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# HISTOMORPHOLOGICAL RESPONSE OF SULFORAPHANE AND RIBOCEINE ON ANNULAR PUNCTURE- INDUCED MODEL OF RABBITS INTERVERTEBRAL DISC DEGENERATION

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## ABSTRACT

**Background:** Intervertebral disc degeneration is one of the leading causes of Low back pain that is regarded as one of the most frequent neurological ailments. **Objective:** To determine the regenerative potential of sulforaphane and riboceine supplements in a rabbit model in annular punctured intervertebral disc degeneration. **Methods:** Forty New Zealand white rabbits (3 to 3.5 kg each) underwent annular puncture of the L2-L3, L3-L4, and L4-L5 discs. Rabbits were sacrificed at 2, 4 and 8 after puncture. For a longitudinal study to assess changes in disc height over time, serial X-rays were performed at 0, 4 and 8 weeks for the rabbits. Upon sacrifice, the whole spinal column and discs were extracted and analyzed with real time reverse transcriptase-polymerase chain reaction, and histological staining. **Results:** The radiology assessment revealeda significant progressive decrease in disc height which was reversed by the administration of sulforaphane and riboceine. Significant disc space narrowing compared to preoperative disc height was observed during the time period ($p<0.05$). The histological grade, collagen type 1 and 2, aggrecan, and matrix metalloprotease-13 mRNA expression and Hematoxylin and Eosin were indicative of degeneration, supporting the results of the radiology. **Conclusion:** Administration of sulforaphane and riboceine in this study revealed the regenerative potential of nutritional supplement in intervertebral disc. The annular puncture model can be used to study and validate the therapeutic potential of drugs and biological materials in the treatments of degenerative disc disease.

**KEYWORDS:** Animal model, intervertebral disc degeneration, sulforaphane, riboceine, histology, radiology.

## INTRODUCTION

Low back pain is one of the most common orthopaedic conditions affecting up to 85% of people at some point during their lives, resulting in healthcare and related costs every year.[1,2] The major contributing factor to low back pain is lumbar intervertebral disc degeneration (IDD).[3,4]

IDD is a chronic disease involving progressive changes in disc composition and structures, leading ultimately to chronic pain and dysfunction.[5] Disc degeneration disease is one of the most frequent neurological ailments[6] and every individual has 80% probability of having degenerated disc in their lifetime.[7] It was observed to be first seen in the age group 11–16 years[7,8] and about 20% teens has a mild signs of degeneration[9] with about 10% of sufferers being chronically disabled.[10] The economic burden of degenerated disc as the main cause of Low Back Pain (LBP) in Africa is enormous and continues to increase[11] with prevalence of LBP in Nigeria being about 46.8% in various occupational activities[12] and about 48% of sick absences from work is due to LBP[13] with about 20.6% of hospital workers suffers from LBP.[14]

Anatomically, the intervertebral discs are partially movable secondary cartilaginous joints that connect each of the vertebral bodies in the spine, functioning both to transfer loads and impart mobility.

The intervertebral disc is a complex structure that contains very few cells embedded in an extracellular matrix. These cells have the essential function of maintaining and repairing the matrix by synthesising matrix macromolecules and synthesising proteinases for matrix breakdown.[15] The imbalance between matrix synthesis and matrix breakdown alters the matrix

composition and organisation and ultimately resulting in inadequate cellular repair responses. Therefore, the degraded matrix can no longer carry load effectively and some of the cells become necrotic, the endplate of the disc calcifies and disc degeneration begins.[15]

Discs degenerate far more rapidly than other tissues in the body. In prolonged disc degeneration, blood vessels and nerves penetrate the previously avascular and aneural disc giving rise to discogenic pain. This, in turn, causes further disc degeneration, which alters the spinal mechanics and leads to painful and disabling conditions.[15]

