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Behavior of Mesenchymal Stem Cells in the Chemical Microenvironment of the Intervertebral Disc

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Abstract

Study Design—Responses of mesenchymal stem cells (MSCs) from 2 age groups was analyzed under chemical conditions representative of the intervertebral disc (IVD) (low glucose levels, acidic pH, high osmolarity, and combined conditions).

Objective—To determine the microenvironmental conditions of the IVD that are critical for MSC-based tissue repair and to determine whether MSCs from different age groups respond differently.

Summary of Background Data—MSCs offer promise for IVD repair, but their potential is limited by the harsh chemical microenvironment in which they must survive.

Methods—MSCs were isolated from bone marrow from mature (4–5 month old) and young (1 month old) rats and cultured in monolayer under IVD-like glucose, osmolarity, and pH conditions as well as under a combination of these conditions and under standard media conditions for 2 weeks. The response of MSCs was examined by measuring gene expression (real-time RT-PCR), proliferation (MTT assay), and viability (fluorescence staining).

Results—Culturing under IVD-like glucose conditions (1.0 mg/mL glucose) stimulated aggrecan and collagen-1 expression and caused a small increase in proliferation. In contrast, IVD-like osmolarity (485 mOsm) and pH (pH = 6.8) conditions strongly decreased proliferation and expression of matrix proteins, with more pronounced effects for osmolarity. Combining these 3 conditions also resulted in decreased proliferation, and gene expression of matrix proteins, demonstrating that osmolarity and pH dominated the effects of glucose. Both age groups showed a similar response pattern to the disc microenvironment.

Conclusion—IVD repair using MSCs requires increased knowledge of MSC response to the chemical microenvironment. IVD-like low glucose enhanced matrix biosynthesis and maintained cell proliferation whereas IVD-like high osmolarity and low pH conditions were critical factors that reduced biosynthesis and proliferation of young and mature MSCs. Since osmolarity decreases and acidity increases during degeneration, we speculate that pH may be the major limitation for MSC-based IVD repair.

Keywords

mesenchymal stem cells; intervertebral disc; microenvironment; glucose; pH; osmolarity

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Low back pain is the most common cause of activity limitation in people younger than 45 years and the second most frequent reason for visits to the physician. In addition, low back pain is the fifth-ranking cause of admission to the hospital, and the third most common cause of surgical procedures in the United States.[1] Therefore, costs for the health care system due to low back pain is estimated to be 100 billion dollars.[2] Low back pain is a multifactorial crisis, and intervertebral disc (IVD) degeneration plays an important role in its epidemiology.[3-5] Recent approaches for biologic repair and regeneration of the IVD are under investigation including cell transplantation, administration of growth factors, and gene therapy.[6,7] Mesenchymal stem cells (MSCs) may be ideal candidates for cell therapies and tissue engineering because of their high proliferation rate[8] and potential for multilineage differentiation.[9]

The microenvironmental niche, characterized by the niche cells itself and their chemical and physical environment, has a strong influence on MSC behavior and differentiation.[10] The harsh microenvironment of IVDs can influence resident cells negatively, and may be particularly critical for MSCs that might be implanted in the IVD. The response of MSCs to the microenvironmental conditions of the IVD under healthy and degeneration conditions is largely unknown, and it is not clear which factors, if any, are critical to successful MSC survival, proliferation, and differentiation. The IVD microenvironment has distinct and extreme chemical characteristics that are the focus of this study, including reduced nutrition, high extracellular osmolarity, and acidic pH.

The response of IVD cells to microenvironmental conditions has been partially explored,[11-20] but very few studies investigated MSCs in the IVD niche.[21,22] Further, there is some evidence that the potential of MSCs may depend on the donor's age, although this has not been tested so far with regards to approaches for the IVD. Recent studies were able to show that their potential for differentiation and proliferation may be higher in younger donors compared with older donors.[23-25]

The aim of this study was to investigate effects of the chemical conditions representative of a healthy or mildly degenerated IVD on proliferation, viability, and gene expression of MSCs. We hypothesized certain IVD-like conditions will increase biosynthesis rates and proliferation whereas others will inhibit expression of important matrix proteins and also reduce MSC viability and proliferation. We also hypothesized MSCs harvested from young rats will be more adaptable to the IVD chemical niche than MSCs from mature rats.

