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D-Ribose-L-Cysteine prevents intervertebral disc degeneration in annular puncture-induced rabbit model

B. Ogunlade[1,2], S.A. Adelakun[1], A.G. Ibiayo[2], A.A. Alao[3], S.A. Ogunlade[4]

[1] Human Anatomy Department, Federal University of Technology Akure, Ondo State, Nigeria, [2] Anatomy Department, College of Medicine, University of Lagos, Nigeria, [3] Union Diagnostic and Clinical Services, Akure, Ondo State, Nigeria, [4] Pharmacy Department, Obafemi Awolowo University, Ile-ife, Osun State, Nigeria

SUMMARY

Degeneration of the intervertebral discs is strongly implicated as the main cause of low back pain. To determine the preventive potential of D-Ribose-L-Cysteine in annular punctured intervertebral disc degeneration in rabbit model. Twenty New Zealand white rabbits (1.5 to 3.0 kg each) underwent annular puncture of the L2-L3, L3-L4, and L4-L5 discs. Group 1 (non-punctured control) were given phosphate-buffered saline; group 2 (model control) were given phosphate-buffered saline immediately after puncture for 4 weeks ; group 3 (punctured treated) were given 150 mg/kg/bw of D-Ribose-L-Cysteine solution immediately after puncture for 4 weeks; group 4 (punctured treated) were given 300 mg/kg/bw of D-Ribose-L-Cysteine solution immediately after puncture for 4 weeks. Rabbits were sacrificed at 4 weeks after puncture. The animals were housed individually in a meshed wire bottom cages with access to water and standard chow ad libitum. The serial X-rays were performed at 0 and 4 weeks for the rabbits and whole spinal column and discs were extracted and analyzed for various histological staining techniques (H&E and HVG), biochemical and immunohistochemical analysis. The X-rays showed a progressive decrease in disc height over timewhich was significantly prevented by the D-Ribose-L-Cysteine administration. The histological grade, collagen type 1 and 2, aggrecan, and matrix metalloprotease-13 mRNA expression and histological analysis were consistently indicative of degeneration, supporting the results of the X-ray data. This study has now documented that D-Ribose-L-Cysteine halts the progression of intervertebral disc degeneration and can be useful as prophylactic agents especially in people prone to disc degeneration.

Key words: Animal model – Intervertebral disc degeneration – D-Ribose-L-Cysteine – Histology – Gene expression

INTRODUCTION

Lumbar intervertebral disc degeneration affects about 85 % of people at some point during their lives with related cost of management amount to 100 billion dollars every year in the United States (Anderson, 1998). Degeneration of the lumber intervertebral discs is strongly implicated as the main cause of low back pain which is first evident among the age group of 16-20 years (Bogduk, 1991; Freemont, 2009). Disc degeneration is usually not commonly present until adulthood (Miller et al., 1991), but structural (such as fissures within the annulus fibrosus) and biochemical (decrease in proteoglycan contents) changes to the cellular microenvironment of the disc often begin just a few years of birth (Boos et al., 2002).

The intervertebral discs are partially movable secondary cartilaginous joints connecting each of the vertebral bodies in the spine, that allows the transfer of loads and impart mobility. The intervertebral disc consists of multiple, structurally distinct

Corresponding author: Adelakun Sunday Aderemi. Department of Human Anatomy, School of Health and Health Technology, Federal University of Technology, PMB 704, Akure, Nigeria. Phone: 0813-335-4835. E-mail: saadelakun@futa.edu.ng

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anatomical regions. The central nucleus pulposus (NP) contains large quantities of the proteoglycan aggrecan, which aggregates along chains of hyaluronan (Urban, 1996) which generate an osmotic swelling pressure within an irregular meshwork of collagen II fibrils. The peripherally annulus fibrosus (AF) contained the NP which has a heterogeneous composition and architecture (Humzah and Soames, 1988). It is made up of highly organized outer regions containing distinct lamellae, which are composed of bundles of collagen I fibers oriented at oblique angles that alternate within each-consecutive lamella to form an angle-ply structure (Cassidy et al., 1989; Marchand and Ahmed, 1990). In addition, the inner regions of the AF has a transition in the arrangement of collagen fiber from collage type I to collagen type II with increasing proteoglycan concentration, giving rise to a less fibrous and less organized structure (Humzah and Soames, 1988). Two thin endplates of hyaline cartilage extend superiorly and inferiorly over the inner AF and NP to connect with the vertebral bodies, and regulate nutrient diffusion between the disc cells and the vertebral bodies (Rajasekaran et al., 2004; Urban et al., 2004).

The use of animal models of intervertebral disc degeneration plays an important role in clarifying the physiopathological mechanisms and testing novel therapeutic strategies.