The etiology of disc degeneration has proven challenging to characterize because it is poorly defined and its progression is closely linked to aging.[16,17] IDD are thought to be caused byboth genetic and environmental, but are probably also related to wear accumulating over a lifetime of normal daily use. Disc degeneration begins with changes to the cellular microenvironment within the substructures of the disc that progress over decades to structural breakdown and functional impairment.[17,18] Biochemically, IDD is associated with loss of collagen type II and proteoglycans, which are the main extracellular matrix constituents of the healthy nucleus pulposus.[19] These are replaced by collagen type I leading to loss of function, secondary damage to the annulus and eventually horizontal clefts through the entire IVD.[20]

In addition, there is growing evidence that age, gender, height, obesity, smoking, occupational exposure, heredity and psychosocial factors may constitute risk factors of intervertebral disc degeneration and ultimately results to low back pain. Furthermore, surgical procedures, such as microdiscectomy and laser or other decompressive procedures, induce intervertebral disc (IVD) degeneration after surgical treatment. Although disc degeneration is not commonly present until adulthood,[21] changes to the cellular microenvironment of the disc begin within just a few years of birth.[22]

In the majority of cases, especially in clinics, disc degeneration is classified using imaging techniques,[4,23,24] while large-scale investigations on the histomorphological changes in the IVD, particularly in clinically well defined surgical material, is sparse.

Several animal models of IDD have been described[25,26] for studying pathogenesis, pathophysiology, and treatment of IDD.[27,28] These models are spontaneous IDD models and experimentally induced IDD models, experimentally induced IDD models involve the induction of structural damage by a blade,[28] needle,[29] or drill. The Annular needle puncture can cause a slow, cumulative degeneration with pathological and biochemical changes similar to those observed in human IDD and remains the commonly used established animal models of IDD due to its reproducibility and reliability.[30]

Current treatments for discogenic low back pain are predominantly conservative, involving, for example, physiotherapy and anti-inflammatory medications, In cases in which surgical intervention is warranted, spinal fusion is performed;[31] 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.[32,33]

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.[33] There is, therefore, a strong need for therapies that both alleviate painful symptoms and restore disc structures and mechanical functions. Present study therefore focused on histomorphological studies of annular puncture-induced model of rabbits intervertebral disc degeneration subjected to sulforaphane and riboceine.

## MATERIALS AND METHODS

### Nutritional Supplement

Sulforaphane (SFN) supplements were purchased from Sigma- Aldrich (St Louis, MO) and Riboceine supplements were obtained from Max International, Salt Lake City, Utah, USA. They were 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).

Forty (40) New Zealand White rabbits (weighing about 3-3.5 kg) were used with institutional animal care committee approval. The surgical technique was performed using Young-Joon, 2013[34] 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 steril-ized 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. The rabbits were placed in their cages after observation for recovery.

The punctured rabbits were then divided into 5 animals per group.

- Group 1 received 50mg/kg Normal saline (Non-puncture) for 4 weeks (positive control group).
- Group 2 received normal saline 50mg/kg 4 weeks after puncture for 4 weeks (negative control group).
- Group 3 received the oral administration of 150 mg/kg of sulforaphane solution after 4 weeks of puncture for another 4 weeks.
- Group 4 received the oral administration of 450 mg/kg of sulforaphane solution after 4 weeks of puncture for another 4 weeks.
- Group 5 received the oral administration of 150 mg/kg of riboceine solution after 4 weeks of puncture for another 4 weeks.
- Group 6 received the oral administration of 450 mg/kg of riboceine solution after 4 weeks of puncture for another 4 weeks.
- Group 7 received the oral administration of 150 mg/kg of sulforaphane and riboceine solutions after 4 weeks of puncture for another 4 weeks.
- Group 8 received the oral administration of 450 mg/kg of sulforaphane and riboceine solutions after 4 weeks of puncture for another 4 weeks.

Lateral X-rays of the lumbar spine was taken before and at 2, 4 and 8 weeks after surgery to measure IVD height. At 8-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 planned time, 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).[35] 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.[35]

**Biochemical analysis**

Two rabbits were randomly chosen before surgery and at 2, 4 and 8 weeks after surgery for RT-PCR.