Materials and Methods

Cell Isolation and Culture

For cell isolation and culture, all reagents were purchased from Invitrogen (Carlsbad, CA). Femurs from 9 skeletally mature (4–5 months) and 9 young (1 month) rats were bilaterally excised and the ends of the bones were removed. The bone lumen was flushed with isolation medium (see Table 1). Isolated cells were seeded in 25 cm^2 cell culture flasks and kept in an incubator at 37°C, 5% CO_2. After 24 hours, culture medium was changed to remove nonadherent cells (using standard medium), therefore identifying MSCs by the colony forming unit-fibroblast assay (CFU-F assay) as first described by Friedenstein *et al.*[26] MSCs were expanded in monolayer for one passage as described above and then seeded in either 24-well plates for measurement of cell viability and proliferation or in 25 cm^2 cell culture flasks for gene expression analysis. Twenty-four hours later, medium was changed and either standard medium or specific medium with an IVD-like low glucose content, an IVD-like high osmolarity or an IVD-like low pH or the combination of these characteristics was used (Table 1). IVD-like glucose medium was prepared by using a DMEM medium similar to standard DMEM,

but with a glucose content of only 1.0 mg/mL, instead of 4.5 mg/mL.[21] IVD-like osmolarity medium was prepared by adding a sterile solution of NaCl (5 M) and KCl (0.4 M) to the culture medium, therefore increasing the osmolarity from 280 to 485 mOsm.[19] For the IVD-like pH medium, the pH was adjusted from 7.6 to 6.8 by adding 1 M HCl and 1 M NaOH.[27,28] As the pH-adjusted medium had to be incubated for 18 hours at 5% CO_2 and 21% O_2 to reach buffer equilibrium, each culture medium was kept in the incubator 18 hours before use. MSCs were cultured under these specific conditions for 2 weeks with medium changes twice a week. MSCs cultured under standard culture conditions served as a control group for all other conditions. Analysis of gene expression, proliferation, and viability were performed on up to 9 different MSC cultures for each age group.

Real Time RT-PCR

After 2 weeks, cells were trypsinized and lysed with β-mercaptoethanol (Sigma, St. Louis, MO). RNA was isolated by use of the GenElute mammalian total RNA Kit (Sigma) and reverse transcribed into cDNA with MultiScribe reverse transcriptase using the TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA). For each sample, duplicate analysis of the mRNA levels were measured using real-time RT-PCR on the GeneAmp 7700 Sequence Detection System (Applied Biosystems) and the TaqMan Universal PCR Master Mix (Applied Biosystems). Gene expression of aggrecan, collagen-1, collagen-2, p53, and 18S-RNA (=housekeeping gene) was measured as previously described.[29,30] Duplicate Ct values for each sample were analyzed, and the relative amount of mRNA was computed according to the comparative Ct-method.

Cell Proliferation

After 2 weeks, the MTT assay was used to determine cell numbers relative to standard conditions, thus being an indicator for cell proliferation. To each well, 500 μL of a sterile solution of MTT (0.25 mg MTT in DMEM, Sigma) was added. MSCs were incubated for 4 hours at 37°C, and then 200 μL sterile DMSO (Sigma) was added to lyse cells. The solution was transferred to a 96-well plate and absorbance of samples was measured in duplicate (80 μL each) at 565 nm (VersaMax Micro-Plate Reader, Molecular Devices, USA). Absorbance of samples was calculated relative to the absorbance of cells cultured under standard conditions.

Cell Viability

After 2 weeks, fluorescence double-staining with Fluorescein-diacetate (FDA, Sigma) and Propidiumiodide (PI, Sigma) was used to analyze viability of cells. MSCs were washed with sterile PBS and 0.5 mL of fresh staining solution were added to each well and incubated in the dark at 37°C for 20 minutes. Staining solution consisted of 20.8 μg FDA (dissolved in acetone) and 16.7 μg PI per 1 mL Ringer. After incubation, cells were washed extensively with sterile PBS and an inverted fluorescence microscope was used to detect red and green fluorescence and therefore determine cell viability under different culture conditions. Green and red cells were counted and qualitatively assessed in a randomly chosen area to estimate cell viability.

