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Hypothermia and Nutrient Deprivation Alter Viability of Human Adipose-Derived Mesenchymal Stem Cells

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Abstract

Purpose—Adipose-derived mesenchymal stem cells (MSCs) are attractive biological agents in regenerative medicine. To optimize cell therapies, it is necessary to determine the most effective delivery method for MSCs. Therefore, we evaluated the biological properties of MSCs after exposure to various temperatures to define optimal storage conditions prior to therapeutic delivery of MSCs.

Design—Prospective observational study

Methods and materials—Adherent and non-adherent MSCs were incubated at multiple temperatures (i.e., 4, 23 and 37°C) in Lactated Ringers (LR) solution lacking essential cell growth ingredients, or in culture media which is optimized for cell growth. Cells were assessed either after

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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the temperature changes (4 hours) or after recovery (24 hours). Metabolic activity of MSCs, cell number and expression of representative mRNA biomarkers were evaluated to assess the biological effects of temperature. We monitored changes in mRNAs expression related to cytoprotective- or stress-related responses (e.g., FOS, JUN, ATF1, ATF4, EGR1, EGR2, MYC), proliferation (e.g., HIST2H4, CCNB2), and extracellular matrix production (ECM; e.g., COL3A1, COL1A1) by quantitative real time reverse-transcriptase polymerase chain reaction (RT-qPCR) analysis.

Results—Our study demonstrates that storing MSCs in Lactated Ringers (LR) solution for 4 hours decreases cell number and metabolic activity. The number of viable MSCs decreased significantly when cultured at physiological temperature (37 °C) and severe hypothermia (4°C), while cells grown at ambient temperature (23°C) exhibited the least detrimental effects. There were no appreciable biological differences in mRNA markers for proliferation or ECM deposition at any of the temperatures. However, biomarkers related to cytoprotective- or stress-responses were selectively elevated depending on temperature or media type (i.e., LR versus standard media).

Conclusion—The biological impact of nutrient-free media and temperature changes after 4 hours exposure persists after a 24 hour recovery period. Hence, storage temperature and media conditions should be optimized to improve effective dosing of MSCs.

Keywords

Mesenchymal stem cell; stem cell therapy; hypothermia; hypoxia; cell stress; connective tissue diseases; musculoskeletal conditions; quality improvement and patient safety; basic science

Introduction

Degenerative diseases of the musculoskeletal system are a major source of chronic pain and disability in the general population and cause a significant burden to health care systems worldwide, particularly in developed countries. The most common problems include primary and secondary osteoarthritis of knee, hip and other joints, degenerative disc disease and spondylarthrosis.[1] All of these disorders significantly limit mobility and cause a decline in quality of life, especially in elderly patients.

Possible treatment approaches of musculoskeletal problems are considered individually, ranging from least invasive (e.g., physical therapy and pharmacotherapy) to more invasive (e.g., injections or surgeries). Our group examines a number of skeletal degenerative diseases that affect cartilaginous tissues in the articular joints and spine [2–4] that may benefit from stem cell therapies [5–7]. In musculoskeletal regenerative medicine, cell therapy is rapidly gaining traction and has become a prevalent treatment modality that may alleviate pain and combat disease progression. Recent studies have demonstrated that therapeutic effects of mesenchymal stem cells may be due to the release of bioactive molecules rather than functioning as a source of new cells incorporated into healing tissues[8]. Additional work supports these findings and suggests that stem cells mitigate degeneration by providing anti-inflammatory or trophic signals [9–11].

Various clinical trials have explored effects of culture-expanded adipose-derived mesenchymal stem cells MSCs [12–15]. In clinical settings, it is important to provide consistent cell doses for proposed therapeutic effect. Similarly the retention of overall MSC quality during the storage, transport and clinical application is crucial for reproducibility of clinical trials. There is a paucity of data on the biological properties of adipose-derived MSCs and how their phenotype may change from the moment when the cells leave a ‘good manufacturing practice’ (GMP) facility until they are injected into patients. In order to eliminate potential detrimental effects caused by environmental factors that may occur during preparation for cell therapy, our group has extensively explored a number of these effects, such as exposure to preservatives [16], contrast agents [17], hypoxia [18], needle passage [19], various growth surfaces [20, 21], as well as the cytotoxicity of local anesthetics [22]. Here, we examined whether the viability and metabolic activity of MSCs may be compromised by other environmental factors. Because MSCs undergo temperature and media changes during the various stages prior to delivery, we considered that these experimental variables could affect the potency and/or dosing of MSCs during the delivery process.

To address the hypothesis that ambient temperatures and media changes during clinical delivery may affect the viability, metabolic activity, and gene expression signatures of MSCs, we examined survival and metabolic activity of MSCs incubated in both nutrient-rich and non-nutritious solutions within ambient temperatures that are commonly encountered in the clinical setting. Temperatures we considered were body core temperature (37°C), moderate hypothermia (23°C), or severe hypothermia (4°C). We demonstrated that metabolic activity and MSC number are altered with changing temperature with a concurrent temperature-dependent change in the expression of stress-response related markers. MSCs are particularly sensitive to temperature changes when suspended in nutrient-free solutions (e.g., Lactate Ringers solution) that are used during clinical delivery. The latter finding may necessitate a reappraisal of standard operating procedures for MSC-based cell therapies.

