Journal of
Stem Cell Research & Therapy

Bendtsen et al., J Stem Cell Res Ther 2014, 4:4
DOI: 10.4172/2157-7633.1000187

Review Article Open Access

An Anti TNF- A Receptor Antagonist does not Augment the Effect of Autologous Mesenchymal Stem Cell Therapy in Experimental Intervertebral Disc Degeneration in Göttingen Minipigs

Bendtsen M[1]*, Zou X[1], Jørgensen HS[2] and Bünger CE[1]

[1]*Orthopaedic Research Laboratory, Aarhus University Hospital, Denmark*
[2]*MR-Research Center, Skejby Sygehus, Denmark*

Abstract

Study design: Intervertebral disc degeneration (IDD) was induced surgically in 16 skeletally mature Göttingen minipigs. Autologous stem cells were transplanted 12 weeks post-operatively. Half f the animals were randomized to Humira® treatment 6 prior to 6 weeks after stem cell transplantation. Total observation was 30 weeks.

Objective: To evaluate whether anti TNF-α antibody administered systemically for a short period augmented the regenerative effect of autologous stem cell therapy.

Summary of Background data: Anti TNF-α treatment have been tested with regard to resorption of disc herniation and sciatica without significant effect. It is a second line treatment in inflammatory bowel disease and rheumatoid arthritis. It is thought as a pain modulator and inhibits chondrocytic differentiation.

Mesenchymal stem cell therapy has some proven efficacy in regeneration of the degenerative intervertebral disc.

Methods: IDD was induced by full thickness scalpel incisions in 3 levels in 16 skeletally mature Göttingen minipigs. Stem cells were harvested and purified from bone marrow and transplanted after 12 weeks. Half of the animals were randomized to short term treatment with anti TNF-α antibody (Humira®, Abbott Laboratories) for 6 weeks before to 6 weeks after stem cell treatment. Total observation was 30 weeks. MRI was performed before stem cell transplantation and before sacrifice. Quantitative real time RT-PCR and histology was performed after sacrifice.

Results: Autologous stem cell transplantation was able to stop and partially reverse the degenerative process with regard to MRI index (p=0.0031), disc height (p=0.021 and 0.04) and ADC value (p=0.023). Quantitative real time RT-PCR found no difference in apoptosis markers between any of the groups. Histology showed partial degeneration in stem cell treated discs. There was no difference in any parameter between groups treated with Humira® or not.

Conclusion: Autologous stem cell therapy is able to stop and partially reverse the degenerative process and survive *in vivo* for at least 18 weeks. Anti TNF-α treatment does not augment the effect and does not slow down the degenerative process in a minipig model of IDD.

Keywords: Autologous stem cell therapy; Intervertebral disc degeneration; Anti TNF-α; MRI; Real time RT-PCR

Introduction

Intervertebral disc degeneration (IDD) is one of the commonest causes of chronic low back pain (CLBP), disability and long term leaf of absence. It places a major economic burden on society and reduces quality of life in the patients.

It is a chronic progressive process with changes in disc composition, morphology and function.

Notochordal cells develop into nucleus pulposus at the embryonic stage and gradually disappear in the second decade where the first signs of IDD can be detected [1]. Notochordal cells are thought to have a regulating role in disc cell metabolism with positive effect on extracellular matrix production [2].

Bone marrow derived stromal cells increase cell proliferation, proteoglycan synthesis and secretion of growth factors by nucleus pulposus cells when cultured with direct cell to cell contact compared to no contact and no stromal cells [3].

TNF-α is found in higher concentrations in nucleus pulposus specimens from degenerative discs and disc herniation obtained at discetomy compared to non-degenerate intervertebral discs [4].

TNF-α increase production of MMP´s (matrix metalloproteinases) and ADAMTS (a disintegrin and matrix metalloproteinase with thrombospondin motifs), which degrade extracellular matrix. TNF-α not only increase degrading enzymes but also decrease the production of extracellular matrix components (aggrecan and collagen II) [5] crucial for maintenance of disc hydration and function.

TNF-α inhibits differentiation of synovial fibroblasts towards chondrogenic phenotypes through the p38 MAPK pathway. Inhibition of p38 MAPK pathway partially restored chondrogenic differentiataion assessed by real time PCR and histology [6].

