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Original Research

The osmolyte type affects cartilage associated pathologic marker expression during *in vitro* mesenchymal stem cell chondrogenesis under hypertonic conditions

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Abstract: Stem cells' fate during in vitro differentiation is influenced by biophysicochemical cues. Osmotic stress has proved to enhance chondrocyte marker expression, however its potent negative impacts had never been surveyed. We questioned whether specific osmotic conditions, regarding the osmolyte agent, could benefit chondrogenesis while dampening undesired concomitant hypertrophy and inflammatory responses. To examine the potential side effects of hypertonicity, we assessed cell proliferation as well as chondrogenic and hypertrophic marker expression of human Adipose Derived-MSC after a two week induction in chondrogenic media with either NaCl or Sorbitol, as the osmolyte agent to reach a +100 mOsm hypertonic condition. Calcium deposition and TNF-α secretion as markers associated with hypertrophy and inflammation were then assayed. While both hyperosmotic conditions upregulated chondrogenic markers, sorbitol had a nearly three times higher chondro-promotive effect and a lesser hypertrophic effect compared to NaCl. Also, a significantly lesser calcium deposition was observed in sorbitol hypertonic group. NaCl showed an anti-proinflammatory effect while sorbitol had no effect on inflammatory markers. The ossification potential and cartilage associated pathologic markers were affected differentially by the type of the osmolyte. Thus, a vigilant application of the osmotic agent is inevitable in order to avoid or reduce undesired hypertrophic and inflammatory phenotype acquisition by MSC during chondrogenic differentiation. Our findings are a step towards developing a more reliable chondrogenic regimen using external hypertonic cues for MSC chondrogenesis with potential applications in chondral lesions cell therapy.

Key words: Hypertonic stress; Hypertrophy; Inflammation; MSC chondrogenesis; Osmolyte.

Introduction

Cartilage is a resilient tissue covering the ends of bones to protect them from being rubbed by shearing and compressive forces. Cartilage extracellular matrix (ECM) is a hydrated matrix surrounding sparse resident chondrocytes, which is mainly composed of collagen type II (Col- II) and proteoglycans (PG) which are essential for biomechanical properties of the tissue. Owing to the highly sulfated polyanionic glycosaminoglycan (GAG) side chains of PG acting like a sponge to electrostatically attract positively charged cations such as Na^+, the osmolarity of cartilage ECM would be typically higher than most other tissues, and is in the range of 350-480 mOsm (1).

Adult mesenchymal stem cells (MSC) are regarded a promising autologous cell source for cartilage cell therapy. They offer numerous advantages over cartilage derived chondrocytes widely being used in ACI (autologous chondrocyte transplantation). Such advantages include offering a one-step surgical procedure which obviates the need for a primary surgery to obtain cartilage tissue as the cell source. Also, MSC can be expanded to give a huge amount of cells required for cell transplantation (2), while maintaining their differentiation capacity down the chondrogenic lineage at higher passages (3). Thus, adult MSC offer an appealing alternative cell source for cartilage repair.

Classically, MSC chondrogenesis is performed in media containing TGF-β, which is normally considered as best practice media for in vitro chondrogenesis. Many studies in the field of cell-based cartilage repair are involved with identifying appropriate biological, chemical or physical cues to maximally promote chondrogenesis while avoiding hypertrophy, angiogenesis and inflammatory responses, all considered as pathologic signs of cartilage degeneration and disease (4, 5). These phenomena should be ideally overcome for a successful regenerative cell therapy purpose. Various bio-physicochemical stimuli have been checked out to improve MSC chondrogenesis. Namely, hydrostatic pressure, electromagnetic fields, dynamic loading and shear stress are well outlined to have synergistic effects on MSC chondrogenesis (6). The inductive effects of hyperosmolarity on phenotype preservation of chondrocytes during *in vitro* expansion as long as enhanced

adoption of chondrogenic fate by stem/progenitor cells is outlined in the literature (7). Though, a few contradictory reports are also available (8).