Methods and materials

Cell isolation

Human adipose-derived MSCs from fat biopsies were harvested for research use from consenting patients during elective surgeries with approval from the Mayo Clinic Institutional Review Board (IRB). MSCs from three donors (#258, #283, #211) were cultured in platelet lysate containing zoonotic free culture media as previously reported [23, 24]. These cells were extensively characterized for multilineage potential [25] and their molecular characteristics by RNA-sequencing [26, 27]. MSCs were expanded in standard tissue culture media optimal for this cell type which is Advanced Minimum Essential Medium (Advanced MEM, Life Technologies, Grand Island, NY), supplemented with 5% human platelet lysate (PLTMax; Mill Creek Life Sciences, Rochester, MN), 2mM Glutamax (Life Technologies), 2U/mL heparin, and 1% Pen-Strep (100 U/mL penicillin, 100g/mL streptomycin; Cellgro, Thermo Fisher Scientific, Dublin, Ireland). Following several passaging steps, the MSCs were frozen for storage in Cryostore cell cryopreservation media (Sigma-Aldrich, St.Louis, MO) in liquid nitrogen. For the purpose of our experiments,

individual frozen batches of MSCs (passage 6 or 7) were thawed and expanded in a cell culture at 95% humidity, 5% CO₂ and standard incubator temperature (37°C). Upon retrieval from liquid nitrogen storage, MSCs were allowed to recover from cell stresses due to the freeze/thaw procedure and expanded for at least 48 hours in 175 cm² flasks in standard tissue culture media until they reached passage 8. The MSCs were detached using TrypLE Express (Thermo Fisher Scientific). MSCs were seeded at 10⁴ cells/cm² in 12 well-plates (Costar, Thermo Fisher Scientific) and allowed to attach and recover for 1 day after which culture media was aspirated prior to experimentation.

Experimental Design for Cell Treatment

Fresh culture media or Lactated Ringers solution were added so that each plate contained triplicates with 1 ml of solution. Plates with control cells were maintained in the incubator for the whole experimental period without any culture media change or other handling. Culture media and LR were prechilled or pre-warmed to the desired temperatures before the experiment, therefore by design causing rapid rather than gradual temperature changes at the time of addition to the cells. MSCs were incubated for 4 hours (hrs) at distinct temperatures and either immediately analyzed or transferred to the incubator to temperature equilibration and recovery for another 24 hrs. These cells were given fresh culture media so that those that contained LR could restore their metabolism with access to standard nutrition optimized for cell growth.

We selected three storage temperatures (i.e., 4, 23, 37°C) that are relevant to clinical settings and are easily reproducible in the laboratory. Temperature-specific environments were simulated with adherent MSC cultured on standard tissue culture plates or with non-adherent MSCs maintained in plastic tubes. Adherent cells were used to reflect standard growth conditions as clinical grade cells in GMP facilities and non-adherent to simulate cells loaded in a syringe for stem cell therapy injection. For adherent cells, one plate was placed on ice in a cooler box (4°C group), one plate at ambient temperature on the bench surface (23°C group), and two plates remained in the tissue culture incubator (37°C groups). The latter two plates were either subjected to mock treatment (to account for effects of handling cells in a tissue culture hood), or always maintained in the incubator. For non-adherent cells, conical plastic tubes (Falcon, Thermo Fisher Scientific) were immersed in temperature-controlled water baths to simulate the effects of temperature on non-adherent MSCs. All plates except for a plate with control cells were sealed with Parafilm (Bemis NA, Neenah, WI) and all tubes were covered with a lid to model limited gas exchange expected in the closed environment of cells stored in syringes. Also, the sealed vessels eliminate potential differences due to reduced oxygen levels in different experimental environments, while control cells were maintained with access to oxygen and 5% CO₂.

In the second experimental setting, culture expanded cells were enzymatically detached and divided in 3×15 ml conical plastic Falcon tubes or in 1.5ml Eppendorf tubes. This measure was taken in order to simulate the storage of cells in the syringe before application in the clinical environment, where the cells are non-adherent. Tubes with MSCs were centrifuged and culture media aspirated. The pellet of cells was re-suspended with 10 ml Lactated Ringers at: 4, 23, 37°C and incubated in these temperatures for 4 hrs in concentrations of 15

-20×10^4 cells per ml. Following the incubation, part of the cell solution was centrifuged and used for RNA extraction and part was used for re-plating for a metabolic activity MTS assay at 10^4 cells/cm². After the initial experimental modulation of temperature, no additional calibration of cell counts was performed. Thus, our end-point results reflect the cumulative effects of both initial temperature changes and the recovery period on the final number of cells.

Metabolic activity assays were performed immediately after the experiment in the adherent cells as well as in the recovery period at 24 hrs. Following the MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) colorimetric assay (CellTiter 96® Aqueous One Solution, Promega), MSCs were fixed overnight using 10% neutral buffered saline (NBF) and stained with Hoechst stain 33258 (Santa Cruz Biotechnology, Dallas, TX) to observe nuclear DNA.

Gene expression analysis

Real time reverse transcriptase quantitative PCR (RT-qPCR) analysis of human adipose-derived MSCs was performed to assess cell stress-related responses, proliferation and extracellular matrix production using mRNA biomarkers. We extracted RNA using Tri Reagent (Zymo Research, Irvine, CA) immediately after experimental incubation. RNA was isolated using Direct-zol RNA MiniPrep (Zymo Research Irvine, CA) and RNA yield was determined by Nanodrop 2000 (Thermo Scientific, Waltham, MA). Reverse-transcription was performed to obtain cDNA using SuperScript III (Invitrogen Carlsbad, CA). Gene expression was quantified with RT-qPCR in CFX384 Real-Time system (BioRad Hercules, CA) using 3.3ng cDNA per 10µl reaction with QuantiTect SYBR green PCR kit (Qiagen Valencia, CA). Primers' sequences are given in Table 1. Results are normalized to *EEF1A1* rather than *GAPDH* within each sample and data analyzed with 2^{-Ct} method. We note that mRNA expression of *GAPDH* (which encodes the glycolytic enzyme glyceraldehyde dehydrogenase) may change with metabolic status, while *EEF1A1* (which encodes the translational elongation factor 1 α 1) is expected to be less variable (see Fig. 5). Our RNA-seq data for adipose-derived MSCs from >50 patients show that mRNAs for *GAPDH* and *EEF1A1* are highly abundant, but *EEF1A1* is more abundantly expressed, has a lower coefficient of variation and is less variable than *GAPDH* when cells exhibit reduced cell proliferation ([27] and unpublished observations).