Furthermore TNF-α is thought to play a role pain modulation

***Corresponding author:** Michael Bendtsen, Orthopaedic Research Laboratory, Aarhus University Hospital, Denmark, Tel: 4521628397; E-mail: michael.bendtsen@ki.au.dk

Received February 22, 2014; **Accepted** April 01, 2014; **Published** April 03, 2014

Citation: Bendtsen M, Zou X, Jørgensen HS, Bünger CE (2014) An Anti TNF-A Receptor Antagonist does not Augment the Effect of Autologous Mesenchymal Stem Cell Therapy in Experimental Intervertebral Disc Degeneration in Göttingen Minipigs. J Stem Cell Res Ther 4: 187. doi:10.4172/2157-7633.1000187

in low back pain. A study with wild type and TNF-α deficient mice showed higher ingrowths of sensory nerves in to nucleus pulposus of wild type mice when injected intra-muscularly [7]. This corresponds to a higher secretion of proinflammatory mediators and cytokines in discs from patients with discogenic low back pain compared to sciatica [8].

A single dose of anti-TNF-α antibody Infliximab did not affect disc-herniation induced sciatica after 1 year or herniation resorption after 6 months follow-up in randomized controlled trials (FIRST II) [9,10].

In a dose-response study with a single intradiscal injection of Etanercept to patients with lumboscaral radiculopathy or discogenic low back pain no difference was found in pain or disability scores in either dose group [11].

Anti TNF-α is widely recognized as second line therapy in rheumatoid arthritis, inflammatory bowel disease (Mb. Chron and Colitis Ulcerosa) when conventional therapies are insufficient [12-14].

Autologous mesenchymal stem cell (MSC) treatment has proven some efficacy in rabbit model. Mesenchymal stem cells were labelled with GFP and transplanted to degenerative discs. MSC`s survived and differentiated towards nucleus pulposus phenotype with restoration of extracellular matrix [15].

Transplantation of healthy cells from the nucleus pulposus three months after discectomy can to some extent reverse progression of IDD in a dog model for up to one year [16]. These results lead to the EuroDisc study with transplantation of retrieved nucleus pulposus cells in human disc herniation patients. Follow up showed improvement in pain related parameters in the treatment group [17].

The hypothesis was that autologous stem cell therapy could halt progression of intervertebral disc degeneration and that anti-TNF-α treatment would augment the potential of stem cell regeneration.

Materials and Methods

Surgery

16 skeletally mature Göttingen minipigs (age 3.5 to 4 years) were included in the study. Intervertebral disc degeneration was induced by full thickness scalpel incisions in the left anterolateral annulus fibrosus. Interventions were applied to levels L2/3 to L4/5 via a left sided retroperitoneal approach. The abdomen was closed in layers.

Mesenchymal stem cells

Bone marrow was harvested from the left proximal tibia. Approximately 8 ml bone marrow was aspirated into a 50 ml syringe containing 10 ml saline and 5 IE heparin.

MSC`s were isolated by Ficoll® gradient centrifugation, seeded in culture flasks and cultivated to passage 2. They were membrane stained with PKH-26 (Sigma Aldrich) fluorescent dye and frozen at -80°C in 10% DMSO.

MSC`s were transplanted 12 weeks postoperatively into 1 disc in all 16 animals by a percutaneous fluoroscopy guided injection via a 18 G needle. Before injection MSC`s were aliquoted in a hydrogel (PhotoFix-HA, Zimmer Biologics, Austin, Texas, USA). 875.000 autologous MSC`s were aliqouted in 0.2 ml PhotoFix-HA hydrogel and then injected into the nucleus pulposus. Levels were randomized for stem cell transplantation, normal control or degenerative control.

Total observation time was 30 weeks.

Anti TNF-α treatment

Anti TNF-α (Humira® was kindly sponsored by Abbott, Denmark) treatment was conducted by injection of Humira® 40 mg subcutaneously every two weeks in eight randomly assigned animals. Anti TNF-α treatment was started 6 weeks prior to stem cell transplantation and stopped 6 weeks after.