Experimentally induced models of disc degeneration involve the induction of structural damage by a blade (Holm et al., 2004), needle (Masuda et al., 2005; Issy et al., 2013), or drill (Lotz, 2004). The annular needle puncture model is simple, reproducible, reliable andcan cause a slow, cumulative degeneration with pathological and biochemical changes similar to those observed in human IDD (Boos et al., 2002; Le Maitre et al., 2007).

Current treatments for discogenic low back pain are predominantly conservative, involving, for example, physiotherapy and anti-inflammatory medications (Mirza and Deyo, 2007). In cases in which surgical intervention is warranted, spinal fusion is performed (Mirza and Deyo, 2007); however, fusion seeks only to alleviate painful symptoms without restoring disc mechanics or structure, recurrent episodes of pain are common and adjacent levels of the spine can experience accelerated degeneration requiring additional surgery (Ghiselli et al., 2004; Hanley et al., 2010).

More recently, disc arthroplasty (artificial disc replacement) has been used to restore mobility; however, these implants do not recapitulate the mechanical function of the native joint, are subject to wear and failure, and resection is a complex surgical procedure (Hanley et al., 2010). There is, therefore, a strong need for therapies that both alleviate painful symptoms and restore disc structures and mechanical functions.

MATERIALS AND METHODS

Nutritional Supplement

D-Ribose-L-Cysteine was obtained from Max International, Salt Lake City, Utah, USA. It was dissolved in phosphate-buffered saline.

Surgical technique

All animal handling and surgical procedures were conducted in accordance with the ethical approval from the Health Research Ethics Committee, College of Medicine of the University of Lagos (CM/HREC/02/17/105).

Twenty (20) New Zealand white rabbits (weighing about 1.5-3.0 kg) were used with institutional animal care committee approval. The surgical technique was performed using Modified Young-Joon (Young-Joon, 2013) procedure. Briefly, each rabbit was anesthetized with intramuscular injection of xylazine (5 mg/kg) and ketamine (35 mg/kg), and the fur was shaved from the mid back and right flank. After anesthesia, a lateral plain X-ray was obtained to establish the pre-injection baseline height of the IVDs. The rabbit was then placed in the lateral oblique prone position, and the injection field was sterilized with an alcohol sponge. Initially, the L5-L6 disc was identified through manual palpation of the interspinous space from the mid back and pelvic rim. After confirmation of the exact level, a 21-gauge angiography needle was inserted 3-4 cm ventral from the midline into the disc space. After brief confirmation of the needle position in the center of the disc space, the needle was held in the disc space for 30 seconds. Before removal of the punctured needle, the needle was rotated 360 degrees. In each rabbit, each of three discs (L3-L4, L4-L5 and L5-L6) was punctured. The L1-L2 and L1-L3 levels were designated as the non-punctured, internal controls. For each level, all procedures for identification and puncture were performed within a calculated time. Special care was taken to minimize contact with the peri-osteal tissues of the vertebrae because this could cause hypertrophy of the soft tissues and bony structures around the discs. Rabbits were monitored for neurological symptoms for duration of 48 hours. The rabbits were placed in their cages after observation for recovery.

The rabbits were divided into 5 animals per group:

group 1 received 50 mg/kg of phosphate-buffered saline orally for 4 weeks (non-punctured group)

group 2 received 50 mg/kg phosphate-buffered saline orally immediately after puncture for 4 weeks (model saline group)

group 3 received the oral administration of 150 mg/kg of D-Ribose-L-Cysteine solution immediately after puncture for 2 weeks

group 4 received oral administration of 300 mg/kg of D-Ribose-L-Cysteine solution immediately after puncture for 2 weeks

Lateral X-rays of the lumbar spine was taken before and after the experiment to measure IVD height. At 4 weeks after the annular puncture, rabbits were euthanatized and the IVDs were assessed. As an internal control, the non-punctured disc (L1/2 and L2/3) was also assessed.

At the end of the experiment, the animals were

sacrificed with intramuscular injection of ketamine (25.0 mg/kg) followed by intravenous injection of sodium pentobarbital (1.2 g/kg).

Radiologic analyses

The rabbits were anesthetized; then a lateral plain radiograph of the lumbar spine was taken with a radiograph machine (collimator-to film distance, 50 cm; exposure, 5 mAs; penetration power, 44 kVp). During the radiographs, special care was taken to minimize axial rotation of the disc space by holding rabbits in the lateral decubitus position while ensuring the X-ray beam is maintained straight. The lateral film was confirmed to be straight by checking for overlap of both transverse processes of the spine. The X-ray was repeated if a straight film is not obtained. To decrease the error from beam divergence, the beam was centred at 4 cm from the iliac crest. In addition, each rabbit was treated with a consistent amount of anaesthesia in order to provide a similar degree of muscle relaxation to minimize differences in disc height.

