chapter **FIVE**

HYPEROSMOLARITY AND HYPOXIA INDUCE CHONDROGENESIS OF ADIPOSE DERIVED STEM CELLS IN A COLLAGEN TYPE 2 HYDROGEL

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ABSTRACT

Apart from soluble growth factors, various other biophysicochemical cues are known to promote chondrogenesis. Under physiological conditions cartilage in the joint comprises a hyperosmotic and hypoxic environment. Therefore, in this study, we examined the inductive effects of hyperosmotic and/or hypoxic conditions on cultured adipose stem cells (ASCs) and compared them with conventional TGFβ1-induction. After encapsulation in collagen type II hydrogels and specific induction, ASCs were assessed for viability, proliferation, morphology and chondrogenic differentiation potential. Viability was similar under all conditions with low proliferative activity. After 4 days, hypoxia and/or hyperosmolarity did not affect round cell morphology in collagen type II hydrogels , while cells were mainly stretched in the TGFß1-induced group. At 21 days, the TGFß1-treated group had aggregated into a cell nodule. Hyperosmolarity mimicked this aggregation to a lesser extent, whereas cells under hypoxia stretched out after 21 days, with a combined effect in the hypoxic/hyperosmotic group. Both individual and combined hyperosmotic and/or hypoxic conditions significantly upregulated SOX5, SOX9, COMP and Link-p gene expression compared with the non-induced group, and to similar levels as the TGFβ1-induced group. GAG synthesis in both hydrogel and medium was increased under hypoxic conditions, whereas hyperosmolarity decreased GAG formation in the hydrogels, but increased GAG formation in the medium. We conclude that in a joint mimicking the three-dimensional (3D) micro-environment, a combination of hyperosmolarity and hypoxia is able to induce chondrogenesis to the same extent as TGFβ1. This might lead to an interesting alternative when considering short-term triggering in a one-step surgical procedure for the treatment of cartilaginous defects.

INTRODUCTION

Cartilage tissue is well known for its avascular and aneural nature. As a consequence, cartilage shows only limited self-regenerative potential. Conventional treatments aimed at restoring or repairing the damaged cartilage tissue result in suboptimal functional repair[1,2]. Recently, regenerative therapies combining cells, scaffolds and biological factors aimed at biological repair and restoration of function are emerging. Mesenchymal stem cells (MSC) may be ideal candidates for these regenerative cell therapies, due to their high proliferation rate, multidifferentiation capacity[3] and superior cartilage quality[4,5]. Besides bone marrow, MSCs have been isolated from other sources like muscle, periosteum, cord blood and adipose tissue[6]. Adipose tissue is an attractive source since it is abundant, easy accessible through liposuction or resection, and contains a large number of stem cells, referred to as adipose derived stem cells (ASC)[7]. These characteristics make them a suitable candidate to apply in the one-step procedure for the treatment of (osteo)chondral defects[8,9].

In general, induction of these cells into the chondrogenic lineage is accomplished by the addition of biochemical cues like (combinations of) growth factors, in particular members of the tissue growth factor β superfamily (TGFβ1 and BMPs), insulin growth factor, or fibroblast growth factor[10]. In addition, biophysical cues like hypoxia[11,12], hydrostatic pressure[13,14], and ultrasound[15] have been found to induce chondrogenesis in MSCs.

Next to the induction by biophysicochemical cues, the behaviour and differentiation of MSC can be influences by the complex biophysicochemical environment in which chondrocytes of articular cartilage reside[16]. This environment is avascular and the residing chondrocytes exists at a decreased oxygen tension of (1 to 7%)[17,18]. In chondrocyte culture systems increased synthesis of extracellular matrix (ECM) by chondrocytes under hypoxia has been shown[19], and this has been extended to stem cells from bone marrow[20] and liposuction-derived adipose tissue [17] undergoing chondrogenesis. Next to a hypoxic environment, chondrocytes experience a unique ionic/osmotic environment, which is attributable to the polyanionic proteoglycans. They carry a high density of fixed negative charges that attract cations into the interstitial fluid[21]. Extracellular osmolality, which arises principally from the free extracellular ions, is in the range of 350–485 mOsmol, depending on cartilage type and zone, with cation concentrations, and hence interstitial osmolarity, following local proteoglycan gradients[22]. In both chondrocytes and intervertebral disc cells improvement in chondrogenic geno- and phenotype was found in an hyperosmotic environment[23,24] and similar results were found in stem cells from bone marrow upon chondrogenic induction[25]. As stated above, this environment cannot only negatively influence resident cells leading to the degeneration of joints, but moreover, may be critical for ASCs upon implantation in the joints for regenerative therapies, e.g. in a one-step surgical procedure for cartilage regeneration[26]. So far, the behaviour of ASC to the microenvironmental conditions of the joint is quite unknown or only partially investigated[11,12,17,27].