Statistical Analysis

Experimental data for proliferation (MTT) and gene expression (real time RT-PCR) of MSCs cultured under IVD specific microenvironmental conditions was normalized to cells cultured under standard conditions from the same rat. Therefore, MSCs cultured under standard conditions served as a control group. A 2-way ANOVA was used with Fisher PLSD to evaluate effects of age (2 ages) and environmental condition (standard + 4 experimental conditions). All analyses were performed using StatView (Version 5.0.1., SAS Institute Inc., Cary, NC) with a significance level of $P < 0.05$.

Results

Gene Expression

Simulating the IVD chemical microenvironment influenced aggrecan and collagen-1 expression of MSCs, with a similar pattern for both age groups. On the basis of a 2-way ANOVA, we found a highly significant influence of microenvironment ($P < 0.0001$) as well as a significant interaction between microenvironment and age ($P = 0.002$) and a trend for age alone ($P = 0.09$) for aggrecan (Figure 1). For collagen-1, statistical analysis indicated a significant effect of microenvironment ($P = 0.006$) and age ($P = 0.03$) as well as a trend for an interaction between microenvironment and age ($P = 0.06$) (Figure 2). Collagen-2 and p53 were under the detection level and therefore could not be analyzed. In general, IVD-like glucose conditions were found to result in an increased expression of matrix proteins, with a 5.4-fold increase for aggrecan expression ($P = 0.0002$) and a twofold increase for collagen-1 expression ($P = 0.02$) in the mature group, but with no significant changes in the young group. In contrast, IVD-like osmolarity resulted in a strong inhibition of gene expression of aggrecan (33-fold mature $P < 0.0001$, 10-fold young $P = 0.001$) and a slight inhibition (twofold) of collagen-1 in both age groups (mature $P = 0.01$, young $P = 0.02$). Independent from age, IVD-like pH showed inhibitory effects on aggrecan expression (threefold mature $P = 0.01$, fourfold young $P = 0.006$), but no significant effects on collagen-1. Under IVD-like combined conditions, MSCs still showed an inhibition in matrix protein expression especially for aggrecan (12-fold mature, fivefold young, both $P < 0.0001$), demonstrating that pH and osmolarity strongly dominated the effects observed by low glucose conditions alone. Significant age differences were observed for low glucose conditions for both, aggrecan ($P = 0.008$) and collagen-1 ($P = 0.0003$) as well as for high osmolarity for aggrecan alone ($P = 0.005$).

Proliferation

Proliferation of mature and young MSCs was similarly influenced by the disc microenvironment in a highly significant manner ($P < 0.0001$), but there was neither a significant effect of age nor an interaction between age and microenvironment (2-way ANOVA). Under low glucose conditions, cell proliferation was slightly stimulated in mature rats ($P = 0.05$) and not influenced in young rats. Proliferation was strongly inhibited under the low pH (twofold mature/young) and high osmolarity (threefold mature, fourfold young) conditions, with more pronounced effects for IVD-like osmolarity (Figure 3). Under combined IVD-like conditions, cell proliferation was strongly inhibited (eightfold mature, 11-fold young), showing that osmolarity and pH dominated effects of glucose. All effects of osmolarity, pH, and combined conditions were highly significant ($P < 0.0001$). No significant age differences were observed with regards to proliferation.

Viability

When staining MSCs with FDA and PI in 2D culture, we were only able to detect green fluorescence, representing viable cells, but no red fluorescence from dead/necrotic cells (Figure 4). Similar to the cell proliferation assay, fluorescence staining showed a decreased number of cells under IVD-like osmolarity and pH conditions as well as under combined IVD-like conditions. The number of viable cells in IVD-like glucose conditions was comparable to standard conditions.