The IVD height was expressed as the disc height index (DHI) (DHI = IVD height/adjacent vertebral disc height) (20). Change in the DHI of injected discs was expressed as percentage DHI (%DHI) and normalized to the measured preoperative IVD height: %DHI = (postoperative DHI/preoperative DHI) × 100 (Masuda et al., 2006).

Biochemical analysis

Rabbits were randomly chosen for real time quantitative polymerase chain reaction using Young-Joon (2013) procedure. The L3/L4 and L4/L5 levels were extracted for the punctured disc while L1/L2 disc was extracted as a non-punctured control. From each disc, the nucleus pulposus (NP) was carefully removed from AF and stored separately. The tissues were immediately placed into liquid nitrogen and frozen at -80°C in preparation for PCR analysis. Genes were selected from the representative forms related to the ECM component (collagen type 1 and 2, aggrecan) and catabolic enzymes (matrix metalloprotease-13, MMP-13). The frozen NP samples were homogenized using Mini-Beadbeater; Bio Spec, Bartlesville, OK, USA in 1 mL Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was extracted in accordance with the manufacturer's instructions. The primers for the rabbit-specific genes were designed in accordance with published sequences in Young-Joon (2013) procedure. Gene expression was analysed by RT-PCR using ABI PRISM 9700 (Applied Biosystems, Foster City, CA, USA). Gene expression levels were calibrated using a constitutively expressed housekeeping gene, glyceraldehyde phosphate dehydrogenase (GADPH). A positive standard curve for each primer was obtained using serially diluted cDNA sample mixture. Quantifications of gene expression were calculated (comparative Ct method) using standard curves and normalized to GAPDH in each sample, and then the expression of treated discs was normalized to control discs.

The sequences were as follows: aggrecan (5' GCTACGGAGACAAG GATGAGTTC 3' and 5' CGTAAAAGACCTCACCCTCCAT-3'), MMP-13 (5' TGCCCCTCCTCAACAGTAAC-3' and 5'GAGCCCGCTGCATTCTTCTT-3'), collagen type II (5'TCAGGAATTTGGTGTGGACATA-3' and 5'CCGGACTGTGAGGTTAGGATAG-3'), collagen type I (5' GGGCAAGACAGTCATCGAATA-3' and 5' GATTGGGATGGAGGGAGTTTA-3'), GAPDH (5´ AAGGCCATCACCATCTTCCA-3´ and 5´ GGATGCGTTGCTGACAATCT-3´).

Histomorphological organization of collagen fibres analyses

Two rabbits were selected randomly before surgery and at 4 weeks after surgery for histological evaluation. The intact specimens, including the annulus fibrosus, the nucleus pulposus, both endplates, and the adjacent vertebral body bone, were be fixed.

The punctured discs and the control discs were harvested for histologic analyses. An electric saw was be used to cut each disc together with the adjacent vertebral body. Tissues were fixed with 10% neutral buffered formalin for 48 hours, Decalcified in decalcification solution (National Diagnostics, Atlanta, GA, USA) for 3 days, Processed for paraffin sectioning. Blocks embedded in paraffin were cut into mid-sagittal sections (4 µm in thickness) with a microtome. Sections were stained with Haematoxylin and Eosin (H&E) and Haematoxylin van Gieson stain. They were analyzed under a light microscope (Nikon Eclipse E800; Nikon, Melville, NY, USA) at magnifications ranging from 40× to 200×.

The degree of IDD were assessed by a histological grading scale (Masuda et al., 2005), with scores ranging from grade 4 (normal) to grade 12 (severely degenerated). This grading scale is based on degenerative changes in 4 regions: the annulus fibrosus, the border between the annulus fibrosus and the nucleus pulposus, the parenchyma of the nucleus pulposus, and the matrix of the nucleus pulposus.

Expression levels of protein (Immunohistochemical analysis)

The expression levels of Bax protein were determined using Le Maitre (Le Maitre et al., 2007) protocol. Formalin-fixed, paraffin-embedded 3 µm thick sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked by incubating the sections with 3% H2O2 for 10 min followed by digestion with 0.01% protease K for 10 min. Non-specific binding sites were blocked by incubation with confining liquid for 10 min after which the sections were incubated with rat polyclonal antibody to Bax (Cell Signaling Inc., Danvers, MA) at 4°C for 12 h. After thorough washing, the sections were incubated with biotinylated goat anti-rabbit IgG at 4°C for 60 min and then in Streptavidin-HRP for 10 min. The final color reaction was developed by incubation with the chromogenic substrate 3,3'-diaminobenzidine (0.5 mg/mL in

Tris). The sections were counterstained with haematoxylin and mounted for examination with an O-max microscope coupled to ImageJ software (National Institutes of Health, USA).

