. Thus, an important challenge and goal for stem cell researchers is to define factors, both in vitro and in vivo, that either negatively or positively affect the ability of stem cells to carry out their paracrine actions. Contrary to our initial hypothesis, our in vitro experiments demonstrate that growth factor production and proliferative capacity of hMSCs are unaffected by short-term high glucose concentrations. This is important to consider given that many clinical conditions where stem cells may be employed therapeutically are characterized by hyperglycemia secondary to comorbid diabetes or acute stress. Furthermore, glucose concentration, both in vitro and in vivo, is relatively easily controlled and manipulated. As such, it is important to determine the effects of glucose on hMSCs so their therapeutic potential can be harnessed and maximized. However, in an effort to define the optimal conditions to promote both the ex vivo expansion and in vivo actions and survival of transplanted hMSCs, glucose levels do not appear to be an important factor in the short term.

**GRANTS**

This study was supported, in part, by National Institutes of Health Grants R01-GM-070628, R01-HL-085595, F32-HL-093987, F32-HL-092718, and F32-HL-092719.

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