The Spine Journal 19 (2019) 896–904

Basic Science

Efficiency of dual siRNA-mediated gene therapy for intervertebral disc degeneration (IVDD)

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Received 26 July 2018; revised 19 October 2018; accepted 19 October 2018

Abstract

BACKGROUND CONTEXT: One of the common causes of low back pain is intervertebral disc degeneration. The pathophysiology of disc degeneration involves apoptosis of nucleus pulposes cells and degradation of extra cellular matrix (ECM). Caspase 3 plays a central role in apoptosis and the ADAMTS5 (A Disintegrin and Metalloproteinase with Thrombospondin motifs 5) gene plays a critical role in ECM degradation. Hence, we hypothesized that if one can silence these two genes, both apoptosis and ECM degradation can be prevented, thereby preventing the progression and even reverse disc degeneration.

PURPOSE: The purpose of this study is to demonstrate the regenerative potential of small interfering RNA (siRNA) designed against Caspase 3 and ADAMTS5 genes in an in vitro and animal model of disc degeneration.

STUDY DESIGN: In vitro study followed by in vivo study in a rabbit model.

METHODS: In vitro studies were done using the human hepatocellular carcinoma (Hep G2) cell line for validating the efficacy of liposomal siRNA in controlling the expression of genes (Caspase 3 and ADAMTS5). Later, siRNA's validation was done in a rabbit annular punctured model by administering siRNA's individually (Caspase 3 and ADAMTS5) and in combination Caspase3-ADAMTS5) for assessing their synergistic effect in down regulating the gene expression in the degenerative discs. Annular punctured intervertebral discs of the rabbit were injected with siRNA formulations (single and dual) and phosphate buffer saline, one week after initial puncture. Magnetic resonance imaging (MRI) scans were done before and after siRNA treatment (1, 4 and 8 weeks) for assessing the progression of disc degeneration. The histopathology and real time polymerase chain reaction (RT-PCR) studies were done for evaluating their efficacy. We did not receive any funding for conducting the study, and we do not have a conflict of interest with any researchers or scientific groups.

RESULTS: The observations made from both in vitro and in vivo studies indicate the beneficial effects of siRNA formulation in down regulating the expression of Caspase 3 and ADAMTS5 genes. The MRI and histopathological evaluation showed that the disc degeneration was progressive in phosphate buffer saline and AT5-siRNA injected discs but the discs that received Caspase

FDA device/drug status: Not applicable.

Author disclosures: ***RRB:*** Nothing to disclose. ***SKV:*** Nothing to disclose. ***GHD:*** Nothing to disclose. ***VP:*** Nothing to disclose. ***MP:*** Nothing to disclose. ***SMV:*** Nothing to disclose. ***GRAV:*** Nothing to disclose. ***MRN:*** Nothing to disclose. ***SGPV:*** Nothing to disclose.

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https://doi.org/10.1016/j.spinee.2018.10.016

3-siRNA and dual siRNA (Cas3-AT5-siRNA) formulation showed signs of recovery and regeneration 4 and 8 weeks after injection. The efficacy of siRNA designed against Cas3 and AT5 was also assessed in both in vitro and in vivo experiments by using RT-PCR analysis and the results showed downregulation of Caspase 3 gene in Caspase 3-siRNA group, but there was no significant downregulation of ADAMTS5 gene in ADAMTS5-siRNA group (ie, indicated by fold change). Synergistic effect was observed in the group that received dual siRNA (Cas3-AT5 siRNA) formulation.

CONCLUSIONS: This experiment suggests that intervention by siRNA treatment significantly reduced the extent of apoptosis in the discs.

CLINICAL SIGNIFICANCE: Delivery of siRNA directly into spinal discs has a potential in treating disc degeneration nonsurgically.

Keywords: Intervertebral disc degeneration (IVDD); Liposomal siRNA; Caspase 3 and ADMTS5 (A Disintegrin and Metalloproteinase with Thrombospondin motifs); Magnetic Resonance Imaging (MRI); Nucleus pulposes, Histopathology.

Introduction

Intervertebral disc (IVD) degeneration is a major pathological process that is implicated in low back pain (LBP), which leads to significant disability in patients. It is estimated that about 75% to 80% of the population experience LBP during their lifetime [1,2]. The etiology and pathophysiology of intervertebral disc disease (IVDD) is complex and poorly understood [3–6]. The IVD is composed of two different layers of cells, the outer annulus fibrosus (AF) and inner nucleus pulposus (NP) [1,3,5]. These two components of NP cells give compressive resistance to the disc tissue [7–10]. Altered metabolism in disc cells causes loss in water retention, decreases in oxygen tension, decreased pH, inflammation, and increased activity of aberrant proteolytic enzymes (such as Caspases, Matrix metalloproteinases (MMP's) etc.) [11–13]. This causes further damage to the IVD [13].