Statistical analysis

Data were analysed using Statistical Package for the Social Sciences version 24.0 computer soft package (SPSS Inc.; Chicago U.S.A.). Longitudinal X-ray data were analyzed using two-way analysis of variance (ANOVA) followed by multiple comparison using Bonferroni method. Quantitative real time-PCR data were analyzed using student t-test. Data were presented as mean ± standard error of mean. The level of significant was considered at $p< 0.05$.

RESULTS

Radiological analysis

Immediately after puncture, the model saline group (group 2) showed a significant narrowing of disc height when compared with that of the non-punctured group (group 1) (approximately 15.0% decrease compared with the baseline percentage disc height index (DHI), $P <0.05$) (Fig. 1). There were no significant differences in the percentage DHI among the treated groups that received D-Ribose-L-Cysteine solution immediately after the puncture (Fig. 1).

Histological analysis

The results of the IVD histology immediately after puncture were shown in Fig. 2. Photomicrographs of histological sections for groups 3 and 4 revealed that the administration of D-Ribose-L-Cysteine solution prevent distortions in the cytoarchitecture and histomorphology of the IVD compared to that of the model saline group (group 2). The observation of the non-punctured group (group 1) showed normal IVD morphology. However, model saline group showed structural disorganization resembling disc degeneration, narrowing of the disc height in which most of the NP contents have been lost and collapsed.

Histological grading scores

To compare the histomorphological findings more clearly and precisely, a previously described histological grading score for the degeneration of IVD was used (5). The scoring grade ranges from 4 (normal) to 12 (severely degenerated), depending on the level of degeneration. In this study, were no significant differences in the histological scores among the treated groups that received D-Ribose-L-Cysteine solution when compared with the non-punctured control (Fig. 3).

Morphometric analysis of chondrocyte-like cells in IVD

Within this present study, one of the major cellular changes was the significant increase in chondrocyte-like cells in the inner AF and NP after D-Ribose-L-Cysteine administration in the treated groups compared to model groups ($P <0.05$) (Fig. 3). In the outer AF, there was no significant difference in the number of chondrocytes-like cells across all the groups in this study (Fig. 4).

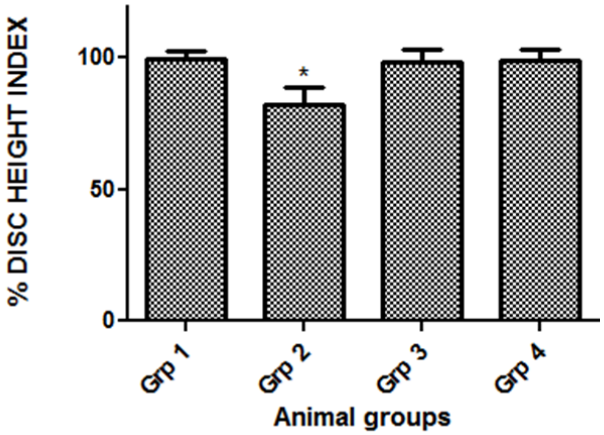

Fig 1. Intervertebral disc height after administration of D-Ribose-L-Cysteine immediately after puncture. Values are mean ± SEM for 5 rabbits in each group. *: $p < 0.05$ as compared to group 1.

Histological sections of organization of collagen fibres

The morphological features in the organization of collagen fibres in the non-punctured group (group 1) were consistent showing oblique concentric direction of collagen fibres, which were continuous and complete within the AF (Fig. 5). The photomicrograph of collagen fibre in the model saline group (group 2) showed disruption in the lamellae structure, partial disorganization in the oblique arrangements of collagen fibrils and loose attachment of the lamellae to the endplate. The treated groups showed nearly similar morphological features in the arrangement of collagen fibres with that of group 1.

Gene expression levels

In this study, the relative gene expression levels of aggrecan and Col2 were decreased ($P<0.05$), while relative gene expression levels of Col1and MMP 13 increased over time after annular puncturein the model saline group (group 2) (Fig. 6; $P<0.05$) compared with the non-punctured group (group 1) although no significant difference in the relative gene expression levels of the treated groups compared to the non- punctured group in response to D-Ribose-L-Cysteine administration immediately after puncture.

Immunohistochemical analysis

The key regulators of mitochondria- mediated apoptosis are anti-apoptotic members such as Bcl-2 implicated in the inhibition of apoptosis and pro-apoptotic members such as Bax which induces apoptosis. The photomicrograph of the histochemical section revealed that the expression level of Bax in the model saline group (group 2) was