Several reports suggest that hypertonicity could induce inflammation. In fact hyperosmolarity is associated with certain inflammatory diseases and disorders (9). Hyperosmolarity can cause inflammation through the activation of NF- kB which induces pro-inflammatory cytokine responses in some epithelial cells. Accumulation of pro-inflammatory factors such as TNF-α can induce fibrosis and hypertrophy (10) both considered to contribute to the pathophysiology of the cartilage (4). As such, it seems necessary to monitor and trace the possible signaling pathways that might be triggered by hypertonic stress of which angiogenesis and inflammatory responses have been proved to be affected by while also being considered as pathologic events in cartilage physiology.

In vitro and in vivo studies have provided evidence of hypertonicity effectiveness for cartilage regeneration. In spite of putative inductive effects, osmotic stress can be a challenge for the function of normal cells. Particular attention should be paid in applying external stimuli to cells not encountered to such cues in their native microenvironment, as is the case for MSC. The question raised is how application of a hyperosmotic condition to MSC already resided, isolated and expanded in a hypotonic environment, compared to that in native cartilage environment, would impact their chondrogenic potential taken into account the induction or suppression of cartilage associated pathologic attributes such as hypertrophy, angiogenesis and inflammatory mechanisms.

So our goal was to clarify if the osmolyte type attributed hyperosmotic stress of the chondrogenic differentiation medium would affect the efficacy of MSC chondrogenesis and the concomitant or upcoming pathologic responses. We used NaCl and sorbitol to increase chondrogenic medium osmolarity to 100 mOsm higher than the control group in order to mimic the cartilage native environment osmolarity (1). We utilized NaCl, since Na+ is the most prevalent cation species in the cartilage (11) and also as it is the most widely used agent to induce hypertonicity.

To our knowledge, scientific reports published regarding the effect of hypertonicity on chondrocytes have only dealt with the chondrogenesis and have neglected other possible pathways that might get ignited up and overwhelm the effectiveness of chondrocytes role and function. It is of high importance to be aware and to consider undesired pathologic pathways that might be triggered when developing or applying external cues. This study was designed aiming to find a more reliable biophysical stimuli for enhanced in vitro chondrogenesis while avoiding undesired effects of inflammation, vascularization, and ossification.

Materials and Methods

Cell culture and chondrogenic differentiation under hypertonic stress

Human AD-MSC (adipose derived-MSC) were obtained from subcutaneous adipose tissue of healthy volunteers undergoing liposuction surgery, with full consent. MSC were isolated from the adipose tissue after vigorous wash in PBS containing gentamicin and streptomycin and digestion with Collagenase I. Cells were expanded in low glucose DMEM (Gibco) containing 10% FBS (Gibco) and used until passage 6. Cells were first assayed for any cytotoxicity attributed to hyperosmolar conditions. Hypertonic media were prepared by adding appropriate amount of NaCl or Sorbitol to make 450 mOsm media. The osmolarity of control culture (DMEM + 10% FBS; 350 mOsm) and the +100 mOsm hypertonic media (relative to control) were confirmed using a Vapour Pressure Osmometer (Knauer).

Hyperosmolar chondrogenic media were made by adding NaCl or Sorbitol to standard chondrogenic medium (TGF-β1 (10 ng/ml, Peproteck), dexamethasone (10^{-7}M), proline (400 µg/ml), pyruvate (1 mg/ml), ascorbic acid (50 µg/ml) and 1% ITS (Insulin, Transferrin, Selenium) (all Sigma). Confluent (70-80%) monolayer cells were treated with standard chondrogenic media (350 mOsm) serving as control (12)or +100 hyperosmolar chondrogenic media obtained by addition of NaCl or Sorbitol. All assays were done after two weeks under chondrogenic induction with or without hypertonic stress.