MRI

MRI were performed on a clinical 1.5 Tesla GE system (Signa Excite) with the following sequences (Scout; T1 sag; T2 sag; T2 axial, LSDI DTI, b-value 1000). DTI data were post processed using MedINRIA software (http://www.sop.inria.fr/asclepios/software/MedINRIA/).

MRI was performed before stem cell transplantation and sacrifice. MRI index was calculated form mid-sagittal T2-weighted slices as the product of area and signal intensity:

MRI index = area x signal intensity

Average disc height was calculated from mid-sagittal slices. Disc height was measured in the middle of the disc and 10% from the margins anterior and posterior respectively.

Real time RT-PCR

After sacrifice spines were removed en bloc and flash frozen in liquid nitrogen and stored at -80°C. While kept frozen tissue samples from nucleus pulposus was obtained with RNase free instruments. The frozen tissues were manually cut into small pieces and homogenized in 1 ml of TRIzol® Reagent (Invitrogen, Taastrup, Denmark) using a mixer mill MM 301 (Retsch, Haan, Germany). Samples were treated with 1 mg collagenase (Sigma-Aldrich, Broendby, Denmark) at 37°C for 1 hour. 0.2 ml chloroform was added and samples mixed before centrifugation. Aqueous phase was transferred to a fresh 2.0 ml tube and RNA was precipitated with 0.6 ml isopropanol and a high salt precipitation solution containing 1.2 M NaCl and 0.8 M sodium citrate. The rest of the extraction followed manufacturer's instructions.

RNA samples were treated with DNase 1 (Ambion, Cambridgeshire, UK) and converted to complementary DNA (cDNA) using the High Capacity cDNA Archive kit (Applied Biosystems, Naerum, Denmark). Real time quantitative polymerase chain reaction (qRT-PCR) was performed on a 7500 Fast Real –Time PCR system (Applied Biosystems,

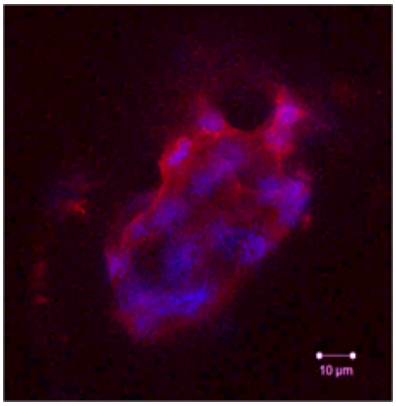


Figure 1: Representative confocal microscopy of Red (PKH-26) and blue (DAPI) stained transplanted stem cells. Both transplanted membrane stained cells and native cells with a blue nuclei only is seen.

Naerum, Denmark). Primers and FAM dye-labelled TaqMan MGB probe was designed by Applied Biosystems Primer Express 3.0 software. mRNA integrity was verified by gel electrophoresis. Primers are shown in Table 1. Data analysis was performed using 7500 Fats System Sequence Detection Software version 1.3 (Applied Biosystems, Naerum, Denmark).

Confocal laser microscopy

Confocal laser microscopy (Zeiss LSM 510, Germany) was performed on motion segments cut in the midsagittal plane to detect transplanted MSC`s. Before microscopy segments were counter stained with DAPI.

Histology

After sacrifice spines were harvested en bloc and cut into motion segments. Motion segments were embedded in cold MMA (methylmethacrylate). Mid-sagittal slices of 7 µm were cut on a Leiden microtome. Slices were stained with Hematoxylin-Eosin (HE), Goldner-trichrome, Safranin-O and Alcian blue-PAS. Degeneration was graded according to algorithm set up by Boos et al. [18].

Statistics

Statistics were done in STATA 9.0 (StataCorp LP, USA). Data was tested for normal distribution with Q-Q plot and histograms. ANOVA was used to detect differences in variance and further explored by unpaired Students t-tests. $P<0.05$ were considered significant.

Ethics

All animal experiments were approved by the Danish Animal Welfare Committee under the Department of Justice J.nr 24 Sept 2004-568/898.

Results

There were no signs of increased infection rate among animals receiving Humira®.

There were no signs of disc herniation, facet joint arthrosis or spondylo-discitis.