In this study, ASC were encapsulated in a collagen type 2 gel to induce chondrogenesis. The effect of two biophysicochemical microenvironmental conditions of the joint were

explored, i.e. reduced oxygen concentration and high extracellular osmolarity. The influence of these two biophysical cues on the chondrogenesis of ASCs were compared with conventional biochemical induction with TGFβ1. The parameters examined were viability, cellular morphology (well-known to influence stem cell lineage commitment[32-34], proliferation, and the chondrogenic differentiation capacity of cultured ASC encapsulated in a collagen type II hydrogel.

MATERIAL AND METHODS

Adipose tissue-derived stem cell (ASCs) isolation and culture
Retrieval of human specimens was approved by the ethical committee of the VU University medical center, and informed consent was obtained.

ASCs were isolated from subcutaneous adipose tissue from patients ($n = 9$) undergoing elective surgical procedures, as described previously[31]. Briefly, harvested tissue was enzymatically dissociated with 0.01 M Collagenase A (Roche diagnostics), decontaminated from erythrocytes by Ficoll density centrifugation, and the remaining stromal cells were plated in 25 cm² tissue culture flasks. Plating and expansion medium consisted of Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS; Hyclone), 100 U/ml penicillin, 100 µg/ml streptomycine and 2 mM L-glutamine (all Invitrogen). Cultures were grown in a humidified incubator at 37°C in an atmosphere of 5% CO_2. On reaching 80-90% confluency, cells were detached with 0.5 mM EDTA/0.05% trypsin (Invitrogen), for 5 min at 37°C, and replated. In this way, a homogeneous population of ASCs was obtained, which was subsequently used for differentiation experiments at passage 3–4.

Embedment of ASC in collagen II hydrogels and in vitro chondrogenic induction.
Collagen type II hydrogels were prepared by mixing six parts of collagen II (chicken sternal cartilage; Sigma), one part of 10x DMEM, one part of reconstitution buffer (2.2 g $NaHCO_3$ in 100 ml of 0.05 N NaOH and 200 mM Hepes) and two parts of cell suspension in culture medium. The final concentration of collagen type II was 2.4 mg/ml and the cell density was 1.5×10^6 cells/ml hydrogel. All the components were mixed on ice; 100 µl mixtures were seeded in 6-well plates and collagen hydrogel lattices were formed by placing the plates in a humidified incubator at 37°C in an atmosphere of 5% CO_2 for 4 h. Thereafter constructs were divided in 5 different experimental groups, in which different media under various culture conditions was carefully overlaid:

1. T*he non-induced group* in plain medium [DMEM, containing ITS+™ Premix (BD Biosciences), 1% FCS (Hyclone), 25µM ascorbate-2-phosphate (Sigma), 100 U/ml penicillin, 100µg/ml streptomycin and 2 mM L-glutamine], under normoxic and iso-osmotic culture condition.
2. *The positive control group* in chondrogenic, iso-osmotic medium [plain medium supplemented with 10 ng/nl transforming growth factor- β₁ (TGFβ₁, ITK-diagnostics)] under normoxic culture condition. 3. *The hyperosmotic group* in plain, hyperosmotic medium (500 mOsm) under normoxic culture conditions.

3. *The hyperosmotic group* in plain, hyperosmotic medium (500 mOsm) under normoxic culture conditions.
4. *The hypoxic group* in plain, iso-osmolar medium under hypoxic (1% oxygen) culture conditions.
5. *The combined group* in plain, hyperosmotic medium under hypoxic culture conditions.

Osmolarity was adjusted by adding 5.4 g/L NaCl to the plain DMEM medium (320 mOsm), and measured using an osmometer (KNAUER, Germany). The hypoxic and combined groups were exposed to hypoxic conditions in a 5% CO_2 / 1% oxygen custom-designed hypoxia workstation (T.C.P.S. Rotselaar, Belgium) at 37°C in a humidified atmosphere; values for hyperosmolarity and hypoxia were based on previous experiments and experiments performed by others[9,24,25]. Hypoxic and combined hypoxic and hyperosmotic media were preincubated for 2h to reach the designated oxygen level prior to application. Medium (2 ml per well) was refreshed every 3–4 days until day 21.