Discussion

This study characterized gene expression, proliferation, and viability of MSCs from young and skeletally mature rats under a variety of IVD-like chemical microenvironment conditions to evaluate the MSC responses and isolate critical factors after 2 weeks. The 2-week time point was chosen to assure stability of cellular responses beyond their immediate adaptation. The

2D culture allowed high proliferation rates and high cell yields enabling evaluation of several environmental conditions while keeping passage number low. Cell expansion and consequently gene expression results are best interpreted as a measure of general biosynthesis rate rather than specific phenotype. Collagen-2 levels were not detectable, consistent with the findings that monolayer culture is known to decrease collagen-2 expression and increase collagen-1 expression[31]; however, the primers/probe were shown to be specific and efficient in previous studies measuring collagen-2 expression in rat IVD tissue.[29,30]

Results indicated that IVD-like glucose conditions increased MSC expression of aggrecan and collagen-1 and also increased/maintained proliferation. In contrast, high osmolarity, low pH, and combined conditions strongly inhibited MSC cell proliferation and significantly decreased anabolic gene expression, indicating that osmolarity and pH dominated the glucose effects and were critical factors that must be overcome for MSC cell and tissue engineering therapies. Finally, chemical microenvironment conditions had similar effects on MSCs of young and skeletally mature animals with differences only for gene expression (especially for low glucose conditions), but not for proliferation.

Values for media conditions were chosen as in a healthy or mildly degenerated disc, with particular reference to the nucleus pulposus. As discs degenerate, glucose and pH levels decrease, creating a harsher environment; yet, osmolarity decreases during degeneration because of a loss of GAG, creating a less harsh environment. From a tissue engineering point of view, it is crucial that MSCs survive, proliferate, and synthesize the appropriate matrix *in vivo* after implantation into the target tissue whose degeneration grade can differ widely. Healthy or mild degeneration conditions were considered reasonable since early intervention is likely to offer the greatest promise for cell therapies, and this choice also allowed comparisons with prior studies on IVD cells. In general, similar effects of chemical microenvironment were found on MSCs in this study and IVD cells in the literature (Table 2), with some differences in extent. However, a lack of studies in this research area requires comparisons of different culture systems and species. When investigating the effects of a fairly healthy or only mildly degenerated disc, similar effects of chemical microenvironment were found in general on MSCs in this study and IVD cells in the literature (Table 2), with some differences in extent. However, a lack of studies in this research area requires comparisons of different culture systems and species and future studies on MSCs will also need to clarify their responses to the conditions found in a more degenerated disc. As discs degenerate glucose and pH levels decrease, creating a harsher environment, yet osmolarity decreases during degeneration because of a loss of GAG, creating a less harsh environment. To better understand progressing degeneration, dose-response studies investigating decreasing pH, glucose, and osmolarity levels on the different variables will need to be performed.

Interestingly, low glucose did not negatively affect MSC proliferation and gene expression. However, this is consistent with the literature, which shows a decrease in apoptosis and an increase in proliferation of MSCs under low glucose conditions.[21,22] Similar to MSCs, glucose levels of 5 mmol/L (5.56 mmol/L = 1.0 mg/mL) are not detrimental to IVD cells; glucose levels need to drop much lower (0–0.5 mmol/L) to reduce cell viability.[12]

IVD-like osmolarity negatively affected MSC proliferation and gene expression, which contrasted the less dramatic response of IVD cells to high osmolarities. Specifically, an increase in osmolarity from 300 to 500 mOm strongly stimulated aggrecan,[20] which is in contrast to the downregulation measured in this study. Viability of IVD cells resulted in increased cell death at high osmolarities, but effects were less pronounced than in this study and were only observed at the beginning of the culture period.[14] The ability of disc cells to adapt to the hyperosmotic microenvironment is potentially based on expression of acid-sensing ion channels (ASIC3).[32] We speculate that predifferentiation of MSCs towards a IVD-like

phenotype may enhance the cells' ability to resist the chemical microenvironment of the disc, although this hypothesis will need to be tested in future studies.

To the authors' knowledge, no other studies have been performed investigating responses of MSCs to an IVD-like pH, and only little is known about responses of IVD cells to an acidic microenvironment. Although results of this study suggest pH effects were less prominent than osmolarity effects, pH may remain a larger factor limiting the use of MSCs for disc repair because the pH in a severely degenerated disc can drop to as low as 5.7.[27] In this study, we have chosen a pH of 6.8, which represents a healthy or only mildly degenerated disc.[16,27,28] It can be anticipated that MSC responses to a lower pH as found in a moderately or severely degenerated disc would be more substantial than measured in this study, especially considering the log nature of the pH scale, and we subjected MSCs to pH values as low as 6.5 in a follow-up study and found severe effects on gene expression, proliferation, and viability.[33] For pH, cellular responses seem to be more substantial for MSCs than for IVD cells. Bovine nucleus pulposus cells decreased synthesis of sulfated GAG below pH 6.8[17,18] and Bibby *et al* found a decrease in cell viability at pH to 6.7 and a more obvious decrease at pH 6.2, especially when culturing cells under nutrient deficit conditions.[12] Matrix acidity, therefore, is critical for MSC gene expression and proliferation and may also accelerate disc degeneration by negatively affecting resident cells. However, ASIC3 expression has been reported for IVD cells, which may enable them to adapt more easily to an acidic environment[32] than undifferentiated MSCs.