Modic Type II changes were found in 5 animals (3 in the Humira® group and 2 in the non-treated group). None of these progressed or regressed regardless of Humaira® administration or not.

Autologous MSC`s were found alive (blue DAPI stained nucleus surrounded by red PHK-26 stained cell membrane) in all transplanted discs in both Humira® and control groups (Figure 1). No MSC`s were found in vertebrae or non-transplanted discs.

There was no statistical difference in MRI index, apparent diffusion coefficient, fractional anisotropy or disc height in the discs before randomization to either stem cell transplantation or degenerative control. At sacrifice levels treated with stem cell injection regained MRI index, ADC value and some disc height. Degenerative controls continued to deteriorate. There was significant difference between normal and degenerative controls, and stem cell compared to degenerative controls with regard to ADC value (normal vs degenerative $p=0.023$), MRI index (normal vs degenerative $p=0.0031$, stem cell vs degenerative $p=0.0481$), and disc height (control vs degenerative control $p=0.021$, stem cell vs degenerative control $p=0.04$). There was no difference between any groups with regard to fractional anisotropy. No statistical difference was found between Humira® and non-treated groups. Data is summarized in Figure 2. Representative MR T2 sagittal images and corresponding maps are shown in Figure 3.

Histology showed slight chondrocyte proliferation, no mucous degeneration, no cell death or cleft formation in the NP of normal controls. The endplates had structured cartilage without cracks or microfractures. No bone formation or sclerosis was observed. Total score was 5.

In stem cell transplanted discs degeneration was detected with formation of chondrocyte clones, some mucous degeneration, slight cell death and cleft and tear formation. Endplates had localized cell proliferation, cartilage irregularities were observed in some discs. Microfractures but no cracks were found. No new bone was formed. Total score was 11.

In degenerative controls changes were more pronounced with further cell death, chondrocyte proliferation and tears and cleft formation. The endplates were more disorganized with cracks and new bone formation. Total score was 19. Representative Safranin-O stained slices are presented in Figure 4.

Data comparing pure hydrogel (designated Hydrogel-PF) compared to stem cells dissolved in the hydrogel carrier have been reported in reference 26. There was statistical difference between MRI index and histologic score.

In quantitative real time RT-PCR data no differences were found between groups treated with Humira® or non-treated. Statistical difference was found in Caspase 8 ($p=0.023$; 1.196 ± 0.039 vs 1.251 ± 0.087), TRAIL ($p=0.044$; 1.195 ± 0.036 vs 1.238 ± 0.060), but not in CD34 or Endoglin (CD105), or HIF1α (non-treated) (with comparison between normal and degenerative control groups. In the group treated with Humira® significant difference was found in Caspase 8 ($p=0.019$; 1.188 ± 0.034 vs 1.249 ± 0.045), TRAIL ($p=0.039$; 1.267 ± 0.045 vs 1.230 ± 0.052), and SOX9 ($p=0.048$; 1.126 ± 0.067 vs 1.097 ± 0.090). Again

Gene Symbol	Accession number	Forward primer	Reverse primer
TFRC	NM_214001	GTGACCCGTACACACCTGGAT	CTGATGACTGAGAGGGTGGAAAC
β-actin	DQ845171	CTCCTTCCTGGGCATGGA	CATGATGGAGTTGAAGGTAGTTTCG
HPRT1	NM_001032376	CGGCTTGCTCGAGATGTGAT	AGCACACAGAGGGCTACGATGT
Caspase8	AY519263	GACTCAGAACAGACAGAAGCC	TCCGCCTCGTCTGGGATAT
TRAIL	AY639873	CCCTCTGTGTGGCCTTGACT	GGAGTACTTGTCCTGCATCTGTTTC
CD105 (Endoglin)	NM_214031	GCCAGTGTGATAGCATCTTTTGTAG	GCTGCGGTCCTGCAGAGA
CD34	NM_214086	TTGGCCAACGGAACAGAAC	TCAGACTGGTGCTTTTCCAGAA
SOX9	NM_213843	CCCACATTCCAAGACAGAAACA	GCCACTGACTCGCCACAAG
HIF1-α	EF070345	CCATGCCCCAGATTCAAGAT	GGTGAACTCTGTCTAGTGCTTCCA

Table 1: Real time RT-PCR primers. Transferrin receptor, β-actin, and Hypoxantine Phosphoribosyltransferase 1 are house keeping genes. Accession number refers to NCBI (www.ncbi.nlm.nih.gov/sites/entrez) in the Nucleotide database.