The development of novel biological treatments such as gene therapy (siRNA or nucleic acid based drugs), cell therapy, and growth factor therapy and their application in the disc space has the potential to treat disc degeneration [14–16]. Such treatments are performed by altering the gene expression and readjusting the metabolic imbalance within the degenerated discs, but without the morbidity associated with surgery [1,8,9]. Small interfering RNAs (siRNAs) have gained popularity since their discovery due to the mechanism involved in gene silencing [15–19]. Synthetic siRNA's are considered to be a novel tool for targeting non-druggable targets as well as for treating diseases such as neurodegenerative diseases, spinal cord injury, and cancer [19–22]. Though siRNA works efficiently in in vitro systems, its translation for therapeutic purposes has faced several uphill challenges [23]. The most prominent hurdle among these challenges is instability of siRNA in in vivo systems [24–26]. siRNA being polyanionic, has a very short half-life in blood, as many opsonins present in blood rapidly bind to it thereby removing it from the circulation [25]. Effective siRNA delivery strategies are crucial for its stabilization in in vivo system [27]. Several clinical successes in gene silencing have been achieved in treating the eye disease. Being easily accessible and an immune privileged organ, the efficacy of instilled siRNA was significantly enhanced in the eye [28].

Similarly, for IVDD treatment, siRNA can be delivered directly into the gel-like mass of NP of IVD, which would avoid natural serum origin nucleic acid sequestering processes and at the same time also retain the siRNA for longer time inside the IVD for gene silencing. siRNA-mediated gene silencing was demonstrated in NP cells in vitro [24] by direct injection into IVD [8]. Plain siRNA has poor ability to cross the cell membrane and bring about gene silencing in the cytoplasm. Complexing siRNA with cationic liposomes facilitates protection against RNA degrading processes and aids in cell entry [29,30]. Direct injection of siRNA lipid complexes into IVDD resulted 1 in silencing of Caspase 3 gene and led to delayed apoptosis [7]. In the present study, we explored the efficacy of two different siRNA lipoplexes in stalling disc degeneration after direct instilling of these formulations in NP etiology of diseases, especially IVDD, suggests that dysregulation of more than single pathway could be responsible factor leading for the disease. The siRNA were first validated in cell lines and tested later in rabbit model for IVDD. siRNA against Caspase 3 (Cas3) and ADAMTS5 (AT5) genes involved in apoptosis and matrix degradation, respectively, were also tested.

Materials and methods

HepG2, a human hepatocellular carcinoma derived cell line was acquired from NCCS, Pune. Following reagents were procured from suppliers mentioned in brackets: siRNA (Dharmacon, Inc.), RT-PCR primers (Bioserve Pvt. Ltd.), SYBR Green Master Mix (Takara Bio USA. Inc.), Cholesterol (Sigma-Aldrich Inc., USA,), cell culture media and components (Thermo Fisher Scientific Inc.) RT-PCR kit (Himedia lab Pvt. Ltd.), RNA later (Sigma-Aldrich Inc., USA), microRNA Sure minikit (Nucleopore, Genetix Biotech Asia Pvt. Ltd.), TRIzol (Invitrogen, Thermo Fisher Scientific Inc.), cDNA synthesis kits (Thermo Fisher Scientific Inc.), Total RNA purification kits (Genetix Biotech Asia Pvt. Ltd.).

Ethanol, Paraformaldehyde, Xylene, Harris Hematoxylene and Eosin stain were purchased form Merck India. All other reagents used are of analytical grade and above.

In vitro studies

The HepG2 cell line was chosen as an in vitro model for evaluating the efficiency of siRNA in controlling the cells from undergoing apoptosis. HepG2 cells were maintained in a Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (D-MEM/F-12) with 10% fetal bovine serum. The cells were cultured in humidified environment at 37023 C with 5% CO2. A solution of 0.05% trypsin, 5.56 mM glucose, and 0.1% EDTA in Calcium and magnesium-free phosphate buffer saline (PBS) was used for cell detachment.

Preparation of lipoplexes

Cationic lipid N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium chloride was synthesized in Indian Institute of Chemical Technology, Hyderabad (IICT) as described earlier [27,29]. Liposomes containing 1:1 mole ratio of N,N-di-n-hexadecyl-N,N-dihydroxyethylammonium chloride and cholesterol were prepared by a thin film hydration method. Briefly, the lipid mixture in chloroform was dried in a round-bottom glass tube and the solvent was gently removed by flushing N2 gas and by warming. The lipid film one was hydrated to a final concentration of 2 mM of each lipid. Multilamellar vesicles formed were sonicated using probe sonicator (Branson sonifier) and filtered through a 100 nm polycarbonated filter using a syringe extruder. Following siRNAs were employed in our study Caspase 3 siRNA (length: 21 bps) Sense: 5′ A.A.A.U.G.A.U.C.U.U.A.C.A.C.G.U.G.A.A.U.U3′; Antisense: 5′-P.U.U.C.A.C.G.U.G.U.A.A.G.A.U.C.A.U.U.U.U.U 3′. AT5 siRNA AT5 (length: 21 bps)- Sense: 5′ G.G.G.C.A.U.C.A.U.U.C.A.U.G.U.G.A.C.A.U.U 3′; Antisense: 5′-P.U.G.U.C.A.C.A.U.G.A.A.U.G.A.U.G.C.C.C.U.U 3′