Metabolic activity and numbers of cells

Metabolic activity testing was performed using colorimetric assays (MTS assay Kit, Cell Titer 96 Non-radioactive cell proliferation assay, Promega). Cells were rinsed with PBS (Phosphate Buffered Saline) and incubated with MTS reagents for 1.5–2 hrs. After incubation, 100µl of media was transferred to a 96-well plate and evaluated by absorbance measurements at 490nm using the Spectra Max Plus Plate Reader (Molecular Devices, Sunnyvale, CA). Signals obtained for each type of media were used as blank background readings and subtracted during numerical analysis. Metabolic activity of experimental groups was normalized relative to that of control cells and presented as percentage.

To assess the number of cells remaining in the wells after incubation, nuclear staining was performed using Hoechst 33258 (Santa Cruz Biotechnology, Dallas, TX) after MTS assays and/or Live Dead Cytotoxicity assay (Thermo Fisher Scientific) with a separate set of MSCs plated for this staining only. Cells were analyzed immediately with Live solution from Live/Dead cytotoxicity assay or rinsed with PBS and fixed using 10% Neutral Buffered Formalin for 24 hrs. In cells that were analyzed using Live stain, the solution was diluted in PBS and added to the wells so that final concentration of the stain is 0.5 ul/ml. For Hoechst stain, following another PBS wash, Hoechst 33258 nuclear stain diluted in PBS was used at 0.1µl/ml concentration. Fluorescence intensity was quantified with a fluorescence reader (Infinite Pro 2000; Tecan, Austria) at wavelengths of 340/460 for Hoechst and of 465/633 for Live stain. After subtracting background values of each sample, the overall level of Hoechst staining and Live staining was normalized by expressing values as a percentage of control cells.

Statistical analysis

All biological analyses were performed using biological triplicates from multiple cell lines of adipose-derived MSCs. Statistical analysis was performed using Graph Pad Prism Version 7.05 (GraphPad, La Jolla, CA). One-way ANOVA and Tukey post-hoc test were used to compare proportions of surviving cells and metabolic activity and RNA expression levels. The p-value 0.01 was considered as significant. The results were presented as mean and standard deviation.

Results

The logistic path for delivery of MSCs in clinical gene therapies is that MSCs are dislodged from monolayers and stored in syringes on ice until injection into patients. This clinical scenario was simulated in our experimentation by generating MSCs that were left in suspension in nutrient-poor Lactated Ringers and exposed to temperatures of 4, 23, 37°C. MTS metabolic assays were performed to assess changes in metabolic activity of these non-adherent nutrient-deprived MSCs. To dissect the effects related to temperature, nutrient deprivation and cell adhesion, we also carried out MTS assays with adherent cells in standard nutrient-rich culture media that is used for GMP production of clinical grade MSCs (i.e., advanced MEM with 5% platelet lysate). Measurements for biological activities and molecular parameters were made immediately after 4 hr and after a subsequent 24 hr period of recovery.

Acute effects of temperature and media conditions on metabolic activity and cell number

We first examined the metabolic activity of adherent MSCs in Lactated Ringers solution immediately following a 4 hr exposure to different temperatures. When cells are switched from standard culture media (M Control 37) to Lactated Ringers solution (LR) but maintained at 37°C, there is a clear decrease in the metabolic activity of adherent MSCs (Fig. 1A). This effect is due to the removal of essential metabolic ingredients (e.g., glucose and amino acids) that isotonic biochemical solution Lactated Ringers lacks. When temperatures are lowered to 4 or 23°C (LR4 and LR23), we observe that the decline in metabolic activity is less pronounced. Thus, the deleterious effect of Lactated Ringers

solution is more prominent at normal physiological temperatures. To assess effects of Lactated Ringers solution on cell number at different temperatures, we monitored the number of viable cells using Live/Dead cytotoxicity staining (Fig. 1B). Regardless of temperature, incubation in Lactated Ringers solution reduced cell numbers, indicating that cells are perishing due to the lack of nutrition. Remarkably, while cell death due to nutrient deprivation is prominent at 4 and 37°C (LR4 and LR37), cells appear to be modestly protected at ambient temperature (LR23), while maximal cell loss (to 40–50%) is observed at 4°C. These data could suggest that hypothermic storage of MSCs in Lactated Ringers solution at 4°C may dramatically reduce the effective dose of MSCs.

Assessment of metabolic recovery after hypothermia followed by extended incubation under standard culture conditions.

To assess whether MSCs are temporarily or permanently compromised in their metabolic ability, we incubated cells for 24 hours in standard culture media after initial exposure to Lactated Ringers solution at varying temperatures. Cells exposed to temperatures of, respectively, 4°C and 23°C (LR4 and LR23), did not return to baseline metabolic activity after 24 hours of post-incubation under standard cell culture conditions (Fig. 2A). However, MSCs that were initially incubated at 37°C in Lactated Ringers solution (LR37) displayed a heterogeneous response with some cell cultures having only partial to no restoration of metabolic activity while others demonstrated full recovery (Fig. 2A). Analysis of Hoechst staining revealed that the low temperatures groups (LR4 and LR23) appeared to regain cell numbers to some extent, while the physiological temperature group (LR37) did not (Fig. 2B). Regardless of temperature, it appears that cells have a limited capacity to restore metabolic activity upon pre-incubation in Lactate Ringers solution and that nutrient deprivation at physiological temperature (LR37) is least conducive for maintenance of metabolic activity and cell viability. Notably, cell storage at ambient temperatures in nutrient poor media (LR23) may not only protect cells from metabolic activity loss (see Fig. 1), but perhaps also be beneficial for more optimal recovery.