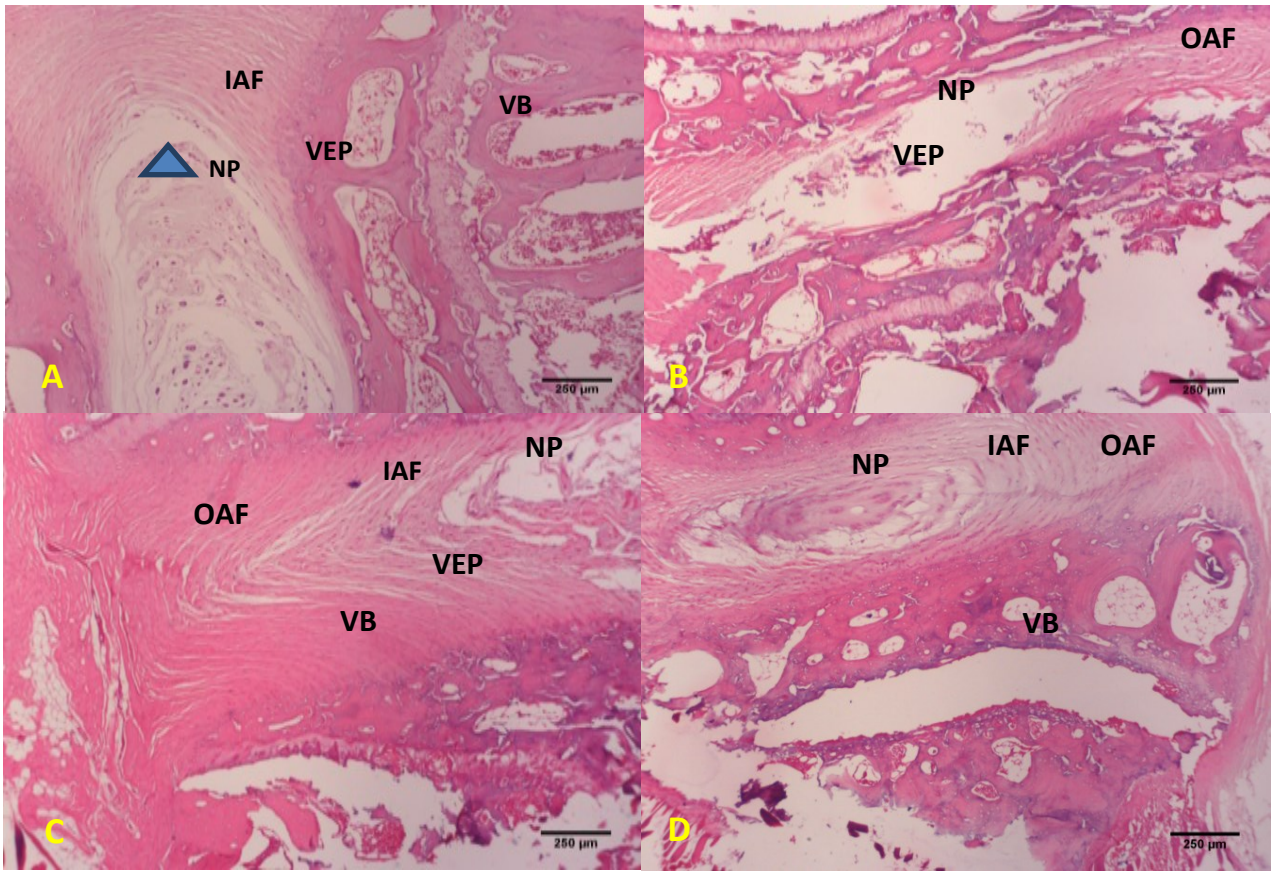

Fig 2. Photomicrograph of intervertebral disc histology administered with D-Ribose-L-Cysteine immediately after puncture. A: Group 1, showing normal IVD morphology with intact CLCs (arrow); B: Group 2, showing decrease in disc height, devoid CLCs; C: Group 3 and D: Group 4 showing similar features with group 1. CLCs: chondrocytes like cells; VB: Vertebral Bone; VEP: Vertebral end plate; NP: Nucleus pulposus; IAF: Inner annulus fibrosus; OAF: Outer annulus fibrosus; H&E staining, x100, scale bars: 250 µm.

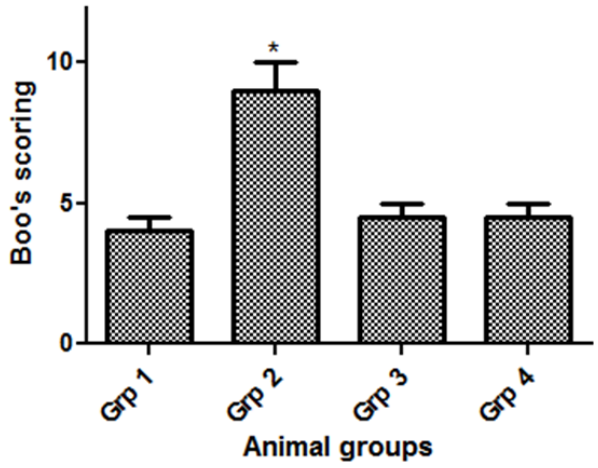

Fig 3. Histological grading scores for level of degeneration of rabbit intervertebral disc after D-Ribose-L-Cysteine administration. *: $p < 0.05$ as when compared to group 1; #: $p < 0.05$ as when compared to group 6.

higher compared with the non-punctured group (Fig. 7) while the treated groups were reduced when compared with group 2 and similar with that of non-puncture group (group 1).