Real time PCR (RT-PCR)

siRNA specific to GAPDH or Caspase 3/ AT5 siRNA at 50nM was added to the liposomes at 3:1 charge ratio for lipoplex formation. siRNA-lipoplexes were added to HepG2 cells in a six-well plate. After, 48 hours of transfection, total RNA was isolated from the cells using RNASure kit. cDNA synthesis was performed with Verso cDNA synthesis kit from Thermofisher. Mean value of GAPDH was normalized with Beta-actin (ACTB) and calculated using δCt method. Following primers were used for qRT-PCR: Caspase-3 Forward (TTAATAAAGGTATCCATGGA-GAACACT); Caspase-3 Reverse (TTAGTGATAAAAA-ATAGAGTTCTTTTGTGAG); ADAMTS-5 Forward (TGT-GCTGTGATTGAAGACGAT); ADAMTS-5 Reverse (GACT-GCAGGAGCGGTAGATCG); GAPDH Forward (TGAG-GTGACCGCATCTTCTTG); GAPDH Reverse (TGGTAACCAGGCGTCCGATA).

The RT-PCR reactions were carried out in a final volume of 20 μl containing 10 ul Dynamo flash SYBR green mix (2x master mix [contains hot-start version of a modified *Tbr* DNA polymerase, SYBR Green, optimized PCR buffer, 5 mM $MgCl_2$, dNTP mix including dUTP]), 0.4 ul ROX dye, 0.4 ul Yellow sample buffer, 0.2 μl forward and 0.2 μl reverse primers, 2 μl of template and 6.6 μl nuclease free water. The template was denatured for 10 min at 94˚C, followed by amplification cycles at 94˚C for 30 seconds, 57˚C (Caspase3, AT5, and GAPDH) for 30 seconds, and 72˚C for 30 seconds, final extension step 7 min at 72˚C.

In vitro siRNA transfection

Cells were seeded at a density of 0.15 million cells/ml in tissue culture plate to attain 80% confluence a day prior to treatment. siRNA and cationic liposomes were diluted in DMEM and mixed to form the siRNA-lipoplexes, at defined charge ratio. After brief incubation at 37˚C, the complexes were added to the cells and incubated for 4 hours followed by washing with PBS and one then suspension in fresh Dulbecco's modification of Eagle medium (DMEM) containing 10% fetal bovine serum.

In vivo studies

As a model for IVDD, annular puncture model in rabbit were used. All studies were performed in humane conditions and by experienced technicians and clinicians of Suven Lifesciences Pvt. Ltd. The animal study was approved by the Institutional Animal Ethics Committee (*IAEC approval no:CPCSEA/IAEC/JLS/003/12/15/006*)formed under the national body CPCSEA, Government of India. For this purpose, New Zealand white rabbits (n = 12, 6 months old, 3.5 kg) were anesthetized by administering 1 mL of Xylazine, 1 to 5 mg/kg, and Ketamine 10 to 15 mg/kg in combination through intravenous injection. The animals were grouped as given in Table. Under deep sedation and analgesia lateral plain radiographs were first obtained to determine the baseline values for lumbar IVD heights before injury to the discs.

Table
List of the experimental groups—in vivo studies

Experimental groups (5 discs/group)				
Control	PBS	Caspase (Cas3)	ADAMTS5 (AT5)	Caspase3-ADAMTS5 (Cas3-AT5)

PBS, phosphate buffered solution.

The rabbits were then positioned so that a postero-lateral-retroperitoneal approach could be used to assess the four consecutive lumbar IVD's (L2–L3 to L5–L6) under radiological guidance. An 18-gauge needle was used for the initial puncturing of the three noncontiguous discs (L2/3, L4/5, and L5/6), and one intact disc between L3/4–L5/6 is considered as a control. An annular puncture model was established using 18-gauge needle with defined depth of 5 mm as previously described [7–9] (Fig. 1).