The L3-L4 and L4-L5 levels were extracted for real time quantitative polymerase chain reaction (PCR) analysis. The L1-L2 disc was also 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 with a homogenizer (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. cDNA was generated with Moloney murine leukemia virus reverse transcriptase (RT)(Invitrogen). The RT reaction was amplified in triplicate with real-time PCR in a final volume of 10 µL using SYBR Green Master Mix reagent at a final concentration of 1× (Applied Biosystems, Foster City, CA, USA).The PCR conditions used were specific to each target transcript. Aggrecan: denaturation for3 min at 94°C, followed by 36 cycles of 94°C for 30 s, 61°C for 45 s, and 72°C for 1 min, and a final extension at 72°C for 6 min; type II collagen (*Col2*): denaturation for 3 min at 94°C, followed by 35 cycles of 94°C for 30s, 60°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min; type I collagen (*Col1*): denaturation for 3 min at 94°C, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 45 s, and a final extension at 72°C for 7 min. Results were obtained and evaluated with Microsoft Excel.

The primers for the rabbit-specific genes were designed in accordance with published sequences available in GenBank. The sequences were as follows: aggrecan (5' GCTACGGAGACAAG GATGAGTTC 3' and 5' CGTAAAAGACCTCACCCTCCAT 3'), MMP-13 (5' TGCCCCTCCTCAACAGTAAC 3' and 5' GAGCC CGCTGCATTCTTCTT 3'), Type II collagen:(5'-TCAGGAATTTGGTGTGGACATA-

3'and5'CCGGACTGTGAGGTTAGGATAG -3') Type I collagen (5' -GGGCAAGACAGTCATCGAATA- 3' and 5' -GATTGGGATGGAGGGAGTTTA- 3')

**Histomorphological analyses**

Two rabbits were selected randomly before surgery and two each at 2, 4 and 8 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 hematoxylin and eosin (H&E) and then, 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,[35] 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.

## RESULTS

**Radiological analysis**

The radiology results revealed statistically significant difference ($p<0.05$) in the treated groups that received the supplementsas the percentage disc height index (DHI) increased across the experimental groupas compared to that of the negative control group (figure 1).

The combined administration of the two drugs induced restoration of disc height to the level approaching (85%) the non-punctured disc(98.8%) with the greatest significance level at the high dose (Figure 1). While the single dose administered group also showed a significant recovery of disc height compared with the vehicle group (group 2), it was not more than 75% when compared to that of the non-punctured disc ($P <0.05$ versus group 1).

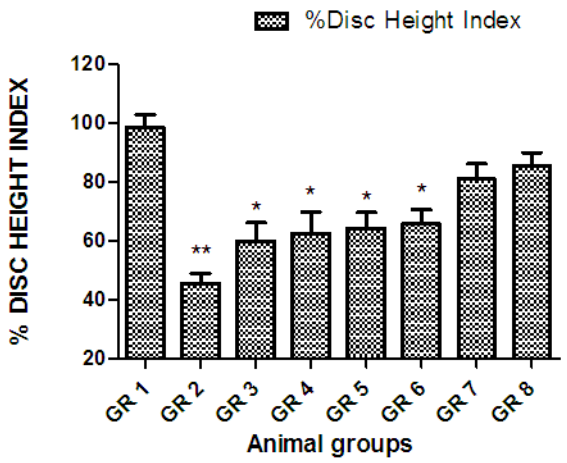


**Figure 1: Values are Mean±SD for 5 rabbits in each group. *: p < 0.05 as compared to group 1(control); **: p < 0.05 as compared to group 8.**

**BIOCHEMICAL ANALYSIS**

Administration of sulforaphane and riboceine revealed a significant statistical increase in the expression level of collagen type 1, collagen type 2 and Aggrecanase as compared to the control group. However, the sulforaphaneandriboceine supplements administered revealed overall significant ($p<0.05$) decrease in the Matrixmetalloproteinase compared to the control group at varied dosage after the annular puncture (figure 2 ).