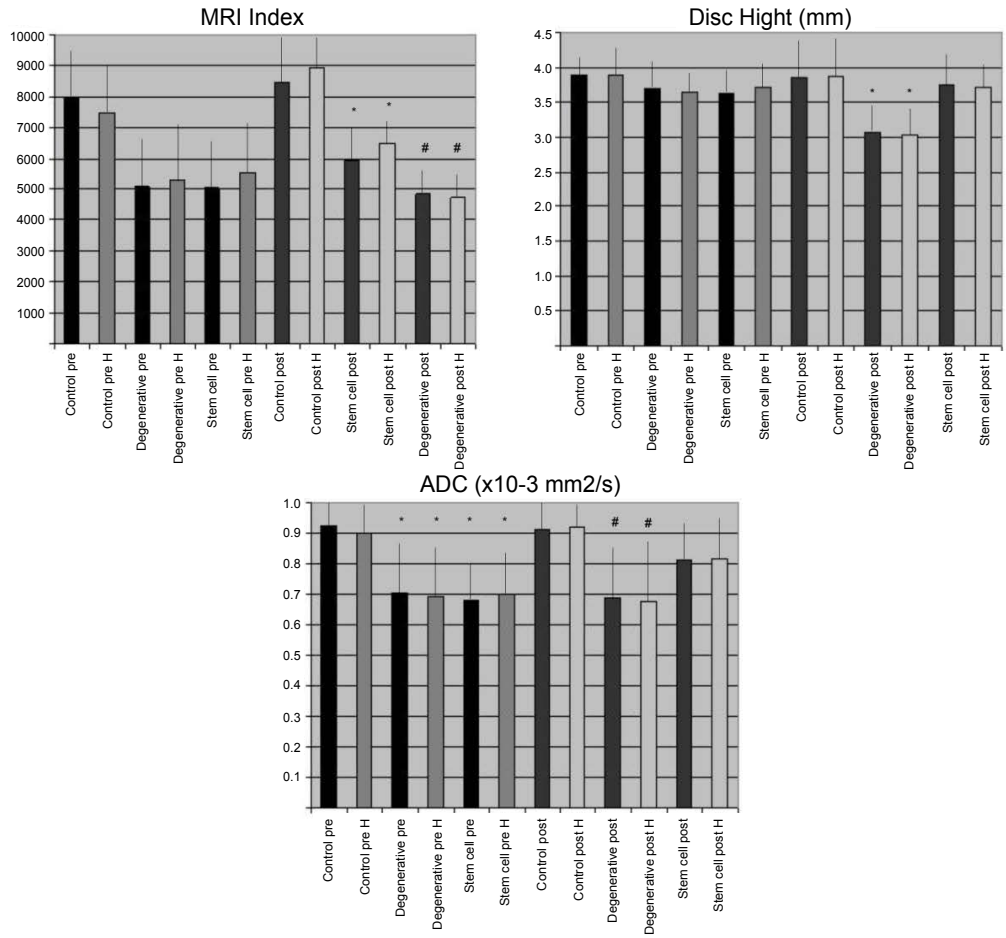


Figure 2: Graph's representing MRI Index, Disc Height and ADC value (mean ± standard deviation). Pre means that data is before the 12 week intervention. Post means that data is before sacrifice (30 weeks observation). Suffix H means that the group is treated with Humira®.

a) *significant different from control $p<0.05$. #significant different from control and stem cell groups (Post) $p<0.05$.

b) *significantly different from control and stem cell groups (Post) $p<0.05$.

c) *statistical different from control (Pre) $p<0.05$. # significant different from control (Post) $p<0.05$).

there was only difference when normal and degenerative controls were compared. Data presented as the ratio between target mRNA and mean of the 3 house keeping genes.

Discussion

Animal models of intervertebral disc degeneration (IDD) are essential in the attempt to regenerate the degenerative disc.