Proliferation assay

MSC viability was assessed over a week using MTT assay, a colorimetric method based on the cleavage of the yellow tetrazolium salt (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide) to purple formazan crystals (13). At specified time points, cells grown in a 24-well tissue culture plate with normal or hypertonic expansion media doped with either NaCl or Sorbitol were incubated with the MTT solution (0.5 mg/ml) for 4 hours. After this incubation period the water-insoluble formazan crystals were dissolved in DMSO and quantitated by obtaining absorbance (570 nm) which directly correlates with cell number.

Gene expression analysis

After a two week incubation in control or +100 mOsm chondrogenic media, total RNA of the cells were extracted using a RNX-Plus kit (CinnaGen) (13). Reverse Transcriptase II (Takara) was used to make cDNA. Expression of some chondrogenic and hypertrophic markers, i.e. Sox9, type II Collagen (Col-II), Aggrecan and type X Collagen (Col-X), MMP1 and MMP3 were assessed using SYBR Green Master Mix (Applied Biosystems) by quantitative Real-Time PCR using primers as reported previously (14, 15). The relative expression of the genes were estimated as the relative ratio of target gene to b-actin and expressed using ΔΔCt in sorbitol to NaCl cultures.

Calcium content assay

Any possible deposited calcium was extracted from the monolayer cultures by incubating them overnight in 10% acetic acid at RT. A calcium content assay kit from Pars-Azmun was used to estimate the deposited calcium in the cultures, as per manufacturer's instructions. The absorbance was read at 570 nm on a micro plate reader.

Evaluation of inflammatory potential

The secretion of TNF-α, as a pro-inflammatory cytokine, was assessed using mini ELISA development

kit (Peprotech). After two weeks of induction in either control or +100 mOsm hyperosmotic chondrogenic conditions, culture media were collected and tested for TNF-α secretion as per manufacturer's instructions using the standards provided in the kit. There were at least three biological replicates and two technical replicates for this assay.

Statistical analysis

Statistical analyses were carried out using one-way analysis of variance (ANOVA) and the differences were regarded as significant if *P* value was less than 0.05. The presented data are mean ± standard deviation. All samples were run in triplicate and the experiments were repeated two times.

Results

Proliferation pattern under hyperosmotic condition

We first performed MTT assay to determine whether stem cells would get affected by the osmolyte type used to produce the +100 hyperosmolar condition. Phenotypically, cells were looking similar in control and hypertonic conditions over a one week period. As dedicated by the growth profile of cells (Figure 1), MSC facing hypertonic conditions had a delayed growth in the first 4 days, when compared with control samples having expansion media. After adaptation to hypertonic conditions, the cells started to increase in number with a similar rate as that with control condition. This lag phase was similar in both NaCl and Sorbitol groups and seems rationale as cells were already expanded in normal tonicity (350 mOsm) conditions and needed time to adapt to hypertonic conditions. This 4 day adaptation phase was observed for cultures grown with hypertonic "expansion" media. No significant difference was observed for cells cultured under hypertonic "chondrogenic" media with typical chondrogenic "control" group (data not shown).

Gene expression

Regarding the expression level of major chondrogenic and hypertrophic genes no identical response was observed between the two treated groups. Although both hyperosmolar conditions induced chondrogenic markers and suppressed Col-X as a hypertrophic marker, the level of impact was different between the two treated groups (Figure 2). We noticed a difference in the amplitude of the expression level depending on the nature of the osmolyte. That is, while both conditions induced chondrogenic marker expression, sorbitol caused nearly three times higher expression of Col-II and Sox9 and 1.5 times higher expression of Aggrecan, when compared to salt group. However, exposition to the hyperosmotic condition also slightly upregulated hypertrophic markers Col-X and MMP1 and 3. While NaCl had induced Col-X expression 25 percent more than sorbitol, sorbitol had more inductive effects on MMP1 and 3 expression. Compared to the control differentiation group, salt had induced two-fold upregulation in Col-X with no significant effect on MMP1 and 3, while sorbitol had not affected Col-X expression but causing more than two folds upregulation for MMP1 and 3.