Cell viability and proliferation

Cell viability ($n = 4$) in all groups was tested using a Toxilight® kit (Lonza) after 4, 7, 11, 14, 17 and 21 days according to the manufacturer's protocol. This assay is based on the release of the enzyme Adenylate Kinase (AK) from damaged cells and allows the accurate and sensitive analysis of cytotoxicity and cytolysis. ADP is added as substrate for AK, in which presence it is converted to ATP releasing a signal for assay by bioluminescence. Luminescence was measured using a luminometer (Victor microplate reader).To assess cellular toxicity, 100 μL spent medium was used per condition and time-point, and measured in duplicate. Blank medium and medium from lysed cells served as negative and positive controls, respectively. The results are depicted as luminometer light output (RLU). Proliferation ($n = 4$) of ASC in all groups was determined using CyQUANT® (Molecular Probes) analysis, as described previously[9].

Cytoskeletal organisation

The effects of different culture conditions on the cytoskeletal organization of ASCs was assessed by labeling the actin cytoskeleton, as previously described ($n = 5$)[32]. ASCs were cultured under specific conditions, as described above for 4 and 21 days, and fixed using 4% formaldehyde. F-actin fibers within the ASC were stained with 2.5 Units Alexa fluor 488 Phalloidin (Invitrogen) in PBS in the dark for 1 hour, followed by direct acquisition with confocal laser scanning microscope (Leica TCS SP5) using a 20x Carl-Zeiss lens. In this way, 2D images of the 3D environment of the hydrogel were obtained.

Quantitative Real-time-Polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the tissue using Trizol (Invitrogen) according to the manufacturer's instructions ($n = 9$). RNA isolation and Reverse transcription were performed as previously described[30]. Relative target gene expression of aggrecan (ACAN), collagen type II (COL2A), collagen type X (COL10α1), cartilage oligomeric matrix protein (COMP), link protein (LINK-p), and the early transcription factors SOX-5/6/9 were determined in a

LightCylcer 480 (Roche Diagnostics). Target genes were normalized to 18s housekeeping gene-expression to obtain the relative gene expression. Primers (Invitrogen) used for real-time PCR were previously described[9,30].

Using Light Cycler software (version 4), the crossing points were assessed and plotted versus the serial dilution of known concentrations of the standards derived from each gene using Fit Points method. PCR efficiency was calculated by Light Cycler software and the data were used only if the calculated PCR efficiency was between 1.85-2.0.

Histochemistry
Histochemistry (n = 4) was performed as described previously[31]. In brief, medium was removed from the construct, rinsed with PBS twice and fixed using 4% paraformaldehyde. Then collagen type II hydrogels from each culture condition were stained directly with Alcian blue (Sigma) at acidic pH (1.0) for visualization of proteoglycans after 4 and 21 days.

Extracellular Matrix Biosynthesis
Extracellular sulfated glycosaminoglycans (GAG) deposition in the collagen type II hydrogels was quantified after harvesting at 14 and 21 days. Overlying media were collected after 7, 14 and 21 days (n = 4). The tissue-containing hydrogels were digested in 3% papain solution buffer overnight at 60°C. GAG were measured using a Blyscan kit (Biocolor LTD, Nothern Ireland). Results were depicted as the total glycosaminoglycan production per gel, corrected for the amount of cells (DNA) in the gels using CyQUANT® analysis according to the manufacturer's protocol. Furthermore GAG release in the medium was depicted per ml medium as a treatment over control ratio (non-induced value set at 1).

Statistics
Kolmogorov-Smirnov tests were used to determine the normalcy of measurements and, if appropriate, their logarithmics. Repeated measures ANOVA was used to determine significant differences when increasing time-points in one donor within one variable were compared, followed by Bonferroni post-hoc analysis. If levels of gene expression were below the detection limit (0.05), values were set at 10^{-2} (or log level at -2). All statistical tests used a significance level of α=0.05.

RESULTS

Cultured ASC in collagen type II hydrogel show similar viability and proliferative activity in all groups
Toxicity was 5-fold higher during the first 4 days in all groups (Figure 1). Compared with the positive control (known amounts of lysed ASC) and values of the CyQUANT assay, this corresponded with ± 75 000 cells. Thereafter, toxicity dropped to low levels, and cell number and viability remained constant, indicating a low proliferative activity (Figure 1). No significant differences in proliferative activity could be detected between the various groups at any time point (data not shown).