Donor age was considered an important variable to investigate in the context of MSC-based disc repair, because disc degeneration is an age-related process. Recent investigations showed that the differentiation and proliferation potential of MSCs may depend on the donor's age, with a higher potential in younger donors compared with older donors.[23-25] In contrast, our experiments show only minor biologically relevant age effects (some differences with regards to gene expression, no differences for proliferation), suggesting that pH and osmolarity conditions dominate the effects on age, so that microenvironment must be considered more strongly.

There is currently no commonly accepted marker for disc cells, and it is impossible to prove a discogenic differentiation process, or to precisely know whether MSCs can differentiate into disc cells.[34] The 2D culture conditions are considered to be a more appropriate choice for anulus fibrosus cells,[35] whereas 3D culture is more relevant for nucleus pulposus cells.[35-37] Cells were not cultured under normoxic conditions, and it is known that IVD-typical conditions are hypoxic with specific and important effects on cell metabolism[38,39]; therefore, an added hypoxic challenge is anticipated. It has been suggested that nucleus pulposus cells are specifically adapted to a hypoxic environment, as indicated by normoxic stabilization of HIF-1alpha,[39] suggesting that interactions between chemical microenvironment and hypoxia are important questions deserving further study.

Fluorescence staining of viable cells strongly supported cell proliferation results, as the number of cells under IVD-like pH, osmolarity, and combined conditions was clearly lower than under standard and IVD-like glucose conditions. The ratio of viable and dead/necrotic cells was not possible in this study because no red fluorescence was detectable. Conclusions about viability had to be made based on relative numbers of live cells since extensive washing with PBS to reduce background staining also eliminated detection of dead/necrotic cells. Subsequent experiments demonstrated that extensive washing did not affect live cells but did remove dead/necrotic cells. It was further determined that 3 gentle wash steps with PBS were optimal to remove background without affecting dead/necrotic cells. Expression of p53 (apoptosis marker) was also too low to be detected using the real time RT-PCR technique, but the primers/probe were shown be specific and efficient using RNA isolated from disc material.

In conclusion, the most important findings of this study were that IVD-like osmolarity and acidity resulted in decreased proliferation and matrix protein expression whereas low glucose levels demonstrated biosynthesis stimulation with positive effects on proliferation. Results demonstrated that when chemical microenvironment factors were combined, pH and osmolarity were critical factors dominating the MSC response. Since in advanced IVD degeneration, osmolarity will be decreased because of loss of GAGs but matrix acidity will be worsened, we believe that addressing the effects of pH alterations on MSC survival, proliferation, and biosynthesis is a crucial step that must be overcome for successful cell therapy or tissue engineering treatments. MSCs in this study responded more negatively to a similar chemical niche than IVD cells as reported in the literature, suggesting that inducing discogenic differentiation before implantation (*e.g.*, *via* growth factors[40]) may improve the MSCs resistance to the harsh microenvironment of the disc; however, this hypothesis remains to be tested in future studies exploring the IVD niche.

Key Points

- IVD-like low glucose enhanced matrix biosynthesis and maintained cell proliferation whereas IVD-like high osmolarity and low pH conditions reduced matrix protein expression and proliferation of young and mature MSCs.
- Osmolarity and pH dominated glucose effects in combined media conditions.
- Chemical microenvironmental conditions had similar effects on MSCs of young and mature cells.
- Matrix acidity increases and osmolarity decreases in advanced IVD degeneration so that addressing the effects of pH alterations on MSC survival, proliferation, and biosynthesis is expected to be the most crucial step towards IVD repair.