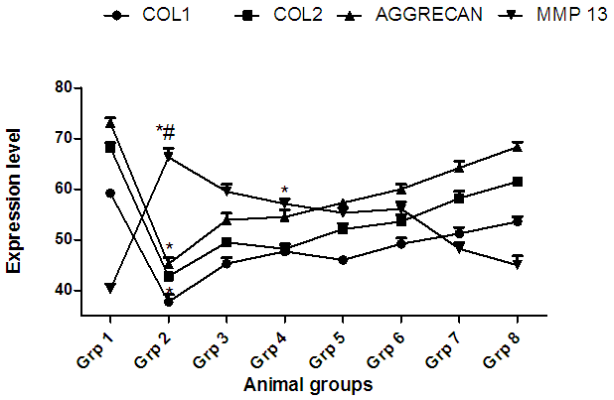


**Figure 2: Phenotypic markers in Nucleus pulposus cells. Analysis of the expression levels of transcripts coding for the phenotypic markers (A)collagen type I (B) Collagen type II, (C) Aggrecan (D) MMP-13 in Nucleus pulposus (NP) cells from IVD of rabbits.**
***: p < 0.05 as compared to group 1(positive control); * #: p < 0.05 as compared to group 8.**

**HISTOLOGICAL ANALYSIS**

The combined administration of sulforaphane and riboceine supplements produced a notable reversal in the distortions to the histoarchitecture of the IVD within the experimental groups compared to the control group.

The observation of the non-punctured group (group 1) (positive control) showed a clear boundary between NP and AF, no cracks or fissures existed in NP, and no cell cluster formation. In addition, there are large numbers of

chondrocyte-like cells, which appear as large cells encircled with pericellular matrix densely stained with histological stain, were found in either the inner AF or the NP (Fig. 3) of the positive control group.

Histological observation in the untreated group (Negative control) (Fig. 4) showed severely degenerated discs in which most of the NP contents have been lost and collapsed, wavy fibrocartilage lamella and associated fibrochondrocyte-like cells of the AF.

In the sulforaphane and riboceine administered groups, (fig. 5, 6, 7 and 8) there were few NP cells surrounded by extracellular matrix and only mild disruption of the lamellar structure was observed in the AF. The same histological findings were observed in the combined dosage of the two supplements in which the structure of both the NP and AF was well maintained compared to that of the single dose (Fig. 9 and 10).

To compare the histomorphological findings more clearly and precisely, a previously described histological grading scale for the degeneration of IVD was applied. In scoring grades from 4 to 12, the grade of degeneration within the NP and AF was significantly less severe in the combined administered groups than in the control groups. However, there were no significant differences in the histological scores among the treated groups between the low and high dosage.

Within this present study, one of the major cellular changes was the increasing number of chondrocyte-like cells in the inner AF or in the NP of the animals administered with combined sulforaphane and riboceine. In the NP and inner AF, the number of chondrocyte-like cells in the treated groups was significantly higher than those of the control groups ($P < 0.05$) (Figure 3). The groups administered with combined dosage of sulforaphane and riboceine showed significantly increased number of chondrocyte-like cells in the NP and inner AF compared with that of the single dose groups ($P < 0.05$).

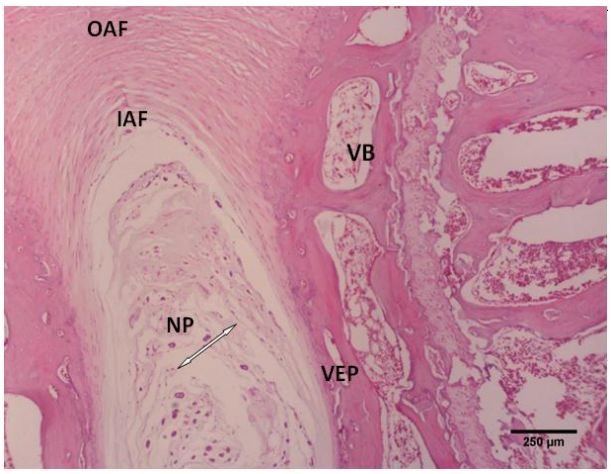


**Figure 3: Photomicrograph of IVD histology (Group 1). Arrow shows chondrocyte like cell within the intact nucleus pulposus and no cleft formation around the end plate: VB: Vertebral Bone; VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus.**