Magnetic resonance imaging (MRI) studies

One week after the initial puncture, MRI scans were taken to confirm disc degeneration. Liposomal siRNA oligonucleotides formulations (Caspase 3, ADAMTS5, and Caspase 3-ADAMTS5 siRNA) from the contra lateral side into the individual discs randomly. The liposomal siRNA formulations were injected at early stage in comparison with other studies [17,18] to reveal the effect of liposomal siRNA formulations during the acute phase of disc degeneration, 25G hypodermic needle was used to inject the siRNA formulations under radiological guidance. The punctured discs were randomized to receive PBS or Caspase 3 siRNA or ADAMTS5 or dual siRNA. All rabbits were sacrificed after 8 weeks postinjection. The siRNA sequences designed for regeneration studies (L2–L3 to L5–L6) and were procured from Dharmacon. The siRNA concentration used for this study was 0.2 μmol/mL, MRI (1.5 Tesla, Phillips) scans were taken before causing puncture to the IVD of rabbit in order to assess the health of the discs and their heights. One week after the initial scan, the scans were taken at regular intervals (first, fourth, and eighth weeks) for analyzing the state of degeneration in the discs and efficacy of the liposomal siRNA formulations (Caspase 3, ADAMTS5, and Caspase 3-ADAMTS5 siRNA; Fig. 2).

Histopathological evaluation

The disc tissues were stained by haematoxylin and eosin (H&E) stain, for studying the tissue morphology of AF and NP cells using standard protocols.

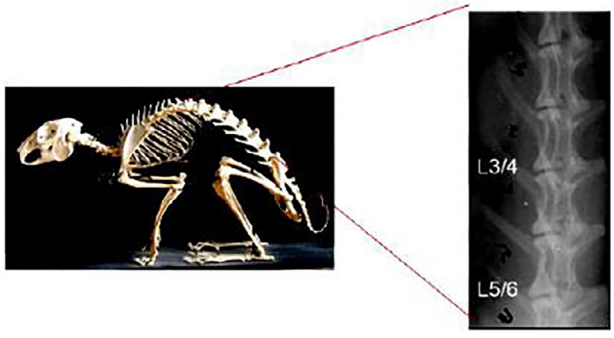

Fig. 1. Anulus puncture sites in the spine of study model. (The image is from http://wildpro.twycrosszoo.org).

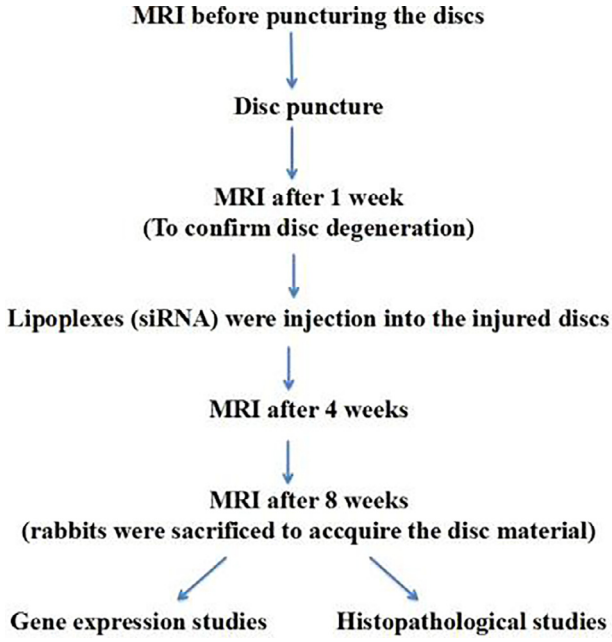

Fig. 2. Schematic representation of radiological assessment before and after introduction of liposomal siRNA.

Statistical analysis

Statistical analyses of multiple groups were performed with analysis of variance (One Way ANOVA), followed by Student's *t* test. p Value less than 0.05 was considered statistically significant.

Results

In vitro studies- HepG2 cells

siRNA directed gene silencing of Caspase 3 and AT5 were studied in vitro using human HepG2 cells. After 48-hour preculture period, human HepG2 cells were transfected with the lipoplexes containing siRNA specific for Caspase 3 and AT5. At 48 hours post-transfection in HepG2 cells, the ADAMTS5 siRNA-transfected cells showed approximately 65% knockdown of ADAMTS5 mRNA compared with the negative (PBS) and positive (Caspase 3 siRNA) controls. The gene expression results for Caspase 3 and AT5 were compared to that of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) expression. RT-PCR analysis has shown the efficiency of siRNA directed against Caspase 3 and AT5 in controlling their expression in in vitro conditions (Fig. 3).