Differences in disc size between humans and animal models play an important role as the distance from cell to nutrient supply increase. Cells rely on nutrition by diffusion through the vertebral endplate into the disc [19].

The minipig spinal anatomy resembles that of the human spine, although it is smaller. The facet joint of the minipig is comparable to human facets and resembles these in a higher degree compared to other animal models [20]. Spontaneous degeneration and Modic type II changes were observed in the current study making it comparable to human IDD.

IDD was induced by disruption of the anterolateral annulus fibrosus in the current study. This might not reflect the process of IDD seen in humans. Still histology of degenerate and normal discs is similar to human findings with disorganization in the form of cleft and tears formation. Even the endplates had cracks and microfracture and new bone formation.

MRI parameters showed progressive degeneration with loss of quantitative disc height, MRI index and apparent diffusion coefficient (ADC value).

In the current study autologous stem cell transplantation to mild to moderate degenerative intervertebral discs were able to stop the progression of IDD and to some extent reverse it with statistical difference in MRI index, disc height, ADC value and histological grading. Where ADC is proportional to proteoglycan content and inversely proportional to collagen content [21].

Transplanted cells were found to be alive by confocal microscopy in all treated discs. This is supported by quantitative real time RT-PCR results in CD 34 and Endoglin (CD105). These are haematopoietic surface markers and expressed in bone marrow derived stem cells. No differences in these two surface markers were found between any of the groups suggesting that transplanted stem cells have differentiated.

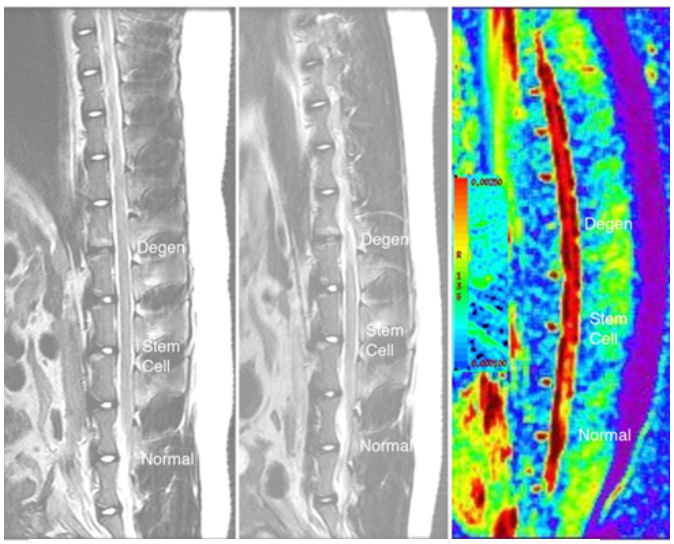
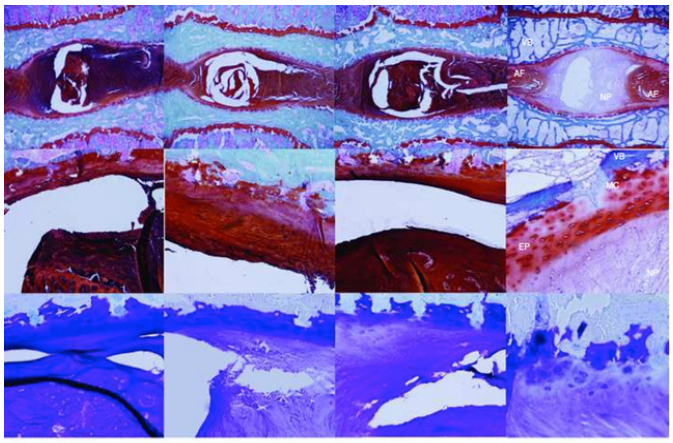


Figure 3: Representative MR images with T2-sagittal before intervention and before sacrifice with corresponding color coded ADC maps. Control designates normal intervertebral disc; Degen– disc with induced IDD, no local treatment, while Stem Cell designates local stem cell therapy. In the degenerative disc the white water filled nucleus pulposus has disappeared compared to a normal disc where the NP is bright and with normal disc height. The Modic Type 2 changes seen in adjacent endplates to the degenerative disc remain unchanged. In the level receiving stem cell transplantation the NP increase a litte in size and brightness.