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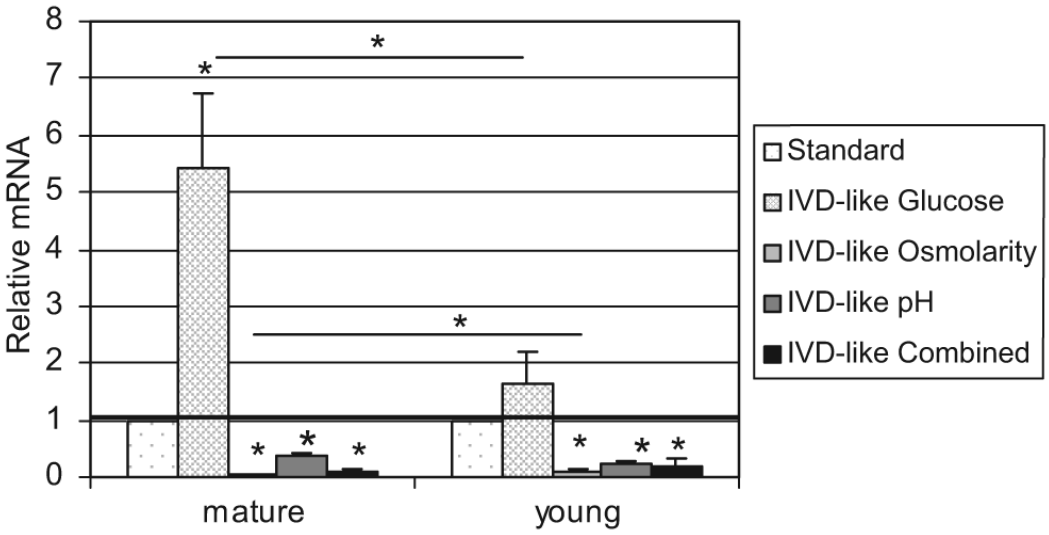

Figure 1.
Relative expression of aggrecan mRNA under different medium conditions, compared with standard conditions. Data are presented as mean ± SEM with $P < 0.05$ for n = 9 in both age groups. Asterisks above bars mark a significant change in gene expression relative to standard conditions; asterisks above lines mark a significant age effect for the respective microenvironment condition.

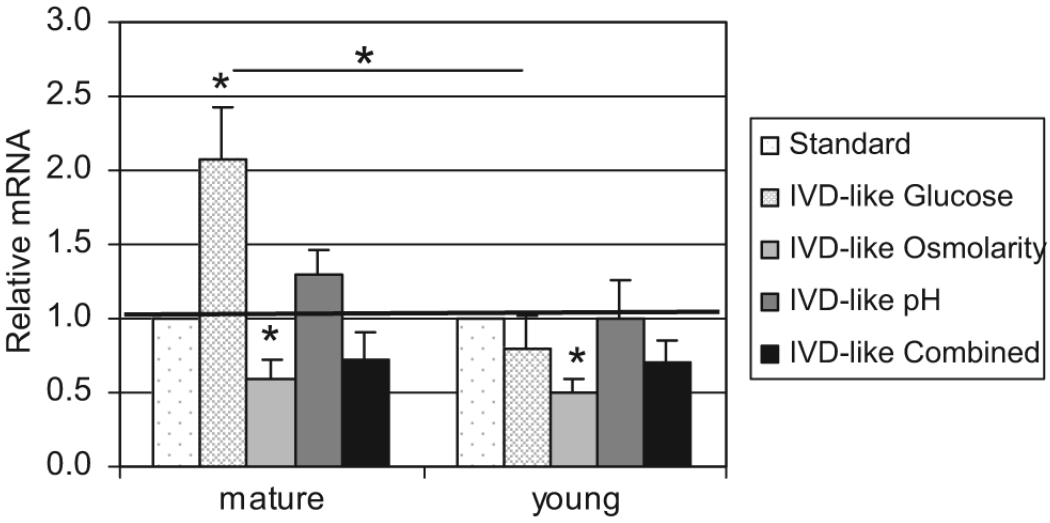

Figure 2.

Relative expression of collagen-1 mRNA under different medium conditions, compared with standard conditions. Data are presented as mean ± SEM with $P < 0.05$ for n = 9 in both age groups. Asterisks above bars mark a significant change in gene expression relative to standard conditions; asterisks above lines mark a significant age effect for the respective microenvironment condition.