A
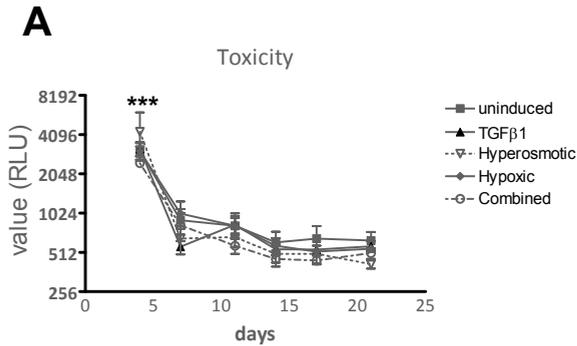
Figure 1. Cellular toxicity under different biophysicochemical conditions of ASCs cultured in collagen type II gel. Cellular toxicity was initially high due to harsh biophysicochemical induction on day 4 in all groups, thereafter decreasing to low values. Results are depicted as mean ± SEM (n=4); ***p<0.001

Hypoxia and hyperosmolarity cause round morphology of ASCs in collagen type II hydrogel during early induction, whereas hypoxia stimulates stretching during later induction

Actin filaments were stained to visualize cell shape or cytoskeletal organisation of the cells (Figure 2). This figure represents 2D images from the 3D situation in the hydrogels. This implicates that not all cells are in the same plane of focus in the gel. The morphological descriptions below therefore may not be captured in the images provided, but may be deduced from full 3D analysis of the gels.

After 4 days, most of the ASC in all groups -except the TGFβ1-induced group- showed round cell morphology in a collagen type II hydrogel with non-stressed or granule-like actin
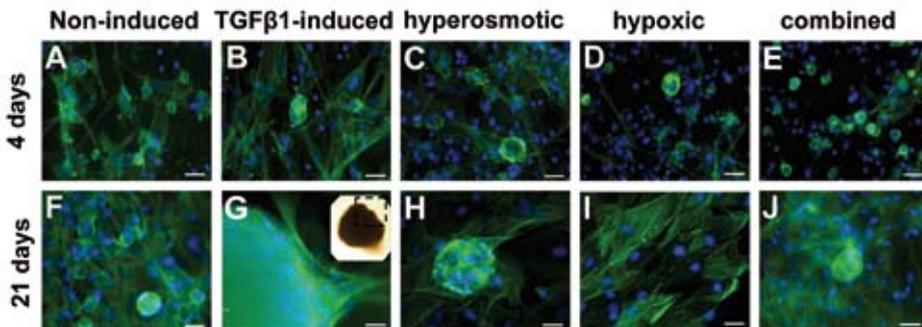
Figure 2. Morphology of ASCs in collagen type II gel after culture for 4 days (A–E) and after 21 days (F–J) under various biophysicochemical conditions. Green fluorescent staining represents actin filaments, blue staining represents cell nuclei. ASCs in collagen type II gel mainly showed round morphology after 4 days under various biophysicochemical conditions, with relaxed actin filaments, except for the TGFβ1-group (upper row). After 21 days of culture under various conditions, the non-induced cells remained round, whereas the TGFβ1-group contracted into a nodule (see insert in G). Cells cultured under hypoxic conditions clearly stretched out after prolonged culture, whereas hyperosmolarity induced small nodules residing in the gel on top of stretched cells with stressed actin filaments. Bars=20μm