To further investigate whether the significant changes in metabolic activity and cell number were due to LR exposure rather than temperature changes, we repeated the previous set of experiments in standard culture media containing a proper nutritional profile. Metabolic activity and viability of adherent cells cultured in LR or standard culture media was assessed 24 hours after exposure to temperature changes (Fig. 3). As expected, cells cultured in growth media and exposed to 37°C (M37) had similar metabolic activity and cell number as control cells. However, MSCs exposed to 4°C and 23°C had significantly lower viability (Fig. 3A) and metabolic activity (Fig. 3B) than the control group in standard culture media. The pattern and extent of these changes resembles that of cells exposed to 4 and 23°C (Fig. 3A, B) in LR solution. Together, these data demonstrate that nutritional differences between LR and standard culture media contribute minimally to the biological changes seen upon temperature variation.

Injections during stem cell therapy are performed with non-adherent cells in suspension. Compared to adherent cells, non-adherent cells may be exposed to additional stress due to substrate detachment and loss of cell-to-cell contact. The effects of this additional handling

and non-adherent storage within the syringe on the biological state of MSCs need to be addressed. Therefore, we examined whether non-adherent cells have the same cellular properties upon temperature changes as adherent MSCs (Fig. 4). We note that non-adherent cells cannot be maintained in nutrient-rich cell culture media, because these conditions produced non-specific cell aggregates that compromise metabolic read-outs (data not shown). Hence, treatment combinations were restricted to culture conditions using Lactated Ringers which most closely mimic current standard operating procedures for MSC delivery. Results from these experiments demonstrated that temperature changes in non-adherent MSCs lead to alterations in metabolic activity (Fig. 4A) and cell viability (Fig. 4B) of non-adherent MSCs that are comparable to those observed for adherent cells and that these biological effects persist for at least one day after the initial exposure (triplicates from two cell lines) ($p < .0001$) (Fig. 4). Similar to results for adherent cells, these results are expected from deprivation of nutrients present in cell culture media and apparently occur independent of cell contact with a substratum. However, recovery appears to be more pronounced if the incubation temperatures are 4 and 23°C. Ambient temperature (23°C) appears to be the least harmful temperature for short term storage of MSCs. Cells at physiological temperature (37°C) displayed a major decline in metabolic activity and viability suggesting that optimal temperatures for cell growth are causing a rapid decline in cell survival of non-adherent cells if subjected to starvation. These data with non-adherent cells are consistent with the previous results obtained by testing adherent cells. Taken together, it appears that LR decreases the metabolic activity of cells in a temperature dependent manner, but this decrease is not further exacerbated by lack of cell adherence.

Analysis of mRNA expression

The temperature changes during cell handling and storage may induce major cell stress and provoke cytoprotective responses and changes in the biological behavior of MSCs that may decrease the effective dosing and therapeutic effects of MSCs. To assess for stress responses due to temperature shifts in standard nutrient-rich media and nutrient-free Lactated Ringers, we performed mRNA expression analysis using RT-qPCR. We focused on expression of stress-related genes and cytoprotective markers that mediate the 'immediate early response' (transcription factors FOS, FOSB, JUN, JUNB, JUND, EGR1, EGR2, MYC) and constitutive proteins (transcription factors ATF1 and ATF4) (Fig. 5; 6A; 7A, B, C); we note that molecular changes and signaling cascades stimulated by expression of stress response genes in response to temperature or nutrient changes may result in cell cycle inhibition, proliferation arrest, and/or apoptosis [28]. We observed distinctly higher expression of multiple genes related to the AP-1 transcription factor complex (i.e., FOSB, JUNB, JUND) in all cells cultured on Lactate Ringers compared to control MSCs in standard culture media. Expression is proportional with increasing temperature and similar under all experimental conditions (Fig. 5 A, B, C). These data show that nutrition deprived cells exposed to physiological temperatures undergo significant cell stress, potentially leading to cell death.

Markers linked to proliferation and cell cycle progression were also evaluated by mRNA expression analysis. For these studies, we monitored the mRNA levels of S-phase related gene HIST2H4, which encodes the nucleosomal protein histone H4 that packages newly replicated DNA, and the mitosis-specific cyclin Cyclin B (CCNB2), which together with the

cyclin dependent kinase CDK1 controls cell division (Fig.6 B,C; 7 E). Both HIST2H4 and CCNB2 exhibited similar patterns of expression in all experimental conditions, although we noted modest changes in the expression of the two genes at 4°C, but these changes did not reach statistical significance. Thus, a temporary temperature change during 4hrs of incubation without nutrition has no appreciable effects on cell proliferation.

Recruitment of MSCs into damaged tissues may modulate extracellular matrix production (ECM) during deposition of fibrotic tissues that provide stress shielding during tissue repair. To evaluate the ability of MSCs to produce extracellular matrix proteins (ECM) following temperature changes, we assessed mRNA expression of COL1A1 and COL3A1 (Fig.6 D, E, 7E). Expression of both COL1A1 and COL3A1 appeared to be moderately increased at 4°C compared to control cells, although again these results did not reach statistically significance. Therefore, we conclude that the ability of MSCs to produce a collagenous ECM is not altered by changes in temperature.

Discussion

Cell therapies in musculoskeletal regenerative medicine employ a wide variety of stem cells. Pluripotent embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSC) may have significant regenerative potential through their ability to generate any tissue cell type (including somatic and germ line cells) however these stem cell types are either encumbered by ethical controversies (e.g., ESCs) or incomplete re-programming (e.g., iPSCs). Somatic multipotent stem/stromal cells such as MSCs have more realistic clinical potential in therapeutic applications, because they are easily harvested from the vascular stroma of bone marrow, adipose tissue or umbilical cord tissue. Adipose-derived MSCs cells are preferentially used since the isolation procedure is the least invasive and relatively painless [29]. Another advantage is their rapid in vitro expansion which reduces risk of their clonal chromosomal and phenotypic changes. These cells represent normal diploid human cells that cease proliferation due to contact inhibition [27]. More importantly, adipose-derived MSCs are cultured in human platelet lysate and their zoonotic free production as high quality GMP-compliant MSCs permits direct clinical applications [23].