DISCUSSION

Therapeutic strategies currently used for patients with disc degeneration remain symptomatic; dedicated mainly to relieving painful symptoms of low back pain. Some preclinical tests in adapted animal models are required before promising biotherapies could enter the therapeutic arsenal. Rabbit was used as a model for IDD due to some important attribute which include easy accessibility with low cost, surgical accessibility was easy with adequate disc size, quick and reliable onset of disc degeneration (Pfirrmann et al., 2001).

The age of the rabbit used for this study ranges between 20-24 weeks since no morphological evidence of degenerative changes of the intervertebral disc was observed in previous study till 24 weeks age in rabbit model (Kim et al., 2005). The results of this study demonstrated that the administration of D-Ribose-L-Cysteine was effective in preventing and restoring disc height in this animal model.

In this study, the annular punctured model exhibited slow progressive disc degeneration as evident by radiology (in the percentage DHI) (Fig. 1) and histology (in the histological score) (Fig. 2) beginning at 2 weeks till at least 4 weeks post-surgery. Thus, this annular puncture model revealed a pro-

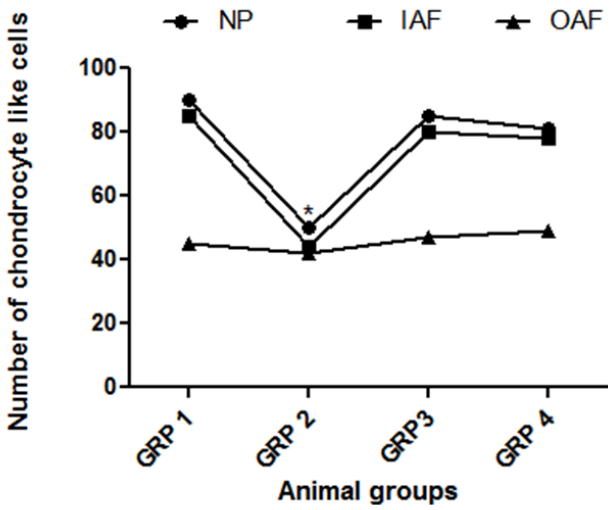

Fig 4. Number of chondrocyte like cells in nucleus pulposus (NP), inner anulus fibrosus (IAF) and outer annulus fibrosus (OAF) of rabbit intervertebral disc after D-Ribose-L-Cysteine administration.*: $p < 0.05$ as compared to group 1.

gressive disc degeneration overtime resembling human intervertebral disc degeneration (IVDD) (Masuda et al., 2005; Freemont, 2009). The disruption in the cytoarchitecture within the annulus fibrosus in several animal models and in humans has revealed the occurrence of disc degeneration (Olsewski et al., 1996; Carragee et al., 2009).

The present study showed that the administration of D-Ribose-L-Cysteine immediately after annular puncture prevented the progression of IDD as evident in the percentage DHI (Fig. 1). Moreover, there were no apparent side effects such as ossification in the IVD and anatomical or physiological changes in NP cells in this study.

Histomorphological outcome is a key tool used in validating the present model which could predict the severity of disc degeneration. The present study revealed various histological alterations, ranging from discrete disruption of the nucleus pulposus, decrease nucleus pulposus cells and decrease lamella disorganization and complete obliteration of its cavity.

Histological observation of the IVD sections of the treated groups after the administration of D-