filaments (Figure 2A-E). Only ASC in the collagen type II hydrogels that touched the bottom of the wells stretched out, showing stressed actin filaments. Combined hyperosmotic/hypoxic conditions seemed to reduce cell surface slightly (Figure 2E). In contrast, TGFβ1-stimulated ASC were mostly elongated with stressed actin filaments (Figure 2B). After 21 days, round cell morphology with membrane ruffles was maintained in the non-induced ASCs (Figure 2F), whereas TGFβ1 stimulation resulted in chondrogenic nodule formation with round cells and dispersed actin cytoskeleton (Figure 2G). Hyperosmolarity maintained round cell shape after 21 days, with low aggregation level resulting in only small round cell masses with relaxed actin filaments and slightly more elongated cells with stressed actin filaments at the hydrogel bottom (Figure 2H). Hypoxic conditions caused elongated cells with stressed actin filaments after 21 days (Figure 2I). A combined pattern could be observed in the hypoxic and hyperosmotic group (further referred to as combined group; Figure 2J).
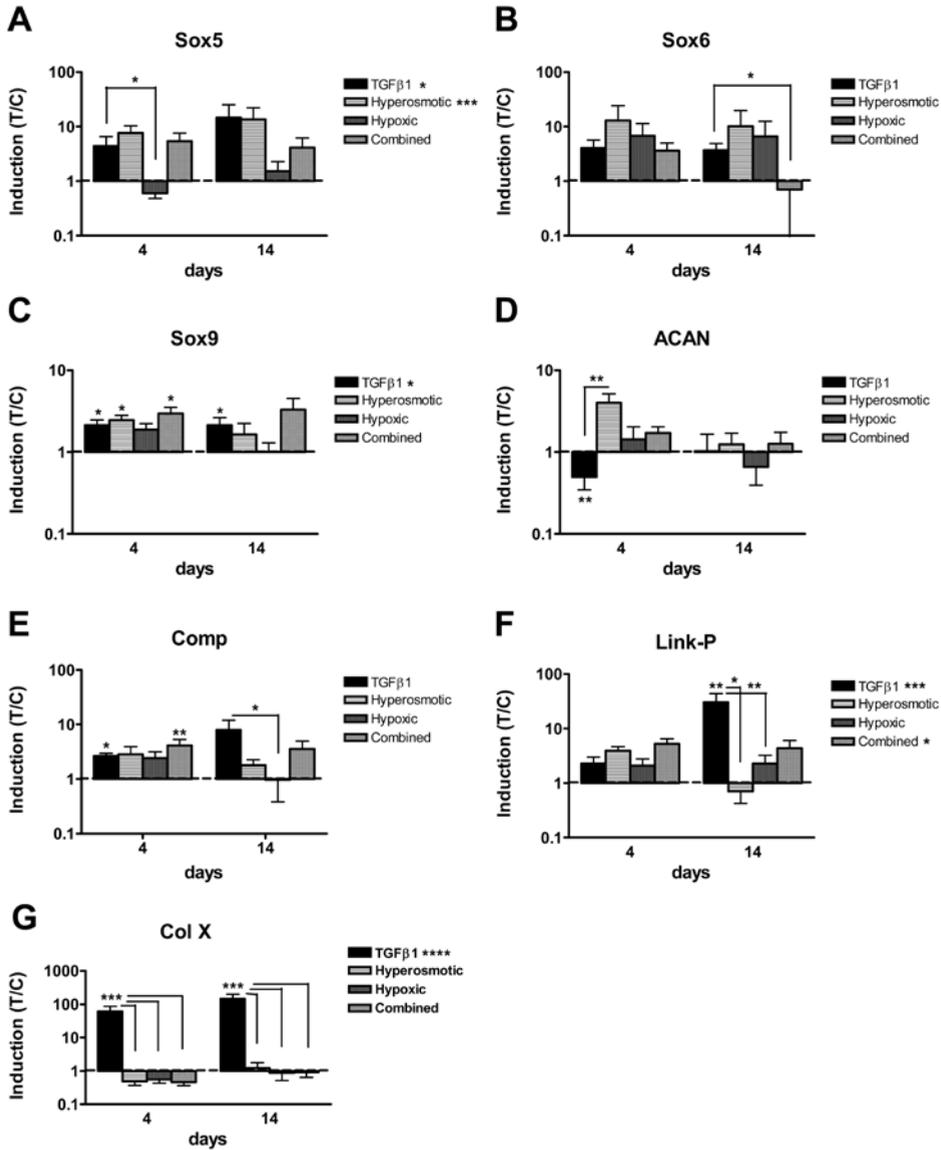
Hypoxia and/or hyperosmolarity stimulate chondrogenic gene expression comparable to TGFβ1-induction

Chondrospecific gene expression analysis showed significant upregulation of the early transcription factor SOX-5 at both time points in the TGFβ1-induced, hyperosmotic and combined groups (Figure 3A), whereas the hypoxic group showed SOX-5 downregulation after both 4 and 14 days when compared to the non-induced group. For SOX-6, only the combined group showed a significant difference (in this case downregulation) compared to the TGFß1-stimulated group after 14 days of induction (Figure 3B). SOX-9 was significantly increased (compared to non-induced control) in the hyperosmotic and combined group after 4 days and at both time points in the TGFß1-induced group. ACAN gene expression was increased in the hyperosmotic group after 4 days and at both 4 and 14 days in the combined group compared with the TGFß1-induced control (Figure 3D). COL2a expression could only be detected, but not quantified, in 5 f 9 donors in all groups (data not shown). The late marker COMP was down-regulated in the hypoxic group, whereas the combined group showed significantly up-regulation of COMP to similar levels as TGFß1-induced controls (Figure 3E). A trend was only visible between the non-induced group and both the TGFß1-induced and combined groups, on both time-points (p=0.08 for both). LINK-p gene expression was upregulated at both time points in the TGFß1-induced and combined groups compared to the non-induced group (Figure 3F). A significant difference in the LINK-p gene expression was observed after 14 days between the positive control and the hyperosmotic, the hypoxic and the non-induced group, but not in the combined group. Lastly, the hypertrophic gene COL10 was clearly expressed significantly more in the TGFß1-induced versus all other groups at both time-points (Figure 3G).