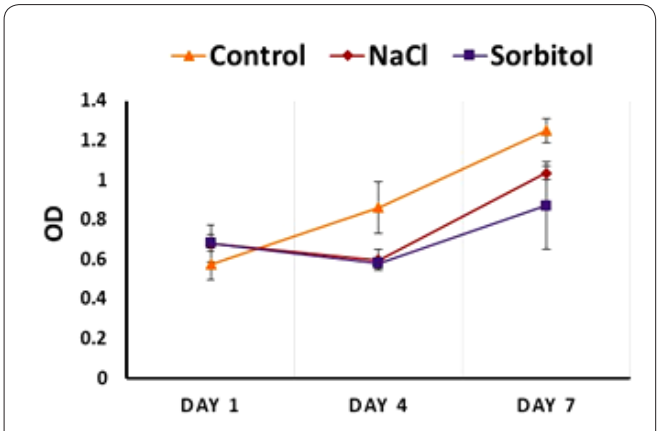

Figure 1. Effect of hypertonic conditions on cell proliferation in expansion media. There was no significant difference between NaCl and Sorbitol groups. Values are triplicate mean ± SD.

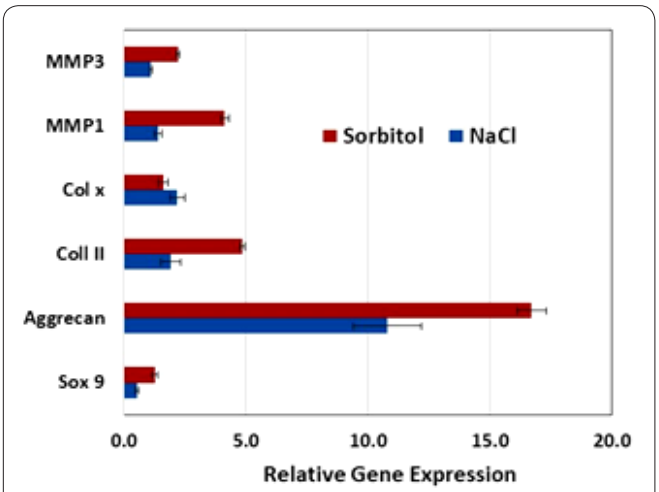

Figure 2. Chondrogenic and hypertrophic marker expression after two weeks of normal or hypertonic chondrogenic culture. Gene expression was calculated using ΔΔCt method and presented as values of Sorbitol or NaCl group relative to control group. Sorbitol had more chondro-promotive and Col-X suppressive effect than NaCl. However MMP1 and 3 showed higher expression in Sorbitol group.

Calcium deposition effect

Calcium deposition was investigated via quantification of mineralized calcium which is an indicative of osteogenesis and hypertrophy. Calcium deposition was

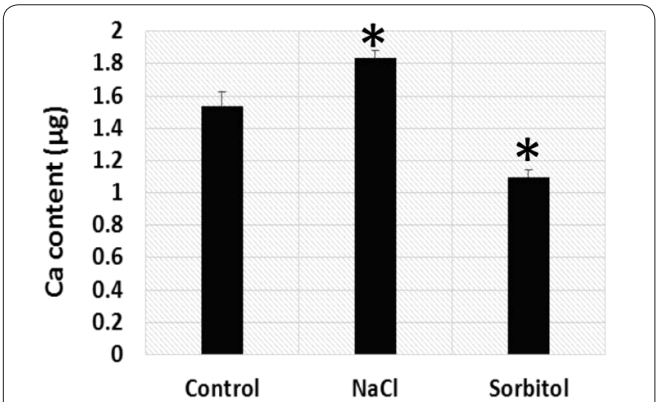

Figure 3. Osteogenic potential of hypertonic stress. The amount of deposited calcium was assayed in cultures after two weeks of induction in chondrogenic medium. While the presence of NaCl had induced calcium deposition, Sorbitol had significantly decreased the osteogenic potential up to 60% when compared to NaCl group. The * implies statistical difference ($P<0.05$) with control group.

significantly but slightly higher in +100 mOsm NaCl group compared to control (Figure 3). Whereas, presence of sorbitol caused a 30 percent decrease in calcium deposition in comparison to control group.