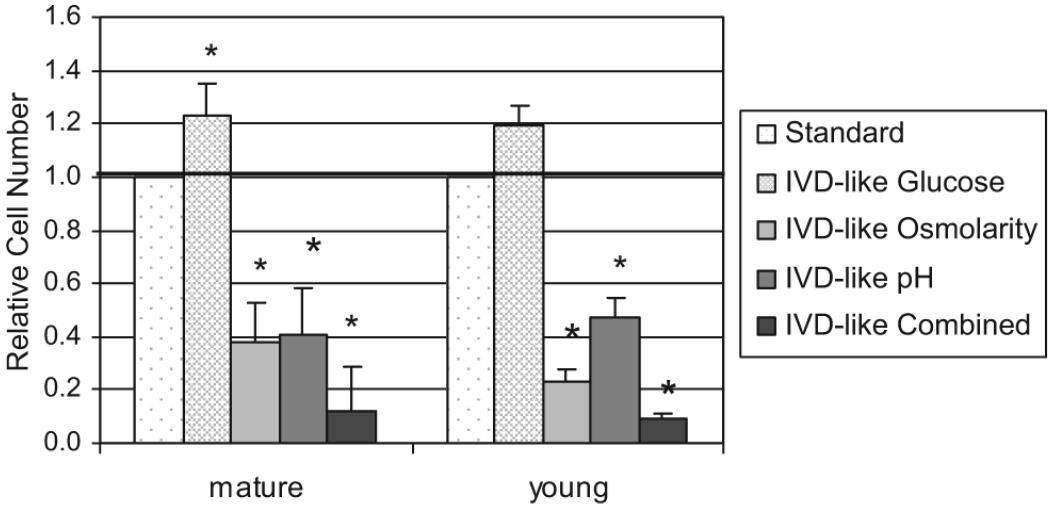

Figure 3.
Cell proliferation under different medium conditions, compared with standard medium. Data are presented as mean ± SEM with $P < 0.05$ for n = 5 (mature rats) and n = 6 (young rats). Asterisks above bars mark a significant change in cell number relative to standard conditions.