The success of cell-based therapies relies in part on certification of clinical grade MSCs that conform to standardized MSC release criteria. However, MSCs are characterized under optimal cell culture conditions and this biological status does not necessarily resemble the status of cells delivered at the point of care. Standard production of MSCs occurs under nutrient-rich, adherent and static conditions that mimic a normal physiological environment at body temperature (37°C). However, when MSCs are delivered during therapy, a number of non-physiological events may compromise the dosing and/or biological activity of MSCs. Potentially adverse steps in the logistic chain of stem cell delivery include (i) hypothermic temperature (4°C) due to storage at on ice, (ii) nutrient deprivation and loss of cell adherence due to suspension of MSCs in Lactate Ringers solution, (iii) potential cytotoxicity of local anesthetics, preservatives and contrast agents during injection, (iv) hypoxia due to storage in syringes, (v) pressure changes due to needle passage, and (iv) environmental interactions with synovial fluid and joint tissues. This study examined the effects of modulating storage

temperature on MSC metabolic activity, viability and gene expression of cytoprotective, proliferation and ECM related genes.

MSCs frequently encounter major changes in temperatures. Beyond rapid freezing in storage medium for long-term storage which may alter MSC viability [16], MSCs are stored on ice for short term storage on the day of the injection. However, storage on ice may induce hypothermia and alter the biological state of the cells in a manner that reduces their effective dosing. There is a robust evidence that hypothermia (<35°C) in general may preserve cells and tissues during hypoxia by decreasing metabolic requirements of cells [30]. Preservation of cells may be facilitated by cell cycle arrest, because hypothermia blocks cell cycle progression ([28, 31, 32]). Hypothermia is widely used for neuroprotection [33] such as for prevention of hypoxic ischemic encephalopathy in newborns [34] or brain damage caused by injury or stroke [35], preservation of transplanted organs [36] and during cardiopulmonary resuscitation [30]. While cellular changes caused by hyperthermia are well understood, our knowledge of the effects of sub-physiological temperatures on cells is limited and detailed mechanisms are not well understood [32]. Cells exposed to certain temperatures or temperatures exhibit a range of molecular changes, including oxidative stress [37, 38], differences in dissolved oxygen [32], as well as the emergence of heat-sensitive factors like heat shock proteins [39] or cold shock proteins [32, 40], that reflect significant cell stress and may induce apoptosis. MSCs exposed to hypothermia experience a major exposure to oxidative stress due to the production of reactive oxygen species (ROS) [37]. This overproduction of ROS may eventually lead to cell apoptosis if it surpasses certain threshold levels [41].

In this study, we evaluated both mild (23°C) and severe (4°C) hypothermia in either nutrient-rich standard culture media or nutrient-deficient Lactated Ringers solution. Our data show that metabolic activity and cell number of MSCs is reduced, and a cellular stress response as measured by mRNA biomarkers is increased, when cells are maintained in nutrient-deficient Lactated Ringers at physiological temperature (37°C). Likewise, metabolic activity and cell number of MSCs decrease, and molecular stress response increase, when cells are maintained in mild or severe hypothermic temperatures (4°C and 23°C). In contrast to these findings, we observed that the detrimental effects of low nutrition were less pronounced in mild hypothermia (23°C).

Our results are consistent with a conceptual model that considers the opposing effects of temperature and nutrient deprivation on the metabolic activity of mitochondria that is linked to oxidative stress responses. First, molecular and oxidative stress is expected to be minimal under normal physiological temperature and nutrient conditions when mitochondria function optimally. However, stress responses are gradually activated as MSCs become severely hypothermic and the normal metabolic functions of mitochondria may be compromised (at 4°C). Molecular and oxidative stress responses due to nutrient deprivation are expected to be most severe at 37°C when mitochondria are primed for optimal metabolic activity but lack essential ingredients (e.g., glucose). Yet, nutritional stress is less apparent when mitochondrial activity declines due to hypothermia. Consequently, maintenance of nutrient deprived MSCs at ambient temperature (23°C) may represent a biological optimum for cell

survival perhaps because mild hypothermia reduces mitochondrial stress linked to nutrient deprivation (Figure 3C).

In hypothermia, as the metabolic activity decreases, the progression of cell cycle and proliferation becomes inhibited. Underhill and colleagues showed that cooling of HeLa cervical carcinoma cells for 24 hrs reduces proliferation by 25% at 32°C and 50% at 10°C [42]. Both mild and severe hypothermia can synchronize cells in a resting phase, because hypothermia prolongs the G1 phase and arrests the cell cycle in the G2 phase [31]. Release of such growth arrested synchronized cells by re-incubating cells at 37°C results in abundant mitotic activity [31]. Our experimentation examined differences in metabolic activity and cell count both immediately after cold exposure and after a 24 hr recovery period at 37°C. We observed increase in cell number upon recovering cells at normal physiological temperature which may be possibly explained by increased mitosis. However, it is plausible that the differences in the ability to provoke a hypothermia-induced cell cycle arrest may be related to differences in cell types [31], especially because HeLa cells are highly robust cancer cells, while MSCs are diploid cells with more normal cell growth characteristics.