In vivo studies – Rabbit IVD model

Radiological analysis of the IVD of spine

MRI assessment was performed on all spinal columns isolated from the rabbits ex vivo at the time death, that is, 8 weeks after the injection of liposomal siRNA formulations at the site of injury. All the MRI's were read by radiologist who was totally blind to the treatment details to

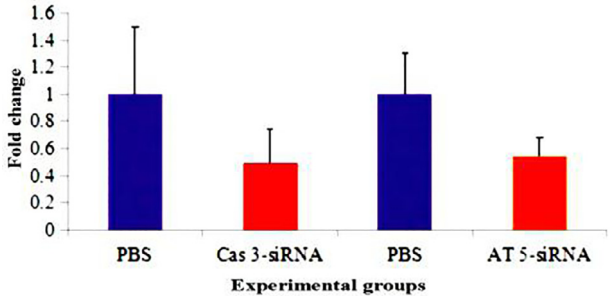

Fig. 3. Graphical representation of gene expression in disc tissue on treatment with specific siRNAs.

eliminate the bias. In the above representative MRI's, the T2 signal intensity in the disc (NP and AF) of the Caspase3-siRNA and Caspase3-AT5 siRNA-injected discs was stronger than that is in the AT5-siRNA injected discs. The MRI scans show the disc tissue as white spot in between two vertebrae, which are indicated with arrows (yellow colored) (Fig. 4).

Effect of liposomal siRNA formulations on gene expression of Caspase 3 and ADAMTS5 (AT5) in disc tissue - RT-PCR analysis

Our findings from Real-time PCR experiments demonstrated the efficacies of siRNA's directed against Caspase 3 and AT5 individually and dual siRNA combination. The customized liposomal siRNA showed ameliorative and regenerative efficiency in the degenerated disc tissues of annular puncture rabbit model. Real-time PCR analysis indicates the fold change in the expression of Caspase 3 and AT5 in the disc tissues, which received individual siRNA or dual siRNA formulation. The fold change of Caspase 3 and AT5 in each disc which was injected with siRNA against Caspase 3, ADAMTS5 and Caspase 3-AT5, were compared with the fold change observed in the disc which received only PBS. The results clearly indicate that the siRNA directed against AT5 does not show any sign of RNA interference (RNAi) in all the disc tissues, which received AT5-siRNA-AT5 formulation and it was also observed that the fold change (two-fold more expression) was more in comparison to the other siRNA formulation (Cas3-AT5).

The discs treated with AT5-siRNA-AT5 were undergoing progressive disc degeneration and loss in annular fibrosus (AF) and NP cells. The discs treated with Caspase3-siRNA-Caspase 3 (twofold less expression) showed effective RNAi in controlling the expression of Caspase 3 in all the disc tissue samples which received Caspase 3-siRNA-Caspase 3 formulation and the disc tissue showed signs of recovery in disc tissues with delayed degeneration. Similar results were found in the disc tissue which received dual siRNA formulation, indicating a possible synergistic effect of dual siRNA in controlling the disc degeneration and

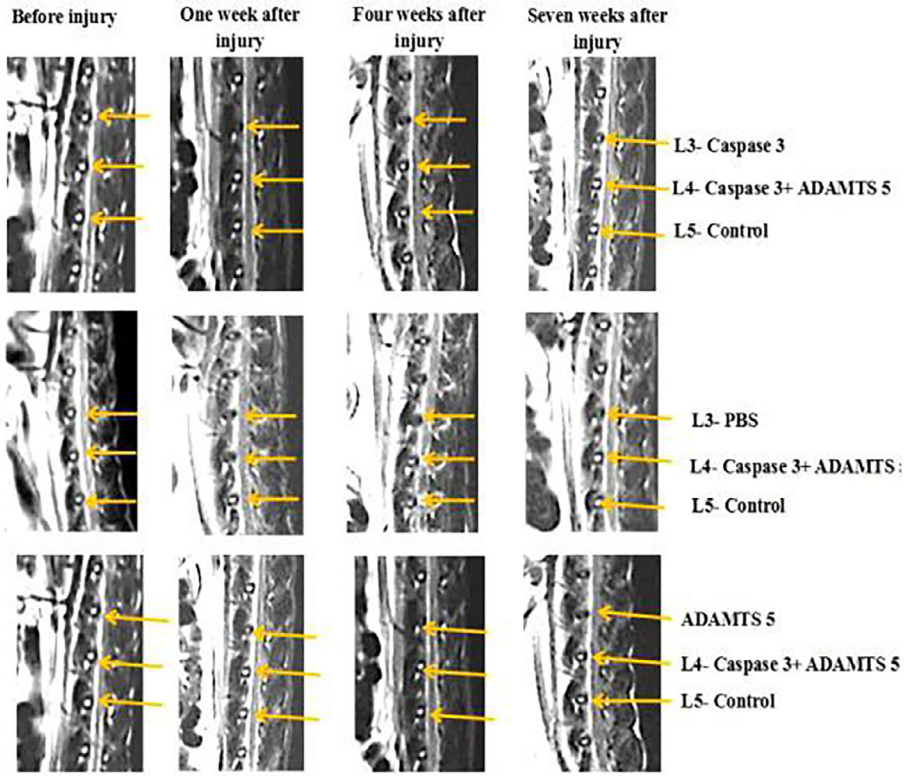

Fig. 4. Magnetic resonance imaging findings before and after liposomal siRNA oligonucleotides injection in the rabbit annular puncture model of disc degeneration. siRNA, small interfering RNA.

helping in the disc regeneration by conditioning one the loss of disc cell expression of Cas3 in the disc that received dual siRNA formulation, it was observed that it was two-fold less in comparison to the discs with single siRNA formulation. Similarly, the expression of AT 5 was found to be 1.5 fold more in the disc which received single formulation in comparison to the expression found in the disc which received dual siRNA formulations (Fig. 5).