Numericals in the right column are AF: annulus fibrosus; NP: nucleus pulposus; VB: vertebral bone; EP: cartilage endplate; MC: microcrack

Figure 4: Representative histologic slides. Top row; x4 Safranin-O stained slides, Middle row; x20 Safranin-O stained slides. Lower row; Alcian Blue-PAS photographed at 20x magnification.

Histology showed increased cell death with severity of degeneration. In degenerative control discs increased levels of TRAIL and Caspase 8 were found. TRAIL is a mediator of TGF-β induced apoptosis while Caspase 8 is a downstream TNF-superfamily death receptor regulator resulting in apoptosis [22].

No difference in any parameter were found in pigs treated with Humira® 6 weeks prior to transplantation and 6 weeks after compared to non-treated animals. This may be due to two reasons: Humira® is a human anti TNF-α antibody or Humira® does not diffuse through the vertebral endplate in sufficient amount. The first hypothesis was tested by adding equal molar concentrations of porcine TNF- α and Humira® with measurement of free porcine TNF- α. This showed that Humira® bound 68% of porcine TNF- α (data not shown). Walters et al showed that cephazolin is able to diffuse into the intervertebral disc but in amounts 50 times less than serum levels and with concentrations 15 times higher in the annulus fibrosus compared to nucleus pulposus [23,24]. The molecular weight of Cephazolin is 476.50 Da while Humira® antibody is constituted of 1330 amino acids with a molecular eight of approximately 148 kDa – almost 300 times the weight of Cephazolin. Roberts et al showed that diffusion of solutes through the vertebral endplate depends on size and shape of the molecule as well as charge. For example only 15 % of a 4 kDa PEG molecule diffused through the endplate at equilibrium [25]. Most of the anti TNF-α drugs have molecular weights around 150kDa except Certolizumab (Cimzia) that has a molecular weight of 91 kDa. Another option is to use an IL-1 inhibitor where Anakinra (Kineret) has a molecular weight of 17.3 kDa, still 4 times larger than the previously investigated PEG molecule [26].

A recent study performed Das et al. [27] concluded that intraveneously administered dextran of varying molecular weights didn`t reach the center of the intervertebral disc. Furthermore the concluded that intradiscal injection seems the most promising way to deliver drugs or growth factors in a high enough concemtration and to ensure dissipation to the entire disc.

Humira® was administered subcutaneously with 2 week intervals as recommended giving a slow release of the antibody. It is thought that this is the reason to insufficient amounts reaching the nucleus pulposus. However concentration was not measured intradiscally during administration.

Furthermore clinical studies showed no effect on disc herniation resorption or pain modulation when anti TNF- α antibody was administered systemically or into the disc itself [9-11]. This raises the question if TNF- α is a potential target in regeneration of the degenerative intervertebral disc or other inflammatory mediators are more important?

Another way to improve cell-based therapy is to use cells already capable of surviving the harsh conditions in the intervertebral disc. Acosta et al. [28] published results with allogeneic juvenile articular cartilage chondrocytes being more efficient compared to allogeneic mesenchymal stem cells in a minipig model. However chondrocytes was from another minipig usually MHC compatible while MSC´s was from Yorkshire farm pigs not MHC compatible. This could be the reason why chondrocytes were superior to MSC´s.The study raises the question if cells dedicated to hypoxic environments are better than others? Such a cell type could be chondrogenic differentiated MSC´s since articular chondrocytes are difficult to harvest without creating donor site morbidity.

This becomes clinically relevant with ongoing clinical trials on stem cell therapy for IDD (ClinTrials.gov Identifier NCT01860417).

Conclusion

The present study demonstrates that autologous stem cell transplantation is able to stop and partially reverse the degenerative process for at least 18 weeks. In this time frame stem cells are able to survive *in vivo*.

Treatment with anti TNF- α antibody systemically does not augment the effect of stem cell treatment and does not prevent progression in non-treated discs.

Autologous stem cell transplantation seems promising in regenerative therapy. However the question on pain relief and how to identify the patient that will benefit from biologic intervention needs further exploration.

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