Conclusion

Our study shows that the metabolic activity and cell counts of MSCs are both severely decreased after incubation in nutrient-deficient Lactated Ringers solution, or upon reduction of ambient temperatures to generate mild (23°C) or severe (4°C) hypothermic conditions. The most optimal condition for MSCs in Lactated Ringers appears to be under mild hypothermia at ambient temperature, because this condition minimizes loss of cells and overall metabolic activity. Clinical grade MSCs are currently delivered for therapeutic use in syringes on ice, a condition that based on our results is not optimal for their delivery. Therefore, our laboratory results suggest that this standard method for therapeutic delivery of MSCs perhaps should be reconsidered to reduce temperature changes and transfer time from GMP production to delivery in the patient.

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Abbreviations:

C	Celsius
CDK1	Cyclin-Dependent Kinase
CO₂	Carbon Dioxide
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix Proteins

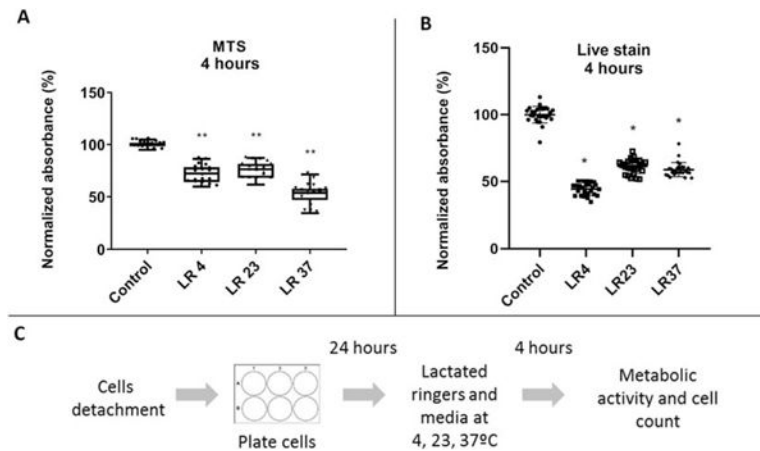
EEF1A1	Elongation Factor 1-Alpha 1
ESCs	Embryonic Stem Cells
g/mL	Gram Per Milliiter
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GMP	Good Manufacturing Practice
iPSCs	Induced Pluripotent Stem Cells
IRB	Institutional Review Board
LR	Lactated Ringers
mRNA	Messenger RNA
MSC	Mesenchymal Stem Cells
nm	Nanomolar
PBS	Phosphate-Buffered Saline
RNA	Ribonucleic Acid
U/mL	Units Per Millilitre
µl	Microliter

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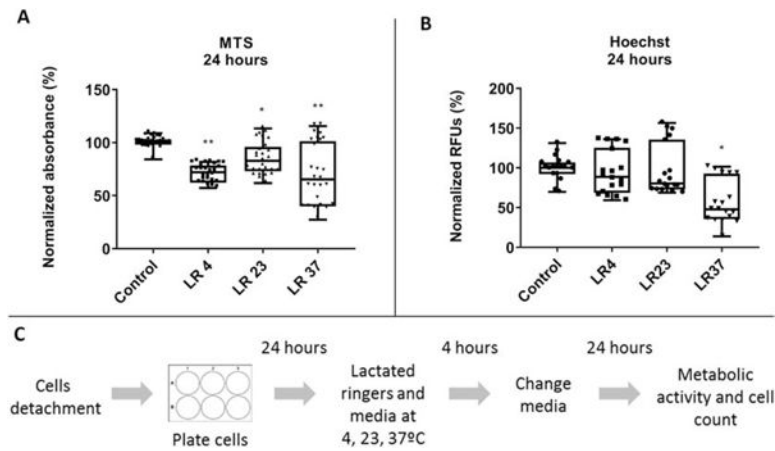
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**Figure 1:**

Effects of temperature changes on adherent MSCs viability and metabolic activity immediately after experimental treatment

This figure illustrates metabolic activity (A) and cell counts (B) immediately after 4 hour exposure to temperatures (4°C, 23°C, 37°C) in Lactated Ringers. The design of the experiment is shown (C). MTS assay was used to assess metabolic activity and Live/Dead stain for cell quantification. The data represent mean values from biological triplicates from two and three cell lines.

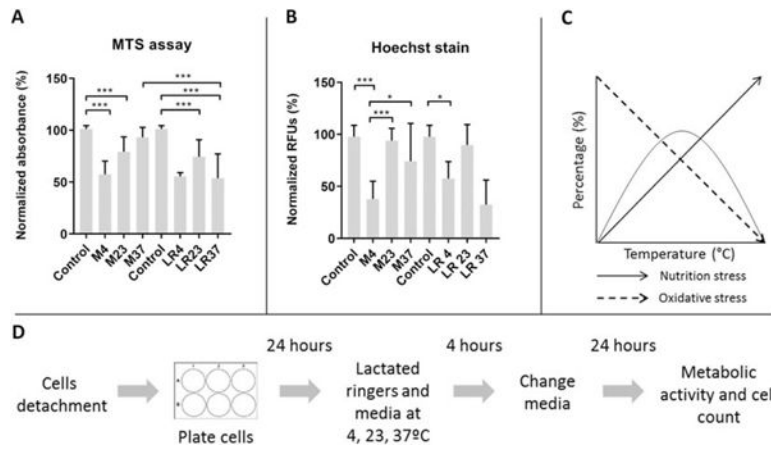
LR= Lactated Ringers, MTS = MTS assay; * $p < .01$, ** $p < .0001$, One-way ANOVA with Tukey's post-hoc test, $n=18$ (MTS, 4hrs), $n=27$ (Live stain as part of the Live/Dead Cytotoxicity assay, 4hrs)

**Figure 2:**

Effects of temperature changes on adherent MSCs viability and metabolic activity at 24 hours after experimental treatment