Histological evaluation of disc tissue

Eight weeks after the administration of Caspase 3-siRNA, AT5-siRNA, Caspase3-AT5siRNAs, and PBS (control) into the injured discs, the control discs (unpunctured) showed normal cell morphology and arrangement (Fig. 6A and B). Whereas the disc's which received PBS (Fig. 6C and D) showed signs of degeneration and loss of AF or NP cells and granulation with disorganized cell morphology. The discs which received the Caspase 3 siRNA, H and E staining demonstrated the maintenance of IVD structure with lightly stained matrix and large cells; the NP and AF cell number increased and appeared to be vacuolated cells filled with smaller chondrocyte-like cells, similar to that of the normal IVD (Fig. 5 either E or F). The discs, which received Caspase 3 siRNA showed degeneration of AF and NP and granulation was observed in the cells (Fig. 6G and H).

The discs that received ADAMTS5 siRNA displayed a granulation of cells with clefts and complete loss of NP tissues, which had been replaced by a fibrocartilaginous tissue (Fig. 6G). The severely degenerated discs that had received the Caspase 3 siRNA showed a loss of proteoglycans and the collapsed, wavy fibrocartilage lamellae typical to the AF with associated fibrochondrocytes (Fig. 6H), and the tissue morphology was similar to that PBS injected disc cells. The discs that received dual siRNA (Caspase 3- AT5 siRNA) combination (Fig. 5I and J) showed signs of regeneration, increase cell number and the AF and NP cells showed normal morphological appearance (Fig. 5I and J). The histopathology grading scores demonstrate that the cellularity and matrix of the AF and NP in the Caspase 3 (Fig. 6 E and F) and Caspase3-AT5 siRNA-treated discs (Fig. 5 I and J) showed significantly lower degeneration and signs of amelioration (ie, showed binucleate cells with regeneration/repair) in comparison to the discs which received PBS (Fig. 6 C and D) and AT5 (Fig. 5G and H).

Discussion

Therapeutic application of siRNA requires accuracy in delivery to the targeted intracellular location so that the RNAi (RNA interference) machinery can interact with the target cell within the target tissue responsible for the pathology. Each of these levels of targeting poses a significant barrier [6]. To overcome these barriers, several strategies have been developed, such as chemical modifications to stabilize siRNA and viral and nonviral nucleic acid delivery systems [8]. Due to the potential adverse effects associated with the use of viral vectors in gene therapy, current areas of research include strategies that reduce the viral load required to produce significant therapeutic end product production, inducible on/off mechanisms, tissue specific promoters, and adeno-associated viral vector constructs [13]. Based on the previously reported literature [1–4], we opted to choose liposome-conjugated siRNA delivery system for the target site.

In the present study, siRNA developed against Caspase 3 and AT5 genes were introduced into the injured discs using