Hypoxic condition stimulates extracellular matrix formation

Production of proteoglycans was assessed qualitatively using Alcian Blue staining after 4 and 21 days (Figure 4A-J), and quantitatively by biochemical measurement of sulphated glycosaminoglycan (GAG) deposition using a Blyscan kit after 14 and 21 days (Figure 5).

    After 4 days only the non-induced cells and the positive control started to produce proteoglycans as visualized by Alcian Blue staining, whereas in the hyperosmotic,

Figure 3. Gene expression profile of ASCs cultured in collagen type II gel under various biophysico-chemical conditions compared to the non-induced group (treatment/control). The addition of hypoxia and/or hyperosmolarity clearly induced chondrogenic differentiation to similar levels to the TGFβ1-induced group, as indicated by significant differences in various early and late chondrospecific genes. The hypertrophic marker collagen type X was upregulated only in the TGFβ1-induced group, but not in the other groups. The results are depicted on a logarithmic scale as mean ± SEM (n=9). Significant differences to the non-induced group at a specific time-point are marked with an asterisk only, whereas differences to the TGFβ1-induced group are marked by connected lines. Overall differences between groups are marked in the legend: *p<0.05; **p<0.01; ***p<0.001
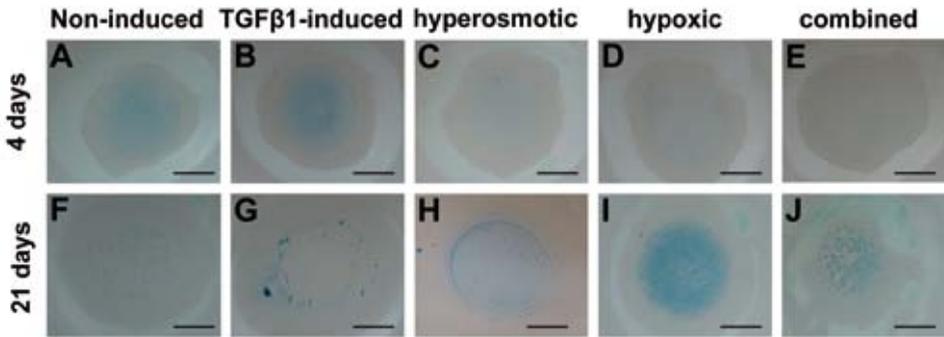
Figure 4. *Alcian blue staining of ASCs in collagen type II gel after 4 days (A–E) and 21 days (F–J) of induction under various biophysicochemical conditions. After 4 days the non-induced and TGFβ1-induced group already showed some Alcian blue staining, whereas not much staining could be seen in the hyperosmotic, hypoxic and combined groups. After 21 days abundant and disseminated staining could be observed in the hypoxic group, whereas more patchy staining was seen in the combined group and lighter staining in the hyperosmotic group. Very bright staining could be seen in the TGFβ1-induced group; however, due to contraction only a vey small nodule was left. The non-induced group stained only very lightly and in a patchy way. Bars=1cm*

hypoxic and combined group less staining could be observed (Figure 4A-E). However, after 21 days abundant proteoglycan formation could be seen [hypoxic > combined group > hyperosmotic group (Figure 4 H-J)]. The TGFß1-induced positive control also stained intensively; however, due to contraction/aggregation, only a very small hydrogel was remaining (Figure 4G), resulting in a localised (non-uniform) staining. This same aggregation was observed in the non-induced control, staining only faintly and in a patchy way (Figure 4F), and, to a lesser extent, a similar patchy staining could be observed in the combined group (Figure 4J).

Quantification of *ECM* formation was performed by measuring the sulphated GAG deposition in the hydrogels and excretion of GAG into the medium. Very little GAG was formed in the extracellular environment of all groups after 14 and 21 days, whereas abundant GAG was released in the medium overlying the hydrogels (Figure 5A vs. 5B). After 14 days a trend towards increased GAG deposition could be observed in the hypoxic group compared with the other groups, however this difference did not reach significance, and decreased after 21 days. In contrast, in the hyperosmotic group and the combined group clearly less GAG was formed in the hydrogels (Figure 5A).