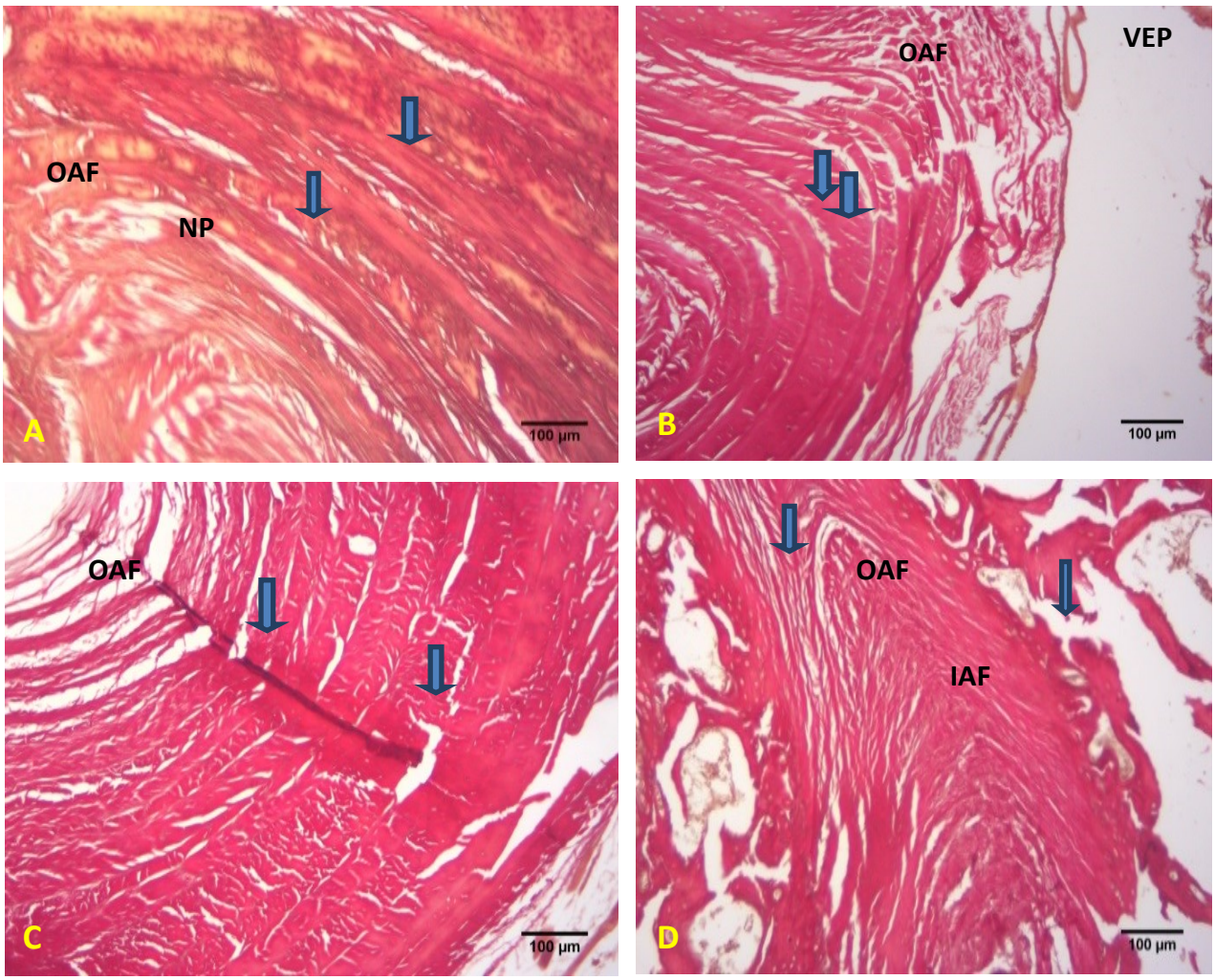

Fig 5. Intervertebral disc collagen fibres of rabbit administered with D-Ribose-L-Cysteine immediately after puncture. A: Group 1; B: Group 2; C: Group 3; D: Group 4 showing presence of collagen fibres in AF (arrows). VB: Vertebral bone; VEP: Vertebral end plate; NP: Nucleus pulposus; IAF: Inner annulus fibrosus; OAF: Outer annulus fibrosus; H&E staining. Scale bars: 100 µm.