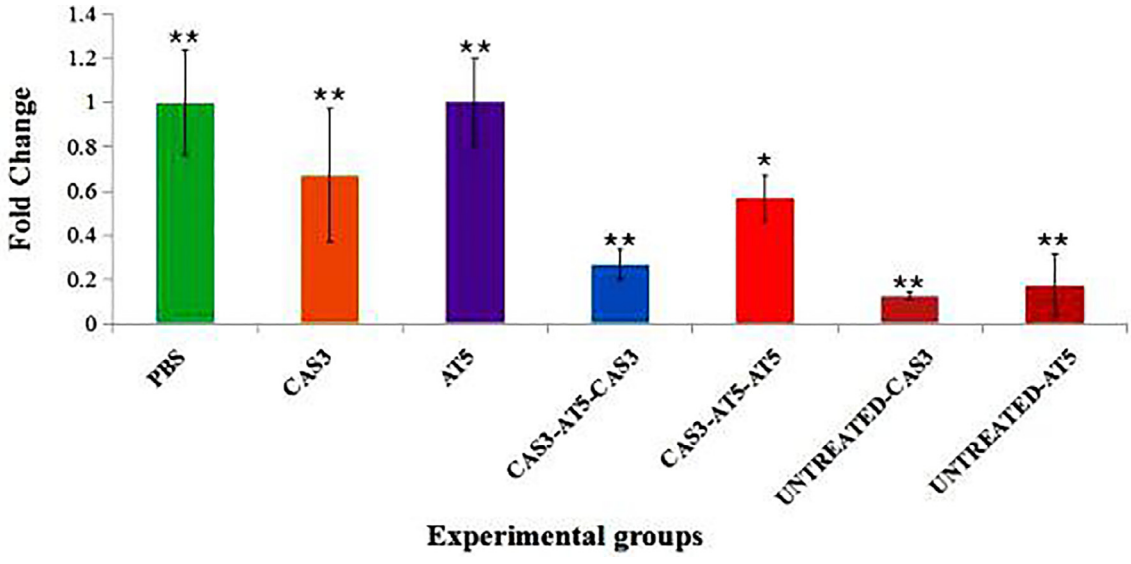

Fig. 5. RT-PCR analysis showing the efficiencies of siRNA's directed against the expression of Caspase 3 gene and AT5 gene in injured disc samples. The samples used in RT-PCR experiment were as follows: negative control (untreated discs)- NC, phosphate buffered solution (PBS), Caspase 3 (Cas3), ADAMTS5 (AT5), dual siRNA-Caspase3-AT5 (Cas3-AT5-Cas3, Cas3-AT5-AT5) with individual primer at a time. RT-PCR, real time polymerase chain reaction; siRNA, small interfering RNA.

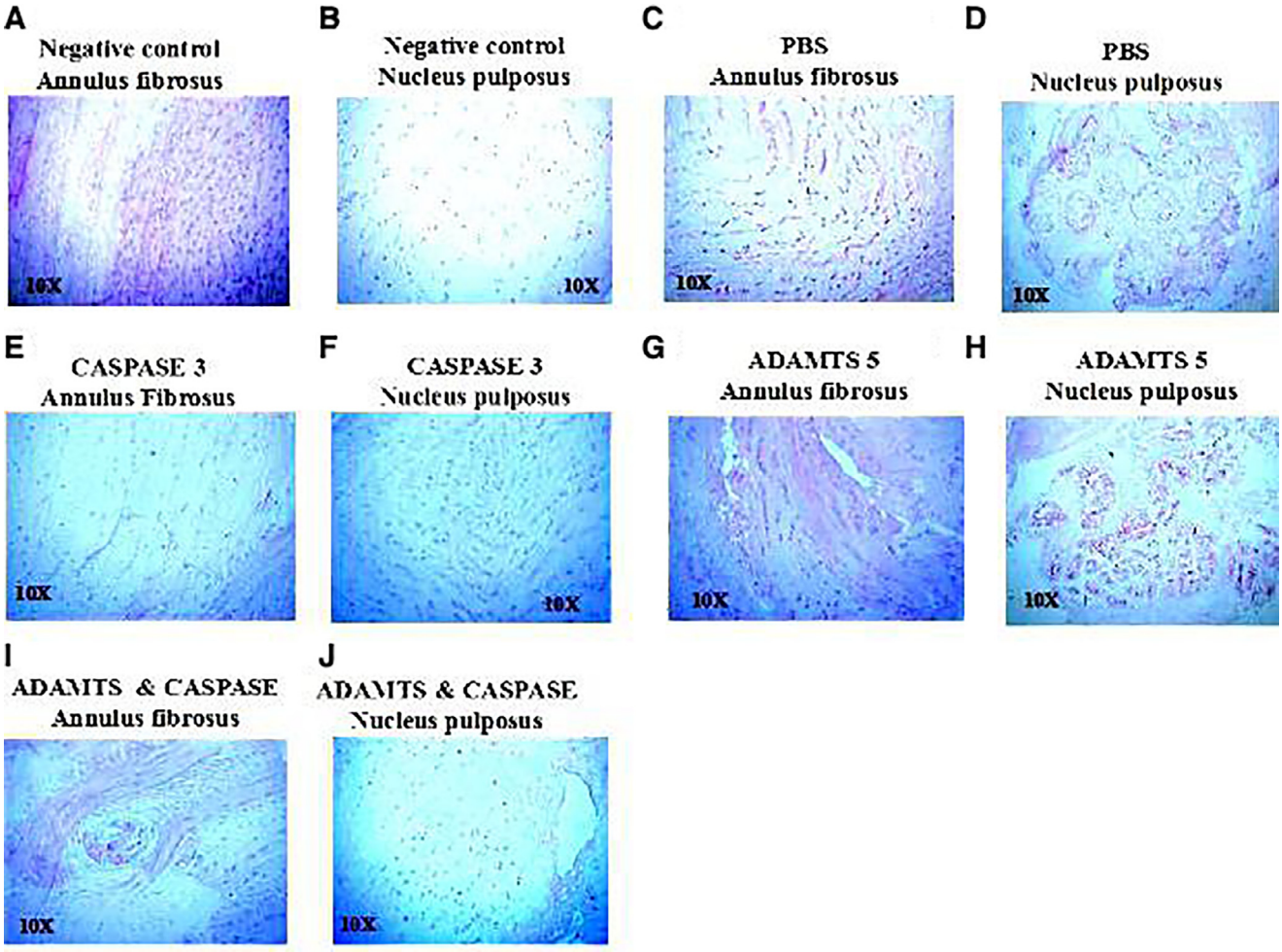

Fig. 6. (A–J): Hematoxylin-eosin stained sections revealing histological changes in IVD when treated with liposomal siRNA formulations in the rabbit annular puncture model for studying disc degeneration, eight weeks after the liposomal siRNA injections (PBS, Caspase 3, ADAMTS5 and Caspase 3-ADAMTS5 siRNA). IVD, intervertebral disc; PBS, phosphate buffered solution; siRNA, small interfering RNA.