Effect of hyperosmolarity on inflammatory responses

We assessed the secretion of pro-inflammatory cytokine, TNF-α, released into the media by cells exposing to hypertonic chondrogenic media. The amount of TNF-α showed an osmolyte-agent dependent manner. While the hyperosmolar condition obtained by adding sorbitol had no inductive or suppressive effect on TNF-α secretion, the presence of salt had caused an inflammatory-suppressive effect compared to normal chondrogenic media (Figure 4), which was almost half of that observed in control group.

Discussion

Although promising, the capability of AD-MSC for cartilage cell therapy has its own limitations in *in vivo* studies. This is in part attributed to AD-MSC having inherent angiogenic and hypertrophic potential (16), and the fact that chondrogenic pathway is usually accompanied or followed by other pathways such as hypertrophy, angiogenesis and inflammations that are undesired when aiming to generate hyaline chondrocytes(17, 18). Hypertrophic chondrocytes start mineralization which causes the cartilage to lose its viscoelastic properties and hydrostatic pressure (4). On the other hand, production of pro-inflammatory cytokines in the neo-tissue could trigger arthritic-like symptoms (19). In order to optimize in vitro chondrogenesis and obtaining chondrocytes with a stable chondrogenic phenotype, various growth factors, chemical and biophysical approaches have been tried out(20, 21). The first goal of this study was to evaluate how the osmolyte dependent attributes rather than osmolarity, such as ionic strength (a in NaCl) or inertness (as in sorbitol) would affect chondro-promotive effects of hypertonic stress and secondly whether the highly acclaimed and highly approved hyperosmolarity would bring about undesired side effects during in vitro chondrogenesis.

By comparing cell propagation under hypertonic and normal expansion media we observed that AD-MSC would not prefer hypertonic media and thus cannot proliferate as those cells cultured in normal 350 mOsm expansion media. However, chondrogenic induction factors present in chondrogenic media (such as TGF-β) can help cells better adapt with hypertonic conditions which then brought about the same growth trend in hypertonic conditions as that under normal chondrogenic cultures (data not shown). This seems rationale regarding the fact that chondrocytes are surrounded by a hypertonic microenvironment in their natural niche and thus MSC receiving chondrogenic signals would better adapt with hypertonic conditions.

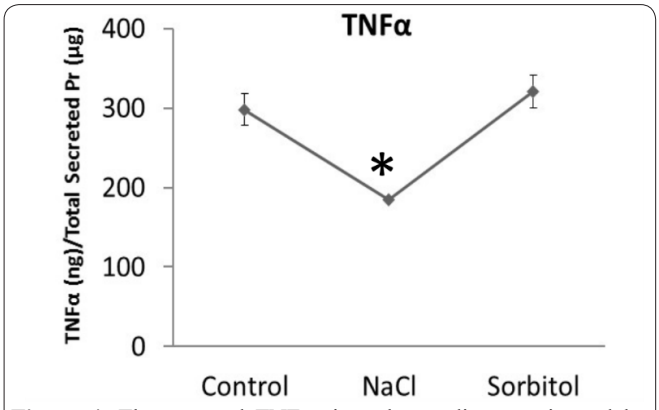

Figure 4. The secreted TNF-α into the media as estimated by ELISA and normalized to total protein. Sorbitol had no effect on TNF secretion, however Salt showed anti-inflammatory effects on chondrogenically differentiated cells. The * implies significant difference with control group (P<0.05).