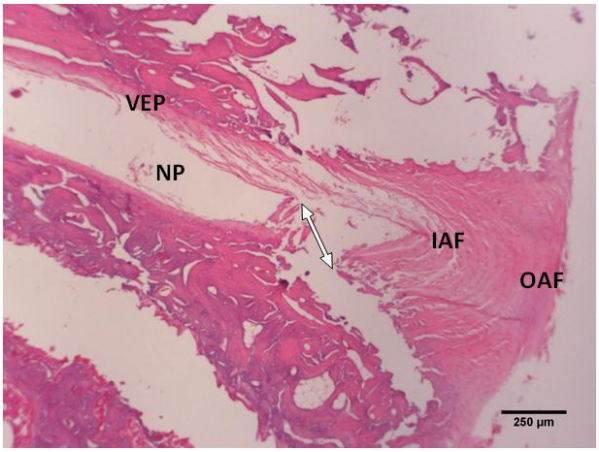


**Figure 4: Photomicrograph of IVD histology (Group 2; Negative control) showing extrusion of the cells within the NP. Arrow shows cleft formation around the end plate VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus Fibrosus;OAF:Outer Annulus Fibrosus; H and E X100.**

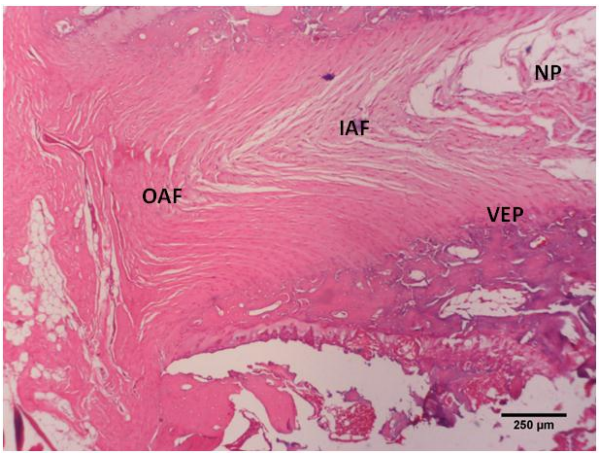


**Figure 5: Photomicrograph of IVD histology (Group 3 ) showing presence of chondrocyte like cells within the partial disorganized NP.VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus Fibrosus;OAF:Outer Annulus Fibrosus; H and E X100.**

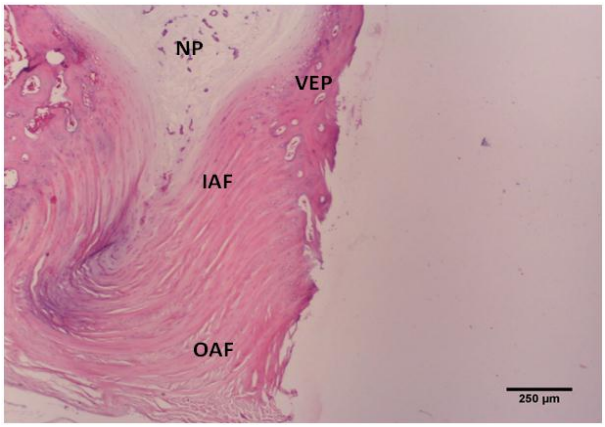


**Figure 6: Photomicrograph of IVD histology (Group 4) showing presence of chondrocyte like cells within the NP.VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus Fibrosus;OAF:Outer Annulus Fibrosus; H and E X100.**