a liposome vector for sustained release in the target cells. Proteoglycan synthesis was significantly increased in NP cells of the injured discs, which was similar to that of control discs (ie, unpunctured discs) when treated with single siRNA formulation. A synergistic effect was observed when compared to cells that were treated with a combination of two siRNA's. Cell cultures that received siRNA showed increase in proteoglycan synthesis [14–16]. Therefore, the development of inducible systems to regulate transgene expression by this strategy is possible. siRNA developed against targets showed down regulation of the gene and in turn helped in amplifying the production of extracellular matrix components.

Several inducible gene expression systems have been investigated, including systems using heat shock proteins, metallothionine, steroid regulatory promoters, tetracycline, and most recently, the insect ecdysone receptor [30]. Most of these inducible systems work by linking a ligand-activated promoter region to the potential therapeutic gene within the vector construct. There are two basic strategies for gene regulation, one could be the exogenously turning on the transgene expression by activating the ligand through administration of an adjuncts. The second approach could be incorporation of an inducible system into adeno-associated virus vector constructs and its delivery into the system [1,3]. siRNA developed against Caspase 3 and AT5 were introduced into both in vitro (HepG2 cells) and in vivo (rabbit IVD) models for assessing its efficacy in silencing the target gene. The RT-PCR results from the in vitro studies demonstrated the efficiency of siRNA in suppressing the gene expression of Caspase 3 and AT5 in HepG2 cells and it was observed that Caspase 3-siRNA - Caspase 3 was more efficient than the AT5-siRNA- AT5 formulation (Fig. 3). The siRNA formulations employed in in vivo models showed similar efficiencies. The MRI scan analysis showed varied observations in the discs which were treated with individual or dual siRNAs (Caspase 3, AT5).

The discs which were injected with Caspase 3-siRNA-Caspase 3 showed effective silencing of the Caspase 3 gene expression in the injured discs and there were also signs of regeneration process. Whereas the discs which received AT5-siRNA-AT5 showed signs of degeneration and loss of disc

cells, this was similar to the degeneration pattern observed in the injured discs which received only PBS. The annular punctured disc that received PBS showed slight increase in the degeneration compared to the pre-injection status a week after puncture. This can be attributed to the second annular puncture [31]. The discs which received the dual siRNA formulation (Caspase3-AT5), showed regeneration of disc material with retardation in the degeneration process (Fig. 4).

The expression of Caspase 3 and AT5 were quantitated in the disc samples using RT-PCR analysis. The RT-PCR results supported the MRI results, indicating the effectiveness of siRNA in regressing the AT5 activity and its help in regeneration of disc (Fig. 5). Histopathology findings also supported the MRI and RT-PCR results. Based on the results, it is evident that siRNA against Caspase 3 has successfully suppressed the Caspase 3 gene and helped in disc regeneration. The disc tissues, which were treated with AT5-siRNA against AT5, were not efficient in suppressing the expression AT5 gene and it appeared that there was progressive disc degeneration. Whereas the discs treated with dual siRNA formulation (Caspase-3-AT5) show signs of regeneration in the disc material indicating the synergistic effect of two siRNAs.(Fig. 6A–J).

The clinical application of siRNA can only be considered after large animal study. One can propose to undertake a pilot study in human patients with chronic LBP exhibiting single level disc degeneration with normal disc height.

Conclusions

The current study provides preliminary results showing synergistic inhibition of Caspase 3 and AT5 genes results in restricting the degeneration in experimentally damaged IVD of degenerated discs. Administering dual siRNA in a protective lipid-based formulation directly into IVD aids entry of siRNA into NP cells and also stabilization of siRNA. This may be responsible for the observed effects. In the absence of any other nonsurgical interventions for IVDD, altering metabolism by gene silencing in NP 1 cells by direct delivery of siRNA has potential in treating IVDD.

Conflict of interest

We do not have any conflict of interest with any one nor did we receive any fund or grants for this study.

Acknowledgments

We would like to extend our thanks to Radiologists and other colleagues from Sunshine hospitals and Dr. Vinod Goyel and his colleagues from Suven Lifesciences Pvt. Ltd., and Dr. Krishnan S. for helping us with the study.

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