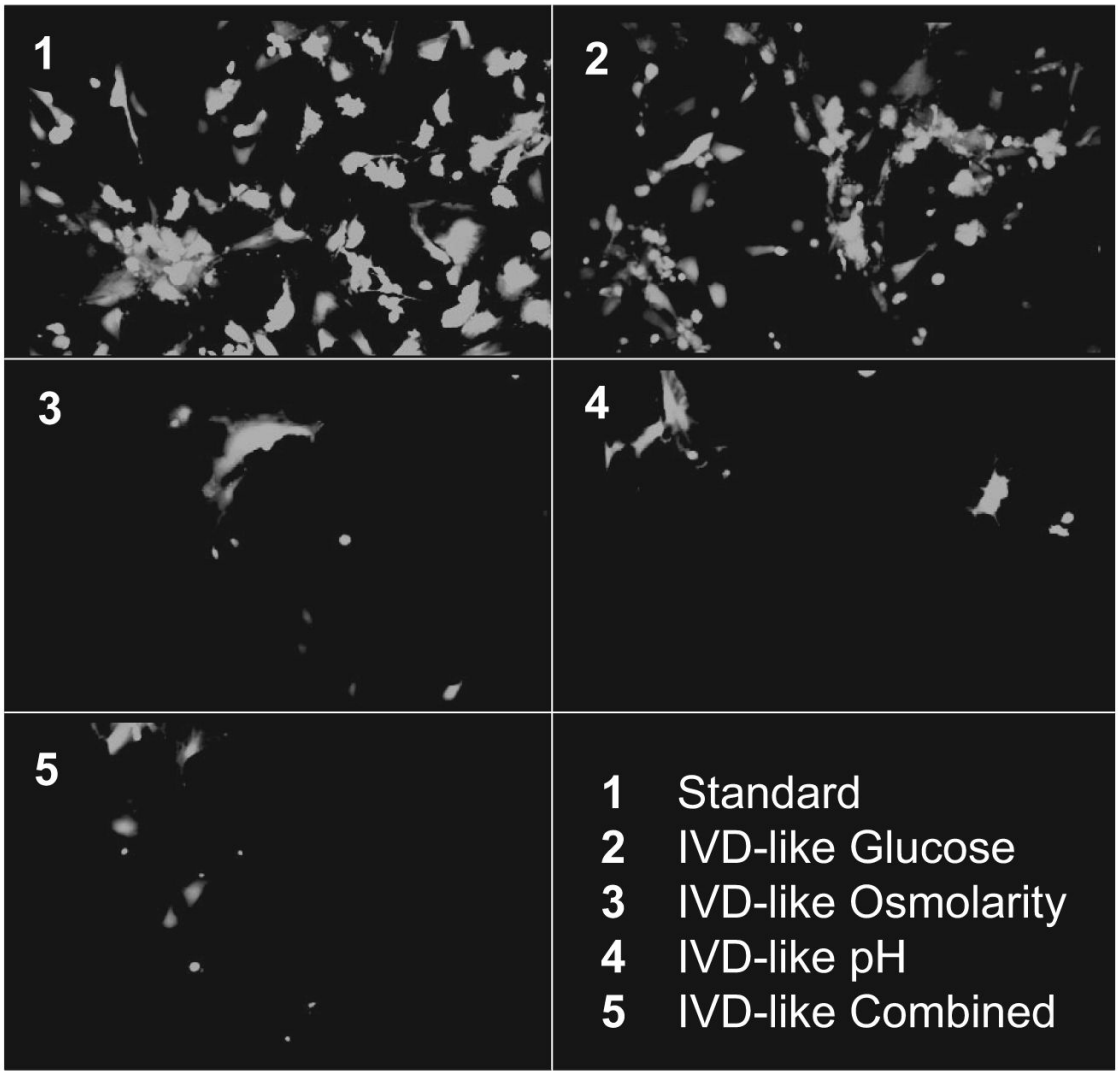

Figure 4.
Cell viability under different medium conditions, compared with standard medium; representative pictures of one donor (mature rat) are shown.

Table 1

Culture Medium

	Isolation Medium	Standard Medium	IVD-like Glucose Medium	IVD-like Osmolarity Medium	IVD-like pH Medium	IVD-like Combined Medium
FCS (%)	5	5	5	5	5	5
Penicillin (U/mL)	100	50	50	50	50	50
Streptoymcin (μg/mL)	100	50	50	50	50	50
Amphotericin (μg/mL)	2.5	1.25	1.25	1.25	1.25	1.25
5 M NaCl 0.4 M KCl (%)				2		2
1 M HCl (%)					3	3
1 M NaOH (%)						0.125
DMEM 4.5 mg/mL Gluc	×	×		×	×	
DMEM 1.0 mg/mL Gluc			×			×
Medium characteristics	4.5 mg/mL glucose (=25 mmol/L) 280 mOsm pH 7.6	4.5 mg/mL glucose (=25 mmol/L) 280 mOsm pH 7.6	1.0 mg/mL glucose (=5.56 mmol/L) 280 mOsm pH 7.6	4.5 mg/mL glucose (=25 mmol/L) 485 mOsm pH 7.6	4.5 mg/mL glucose (=25 mmol/L) 280 mOsm pH 6.8	1.0 mg/mL glucose (=5.56 mmol/L) 485 mOsm pH 6.8

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Table 2

Comparison Between Mesenchymal Stem Cells (MSCs) and Nucleus Pulposus (NP) Cells

Chemical Factor	Variable	MSCs (Rat)	NP Cells (Human*/Bovine#)
Glucose	Gene expression of matrix proteins	↑ (5.56 mmol/L)	N.A.
	Proliferation	⇆ (5.56 mmol/L)	N.A.
	Viability	N.A.	⇆ (5 mmol/L#) [4]
Osmolarity	Gene expression	↓ (485 mOsm)	↑ 500 mOsm*/# [50]
	Proliferation	↓ (485 mOsm)	N.A.
	Viability	N.A.	N.A.
pH	Gene expression	↓ (pH 6.8)	N.A.
	Proliferation	↓ (pH 6.8)	N.A.
	Viability	N.A.	↓ (pH 6.2; first signs at pH 6.7) [4]