By exposing MSC to hyperosmolar chondrogenic media it seems rationale to see cells acquiring a chondrogenic phenotype. However, points usually neglected or disregarded include side by hypertrophic, angiogenic, pathologic and inflammatory pathways that might be induced by the external bio-physicochemical cue alongside or following the main chondrogenic pathway. As such, paying a closer attention to the induction or inhibition of parallel pathways would benefit us when exploiting external bio-physicochemical cues to drive the cells more specifically down a favorable pathway. We analyzed Col-X expression and calcium deposition as markers of hypertrophic differentiation. We observed that compared to hypertonic salt containing media and typical chondrogenic condition (control), hypertonic sorbitol containing culture had more chondro-promotive and hypertrophic prohibitive effects.

Like every healthy tissue, in cartilage there exist a balance between ECM synthesis and degradation. The upregulated activity of matrix metalloproteinase (MMP) is considered a pathophysiologic event in osteoarthritis (22). We observed a significant increase in MMP1 and MMP3 gene expression in sorbitol treated groups in comparison to salt group. In fact salt caused no MMP overexpression compared to the control group but sorbitol caused a four and two fold increase in MMP1 and 3, respectively. Likewise, in corneal epithelial cells hyperosmolarity is shown to enhance MMP expression (23). We thus concluded that in spite of promotive effects of hypertonicity on MSC chondrogenesis, other catabolic pathways might be triggered alongside, and this response is osmolyte type dependent. We did also assay the secretion of VEGF after two weeks in hypertonic chondrogenic cultures. As we did not observe any significant difference between the salt and sorbitol groups we did not include the data herein. Though notably that in both hypertonic conditions the amount of VEGF secretion had shown nearly a 40 percent decrease compared with typical chondrogenic media.

Hyperosmolarity is associated with certain inflammatory diseases and disorders (9). The implication of IL-1 signal transduction pathways under static mechanical compression of articular cartilage(24) and hyperosmotic shock obtained by sorbitol addition has been demonstrated (25). So we questioned whether in MSC chondrogenesis the hyperosmotic condition invoked to act as chondro-stimulatory factor would also provoke inflammatory responses leaving negative consequences on the usefulness of the differentiated cells for cartilage cell therapy. The previously published findings have shown that exposition of cells to hypertonic stress can result in the expression of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α (26). On the other hand

TGF-β can result in anti-inflammatory effects. In fact TGF-β has complicated immune-regulatory effects. TGF-β1 is a potent immune-suppressor. However in an inflammatory environment it plays a dual pro-inflammatory role at first and an immunosuppressive effect at later stages. As such, cells exposed to hypertonic chondrogenic medium face both inflammatory and anti-inflammatory inductions from their environment. Regarding the fact that inflammation and the expression of inflammatory and angiogenic cytokines is associated with arthritic cartilage and is assumed as a pathologic marker in the cartilage tissue, we questioned whether presence of TGF-β could counteract hypertonicity associated inflammatory response or not. We observed that the type and amplitude of the response correlated with the type of the agent utilized to increase medium osmolarity. That is while hyperosmotic condition obtained by sorbitol did not exacerbate or improve the pro-inflammatory marker expression compared with typical chondrogenic group, NaCl nearly halved the secretion of TNF-α. This would suggest that likely the ionic strength of the medium and the TGF-β had an additive effect to suppress the pro-inflammatory cytokines. However, this phenomenon cannot solely be correlated to the ionic strength of the medium in the salt treated hypertonic group. Monovalent ions such as Na^+, K^+ and Cl^- play critical roles in cellular and extracellular processes and it is so likely that physical effects attributed to charge and partitioning, such as ionic strength, contribute to the differential response of the cells under NaCl or sorbitol hypertonic stress.