The overall GAG release in the medium in the non-induced group increased from 2.3 ± 0.9 µg/ml at 7 days to 3.9 ± 1.1 µg/ml after 21 days (data not shown). At the 7-day time-point, GAG release was significantly increased in all groups compared with the non-induced group (Figure 5B). The significance reached $p<0.01$ for the TGFβ1-induced and hypoxic groups, whereas the hyperosmotic and the combined hyperosmotic and hypoxic group reached $p<0.001$. After 14 days only a significant increase could be detected due to the addition of hyperosmolarity or hypoxia (both $p<0.05$) and after 21 days no further significant differences in GAG excretion were detected.

A

GAG/DNA

B

GAG/ml medium
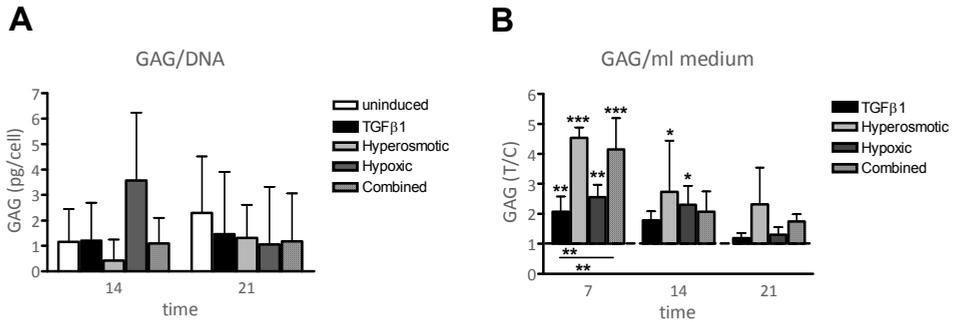
Figure 5. *Glycosaminoglycan (GAG) deposition of ASCs cultured in collagen type II gel under various biophysicochemical conditions. (A) GAG deposition in the gels after 14 and 21 days of induction, corrected for the number of cells. No significant differences in GAG deposition could be observed, although a trend towards increased GAG deposition could be observed in the hypoxic group after 14 days. (B) GAG excreted in the medium after 7, 14 and 21 days of induction corrected to the non-induced group. Since little GAG is retained in the extracellular matrix, most of it is released into the medium. Over time, excretion of GAG into the medium is diminished as the formed GAGs are becoming seques-tered in the extracellular matrix. The results are depicted as mean ± SD (n=4). Significant differences to the non-induced group at a specific time-point are marked with an asterisk only, whereas differences to the TGFβ1-induced group are marked by connected lines; *p<0.05; **p<0.01; ***p<0.001*

DISCUSSION

This study is the first to investigate the effect of an hyperosmotic and combined hypoxic and hyperosmotic microenvironment on the viability, proliferation, morphology and chondrogenic differentiation potential of cultured ASC when encapsulated in a collagen type II hydrogel. The effect of hyperosmolarity was so far only determined in chondrocytes, where it was found to have an inductive effect on chondrogenic gene expressions and proteoglycan biosynthesis[24,33]. Although we realize ourselves that this experimental study represents only a simplified *in vitro* model of the complex *in vivo* joint environment, we are convinced that by varying the biophysicochemical conditions under controlled *in vitro* conditions, the relative influence of each factor on the viability, proliferation and chondrogenic differentiation capacity of the ASC can be determined.

We found that a) viability and proliferation of cultured ASC did not differ significantly between different culture conditions; b) cultured ASC in the collagen type II hydrogel showed mostly round morphology under all conditions except on TGFβ1-induction, which induced strong aggregation of cells into a big cell nodule; c) the 3D environment of the collagen type II hydrogel induced chondrogenic differentiation; d) a combination of hyperosmolarity and hypoxia had similar effects on the induction of chondrogenesis compared with TGFβ1 induction at both the genetic and protein level, as analyzed with qRT-PCR, histology and biochemical assays.

We hypothesized that hyperosmolarity (500 mOsm) in combination with hypoxia could lead to massive cell death due to harsh changes in biophysicochemical environment[25,34].

However, contrary to our expectations, all groups initially experienced high toxicity, whereas from 4 days on toxicity was diminished and cell number remained stable after increasing culturing period (Figure 1). This early toxicity might be rather due to harsh experimental conditions embedding the ASC in the collagen type II hydrogel (e.g. it took 4 hours for the collagen type II hydrogel to solidify, before medium could be added), than to specific biophysicochemical cues. Another explanation can be found in the ontogenic process of chondrogenesis. This process is divided into an early phase of recruited mesenchymal cells aggregation/condensation to form chondroblasts, underlining the importance of cell-cell contact [35-37]. However, in a later phase a shifting occurs from cell-cell to cell-matrix interaction due to the production of abundant *ECM*[38,39] and apoptosis of cells. The toxicity can thus also be (partly) attributed to this developmental process of cartilage formation.