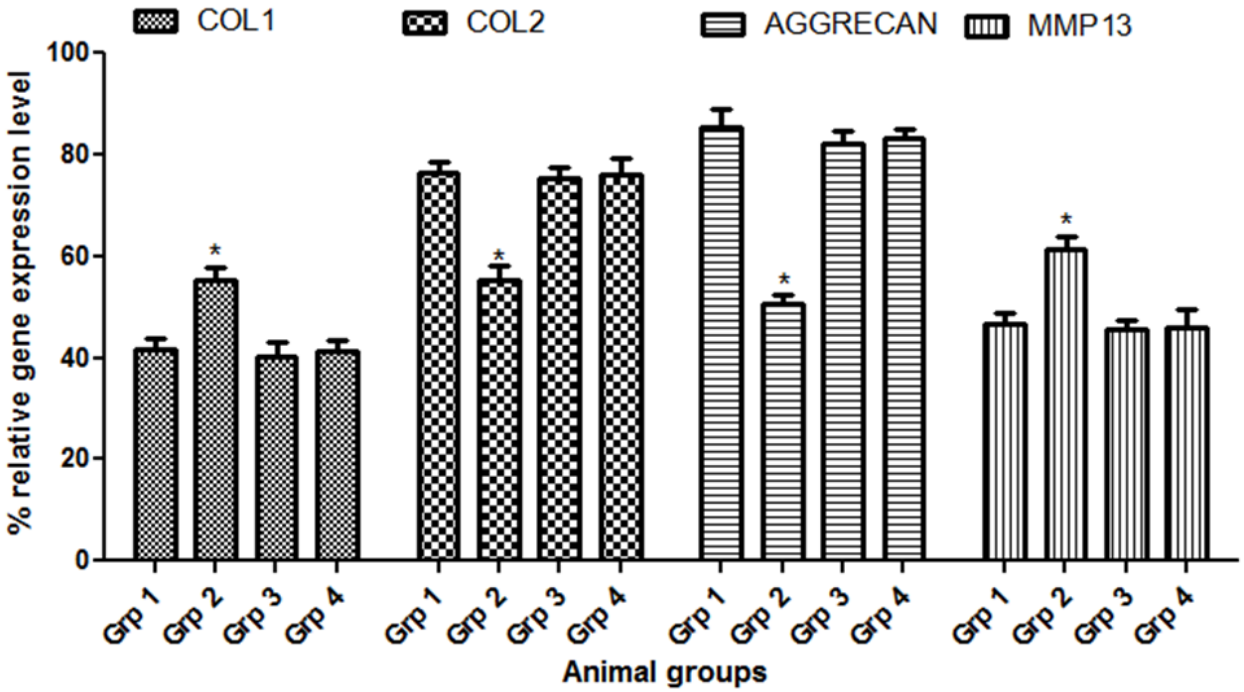

Fig 6. Gene expression level of collagen type 1, collagen type 2, aggrecan and matrix metalloproteinase-13 in rabbits administered with D-Ribose-L-Cysteine immediately after puncture calculated as relative percentages compared with the model non-punctured level.*p<0.05 as compared to model non-punctured control.

Ribose-L-Cysteine revealed clear morphological features compared with the model saline group, showing increasing numbers of chondrocyte-like cells, found in either the inner AF or the NP.

The extracellular matrix of the IVD confers resistance to mechanical loading thereby permitting both rotational and planar mobility. This functional role is attributed to conformational arrangement of collagens, proteoglycans, and non-collagenous proteins within the matrix thereby allowing maximum adaptation for biomechanical functions.

In the present study, it was evident that collagen fibres were more in inner AF than outer AF throughout the groups administered with D-Ribose -L-Cysteine. The difference between the collagen and elastic components of the disc could pose a high risk factor in the etiopathogenesis of disc degeneration and acts as a precursor for degenerative changes thereby causing LBP (Choudhary et al., 2011). Structural differences in the outer and inner AF also affects the strength of the disc, in which the outer AF is weaker and more prone to damage and this is in correlation with clinical observations whereby most of the assault within the IVD occurs in the outer AF (Misterska et al., 2011; Wang et al., 2012).

The structural disorganization of tissues within the nucleus pulposus initiates the degeneration process. Alteration in the NP cell phenotype was carried out on the expression levels of molecules that were previously shown to be modulated during the onset of IVD degeneration (COL1, COL2, AGC and MMP13) (Sakai et al., 2005; Tomoyasu et al., 2007; Alsousou et al., 2012).

The results showed increase in Col1 and MMP-13 relative gene expression level with a concomitant decrease in Col2 and aggrecan in the model saline group when compared to the non-punctured group. In addition, suggesting that NP cells experience a process of dedifferentiation. Dedifferenciation process has been well described in cultured articular chondrocytes (Schnabel et al., 2002) and in osteoarthritic joints (Clouet et al., 2009). Previous research suggested that during the process of disc degeneration, the molecules with a longer halflife (collagen type II) exhibit an early decrease in the corresponding transcript levels (Alsousou et al., 2012). MMP-13 is known to degrade collagens and glycosaminoglycans (Roberts et al., 2000). The increase in MMP13 could therefore be a major contributor of IVD degeneration (Alsousou et al., 2012) as it has been extensively reported in cartilage degradation during osteoarthritis (Sakai et al., 2005).

Bax is a pro-apoptotic protein which is regarded as a key regulator of mitochondrial mediated apoptosis. Bax has been shown to induce apoptosis in several cell lines (Wang et al., 2013). In this study, Bax immunoreactivity was detected mainly in the model saline group in this study (Fig. 7). In addition, the expression levels of Bax among the groups administered with D-Ribose-L-Cysteine were reduced when compared with group 2. It could be deduced that Bax was involved in the process of nucleus pulposus cell apoptosis, thereby affecting the anabolic and catabolic mechanism between cell proliferation and apoptosis ultimately resulting to degen-erated disc (Wang et al.,

2013).

CONCLUSION

The administration of D-Ribose-L-Cysteine was effective in preventing and restoring disc height and increasing chondrocytic cells in the IVD of the rabbit model. The results of this study shows that the administration of D-Ribose-L-Cysteine. This study has now documented that D-Ribose-L-Cysteinehalts the progression of intervertebral disc degeneration and can be useful as prophylactic agents especially in people prone to disc degeneration.

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