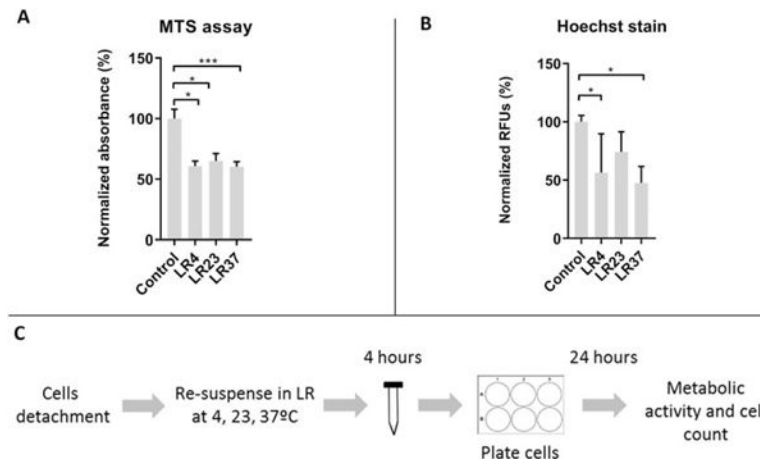
This figure shows metabolic activity (A) and cell counts (B) at 24 hours after exposure to temperatures in Lactated Ringers. The design of the experiment is shown (C). MTS assay was used to assess metabolic activity and Hoechst stain for cell quantification. The data represent mean values from biological triplicates from two and three cell lines.

LR= Lactated Ringers, MTS = MTS assay, Hoechst = Hoechst 33258 nuclear stain; * $p < .01$, ** $p < .0001$, One-way ANOVA with Tukey's post-hoc test, $n=27$ (MTS, 24hrs), $n=18$ (Hoechst, 24hrs)

**Figure 3:**

Effects of temperature changes on adherent MSCs in standard culture media and in Lactated Ringers at 24 hours after experimental treatment

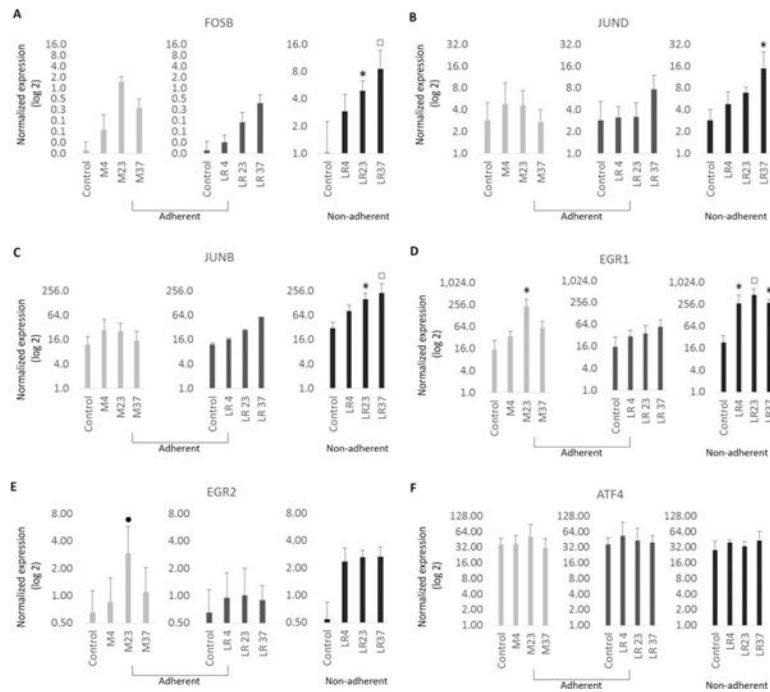
This figure shows metabolic activity (A) and cell counts (B) of the adherent MSCs exposed to 4 hour incubation in temperatures (4, 23, 37°C) in standard culture media and in Lactated Ringers following a 24 hour recovery period. The diagram of nutrition and oxidative stress (C) shows the suggested mechanism behind the observed results where cells in the 4°C and 37°C show lower viability and metabolic activity than 23°C. The design of the experiment is shown (D). MTS assay was used to assess metabolic activity and Hoechst stain for cell quantification. The data represent mean values from biological triplicates of three cell lines. LR= Lactated Ringers, M = standard culture media, MTS = MTS assay, Hoechst = Hoechst nuclear stain 33258; *p<.05, ** p<.01, ***p<.001, One-way ANOVA with Tukey's post-hoc test, n=9 (adherent)

**Figure 4:**

Effects of temperature changes on non-adherent MSCs in Lactated Ringers at 24 hours after experimental treatment

This figure shows metabolic activity (A) and cell counts (B) of the non-adherent MSCs exposed to 4 hour incubation in temperatures (4, 23, 37°C) in Lactated Ringers following a 24 hour recovery period in standard culture media. The data represent mean values from biological triplicates of two cell lines.

LR= Lactated Ringers, MTS = MTS assay, Hoechst = Hoechst 33258 nuclear stain; *p<.05, ** p<.01, ***p<.001, One-way ANOVA with Tukey's post-hoc test, n=6 (non-adherent)

**Figure 5:**

Effects of temperature changes on mRNA expression of MSCs

The graphs show mRNA expression levels immediately after exposure to temperatures for cyto-protective response genes: immediate early response genes (A, B, C), transcription factors (D, E), and constitutive response (F). The data represent mean values from biological triplicates of three cell lines.