The increased sorbitol pathway activity is seen in diabetes mellitus (DM) disease which can cause inflammation associated hypertrophy (27). In DM, extra glucose is reduced by aldose reductase to sorbitol and the resultant increased intracellular sorbitol might damage cells and cause hypertrophy. Clearly, different pathways should be involved in cells exposing to high intracellular sorbitol level in DM disease and stem cells exposed to extracellular sorbitol in *in vitro* chondrogenic conditions which unlike DM, can benefit chondrocytes and cartilage tissue properties. In fact TGF-β with its critical role in ECM synthesis is upregulated by the AR-mediated polyol/sorbitol pathway (27). Cartilage can modify its GAG content, the sulfation level and the active transport mechanisms, generating a Nab-mediated hypertonic microenvironment around the resident cells. This highly regulated mechanism basically gives cartilage its hydrostatic pressure, viscoelastic and load-bearing properties (28). Just like every living cell, chondrocytes use mechanisms to regulate their intracellular electrolyte and osmolyte concentrations. Hdud et al. outlined the differential expression of two osmolyte channels (TRPV4 and BKCa) in equine articular chondrocytes under different osmolarity situations, i.e. hypo- and hyper-osmolarity, compared to endogenous chondrocyte environment (29). Using sucrose as the osmolyte they showed the phosphorylation of p38 MAPK by hyperosmotic stress and outlined its role in protection against hypertonic stress.

Tonicity enhancer binding protein (TonEBP), also known as NFAT-5 is a transcription factor that activates osmo-protective genes serving to protect the cells exposed to the hypertonic microenvironment (9). It plays several roles in maintaining cellular structure and development. It can also activate expression of pro-inflammatory cytokines in some diseases. In IVD, a fibrocartilage tissue between vertebras, TonEBP/NFAT5 regulates the level of non-ionic osmolytes such as myoinositol and sorbitol, in order to maintain the tissue hypertonic environment. The complex interactions between the osmotic receptors, channels and transporters and the various signaling pathway mediators and elements might contribute to the differential and osmolyte-type dependent effects observed here. However, since all the cultures were supplemented with growth factor formulations typically used for chondrogenic cultures, any observed effects in hyperosmotic environment on cell fate and differentiation may be the result of combined osmotic and growth factor effects.

The mechanisms that sense and transduce osmotic stress are poorly understood. But definitely, different pathways might take the major role in sensing and reacting to hypertonic stress, when different osmolytes are involved. Though much more in depth studies are needed to find the best osmolyte and the optimal osmolarity, we aimed to bring to notice the side by responses that normally does not attract researchers' attention.

In the present study we aimed to clarify how the osmotic stress and the osmolyte agent would impact and modulate chondrogenesis and hypertrophy of MSC. We questioned if any patho-mechanism is activated by hypertonic stress in chondrogenic cultures and how would be affected by the osmolyte agent. We paid special attention to undesired hypertrophic and a few of catabolic and pro-inflammatory markers, in the course of MSC chondrogenesis under TGF-β chondrogenic medium in combination with +100 mOsm hyperosmolarity using either NaCl or Sorbitol. We showed that while application of hypertonic stress can both enhance chondrogenesis and suppress hypertrophic and inflammatory markers, the type and amplitude of the response is a function of the osmolyte agent. While sorbitol-dependent hypertonic stress had no inflammatory evoking effect, salt could even decrease the expression of TNF-α to about half that in control group. Also, we observed a sorbitol hypertonicity attributed worsening effect on MMP expression. Basically we wanted to investigate whether hypertonic stress can reliably be used as a biophysicochemical cue for MSCs in cell-based repair strategies of chondral lesions. Though there are a lot to be resolved in details, our results arouse concern about the neglected undesired pathways normally accompanied or followed by chondrogenesis. The mechanisms that transduce osmotic stress to the cell as well as the pathways affected by are not yet fully discovered. Here we did not surveyed the pathways involved in sensing and transduction of osmotic stress signal from environment in to the cells. It is worthy to investigate the signaling pathways involved including the angiogenesis and inflammatory responses**.** Our study was a step towards developing a more reliable hypertonic condition as an external cue to optimize chondrogenesis while avoiding undesired effects of inflammation, vascularization, and ossification.

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Interest conflict

The authors declare there is no conflict of interest.

Author's contribution

SA: Performed the main experiments, MK: Designed and supervised the work, Drafted the MS, NT & SH & MK: Revised and Edited the MS, AH: Did the gene expression analysis, MS: Supported and advised the study AF: Advised the study, HHA: Designed and supervised the work.

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