It is known that chondrogenic differentiation requires a 3D environment. Several methods exist to induce chondrogenic differentiation, like the commonly used pellet method, alginate beads or the use of scaffold materials or hydrogels[40,41]. In this study a collagen type II hydrogel was used which, in concordance with earlier studies, on itself already induced chondrogenic differentiation[42,43]. As proposed earlier by our group and also found in this study, the micro-environment plays a pivotal role in this induction, by influencing cell shape and the aggregation of cells[43,44]. This mechanism could explain why other studies using *ECM* proteins present in articular cartilage found a similar induction[34,45] and underlines the importance of matrix elasticity on differentiation of cells[28,29].

While hypoxia and hyperosmolarity resulted in low proliferative activity of ASC, both single and combined induction enhanced differentiation of the ASCs into the chondrogenic lineage, both on the genetic level, by early up-regulation of SOX-genes and COMP and Link-p gene expression, as well as on the protein level, by enhanced proteoglycan production. The effect of hypoxia on ASC was already demonstrated by others[12,27]; however, contradictory results on the influence of hypoxia on the chondrogenic differentiation of ASC were presented. This might be attributed to differences in the culture and/or induction methods used, including the percentage of serum, the use of dexamethasone, the type of growth factor used, glucose and glutamine concentrations and/or the oxygen tensions applied.

Remarkably, in this study, a high gene up-regulation of collagen type X could be observed in the TGFβ1-induced group, whilst the other groups did not show any upregulation. Although sometimes disputed, this gene might be a marker for hypertrophic chondrogenic differentiation[46,47] and concomitant (too) aggressive chondrogenic induction by the addition of TGFβ1. Thus, the combined chondrogenic induction with hypoxia and hyperosmolarity, resulting in a more gradual chondrogenic differentiation, might be preferred.

ECM deposition was compared by visualizing proteoglycan formation with Alcian Blue staining, and quantifying the sulphated GAG deposition in the ECM of the hydrogels and GAG excretion in the medium. The results of the Alcian blue staining seemed to correlate well with the GAG formation in the hydrogels, the hypoxia group staining most intensely and showing the highest GAG formation of all groups at both time-points, followed by the TGF β1-induced and non-induced group. This is in concordance with other studies, showing increased matrix biosynthesis (GAG production) in both chondrocytes

and mesenchymal stem cells when cultured under hypoxic conditions[12,48]. Interestingly, the GAG excretion patterns were clearly different between groups, i.e. whereas GAG excretion was increased in the non-induced, TGFβ1-induced and hypoxic group over time, excretion showed a clear decrease in the hyperosmotic and combined groups between 7 and 14 days. This might be attributed to a less developed extracellular collagen type II deposition in these latter groups in the early period, thus hampering sequestration of the formed GAG in the ECM as also reported for osteoarthritic cartilage[49,50], whereas after a week the ongoing ECM production allowed more efficient GAG encapsulation and concomitant lowered GAG excretion. However, our experimental conditions did not allow this hypothesis to be tested, since cells were cultured in collagen type II hydrogels. This made it impossible to distinguish between native collagen type II and collagen type II from the hydrogel with (immuno)histochemical visualisation or western blot analysis.

This study demonstrated an increase in chondrogenic genes and proteoglycan biosynthesis in response to combined hypoxia and hyperosmolarity, mimicking normal joint environment. However, osteoarthritic joints are characterized by proteoglycan loss, decreased collagen stiffness, and increased cartilage hydration (resulting in a hypo-osmotic environment). Consequently, the resulting increased cell volume of the residing chondrocytes makes them more sensitive to mechanical loading and cell death[51,52]. Although we did not test hypo-osmotic conditions in this study, other studies have shown that GAG production was low under hypo-osmolarity but high when varying the osmolarity between physiological levels[53,54]. This implies that in cellular therapies aimed at curing the osteoarthritic joint, restoration of a GAG sequestering network and re-establishment of a hyperosmotic microenvironment are both important parameters to be taken into account.

From this study it can be concluded that ASCs in a collagen type II hydrogel can differentiate towards the chondrogenic lineage, as investigated by qRT-PCR and histological and biochemical analysis of proteoglycans. Moreover, the combined application of both hyperosmolarity and hypoxia, two biophysicochemical cues present under physiological conditions in the joint environment, is able to induce chondrogenesis to the same level as induction TGFβ1. Therefore, these data provide valuable insights for the development of stem cell based cartilage tissue engineering therapies. This might lead to an interesting alternative when considering short-term triggering in a one-step surgical procedure for the treatment of cartilaginous defects.

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