* $p < .05$, □ $p < .01$, ● $p < .0001$, One-way ANOVA with Tukey's post-hoc test, $n=9$ for each cell line

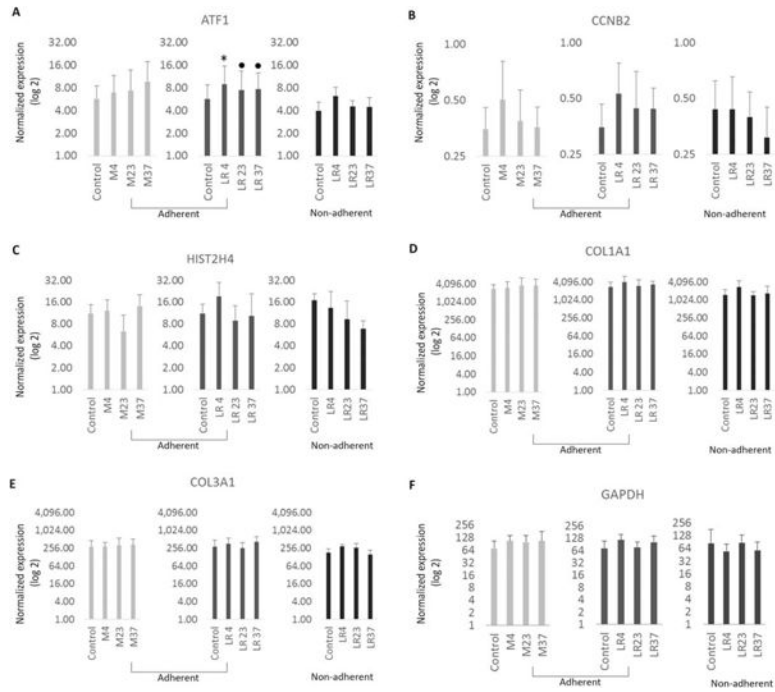


Figure 6: Effects of temperature changes on mRNA expression of MSCs
 The graphs show mRNA expression levels immediately after exposure to temperatures for cyto-protective response genes: constitutive response (A), as well proliferation genes (B, C) and ECM (D, E). The data represent mean values from biological triplicates of three cell lines.
 *p<.05, □p<.01, ●p<.0001, One-way ANOVA with Tukey’s post-hoc test, n=9 for each cell line

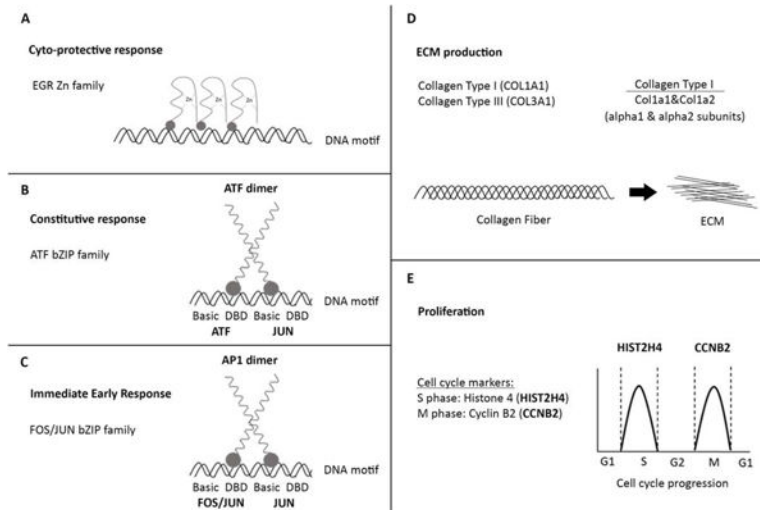


Figure 7:
Diagram of PCR biomarkers

The diagram shows graphical representation of biomarkers for cyto-protective genes (A, B, C), for ECM markers (D) and proliferation markers (E). EGR gene family encodes for TF that bind as zinc finger proteins to DNA. ATF dimer is formed by 2 ATF or ATF and JUN proteins that belong to bZIP family of TF. Genes FOSB and/or JUNB and/or JUND together form an AP1 dimer (activator protein 1) which serves as a TF that regulates gene expression by binding to DNA by bZIP domain. COL1A1 gene encodes for alpha 1 subunit of collagen 1 and together with alpha 2 subunit (COL1A2 gene) they form procollagen 1 molecule. A collagen fiber is made from triple-stranded pro-collagen molecules, multiple fibers form the ECM. HIST2H4 is S phase marker (DNA replication) of the cell cycle and CCNB2 is M phase (mitosis) marker.

ECM=Extracellular matrix, DBD=DNA-binding domain, bZIP (leucine zipper), TF=transcription factor

Table 1.

mRNA primer sequences

mRNA primers sequences	Primers, 5'-3'	Primers, 3'-5'
Gene	Forward	Reverse
GAPDH	ATGTTTCGTCATGGGTGTGAA	TGTGGTCATGAGTCCTTCCA
EEF1A1	TGTCGTCATTGGACACGTAGA	ACGCTCAGCTTTCAGTTATCC
FOSB	GCTGCAAGATCCCCTACGAAG	ACGAAGAAGTGTACGAAGGGTT
JUNB	TGGCCCAGCTCAAACAGAAG	CAGAAGGCGTGTCCCTTGAC
JUND	GTGAAGACCCTCAAGAGTCAGA	GACGTGGCTGAGGACTTTCT
EGR1	ACCCCTCTGTCTACTATTAAGGC	TGGGACTGGTAGCTGGTATTG
EGR2	ATTCTGAGGCCTCGCAAGTA	GCTTATGCCCAAGTGTGGATT
COL1A1	GTAACAGCGGTGAACCTGG	CCTCGCTTTCCTTCTCTCC
COL3A1	TTGAAGGAGGATGTTCCCATCT	ACAGACACATATTTGGCATGGTT
ATF1	CTGGAGTTTCTGCTGCTGTC	GGCAATGGCAATGACTGTC
ATF4	ATGACCGAAATGAGCTTCCTG	CTGGAGAACCCATGAGGTTTG
CMYC	CGGATTCTCTGCTCCTCGAC	CCTCCAGCAGAAGGTGATCCA
HIST2H4	AGCTGTCTATCGGGCTCCAG	CCTTTCCTAAGCCTTTTCC
CCNB2	CCGACGGTGTCCAGTGATT	TGTTGTTTTGGTGGTTGAAC