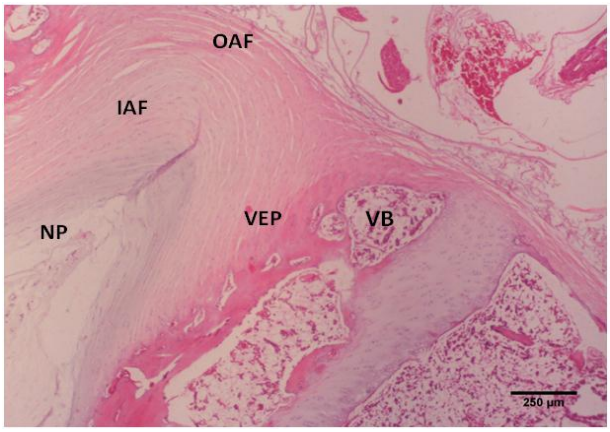


**Figure 7: Photomicrograph of IVD histology (Group 5) showing presence of chondrocyte like cells within the NP.VB: Vertebral Bone; VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus Fibrosus;OAF:Outer Annulus Fibrosus; H and E X100.**

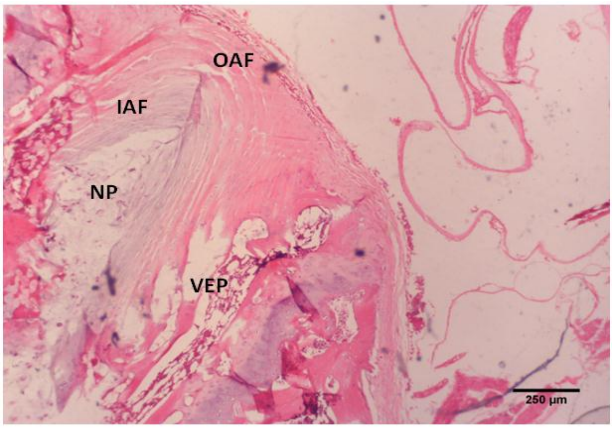


**Figure 8: Photomicrograph of IVD histology (Group 6) showing presence of chondrocyte like cells within the distorted NP. VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus Fibrosus;OAF:Outer Annulus Fibrosus; H and E X100.**

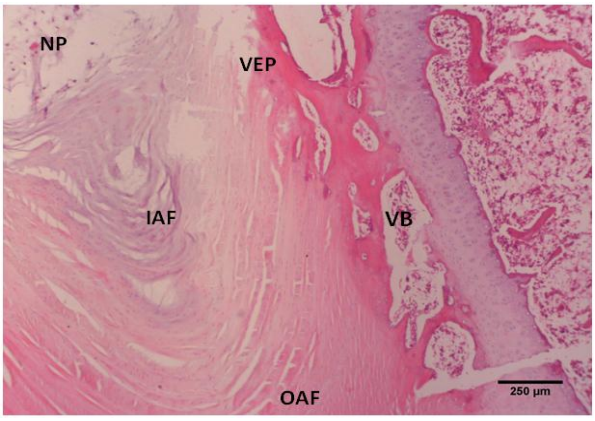


**Figure 9: Photomicrograph of IVD histology (Group 7) showing presence of few chondrocyte like cells within the NP, IAF fibers revealed partially disorganised.VB: Vertebral Bone; VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus Fibrosus;OAF:Outer Annulus Fibrosus; H and E X100.**

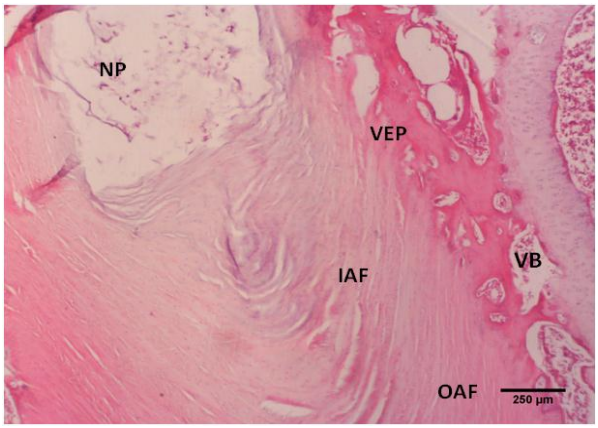


**Figure 10: Photomicrograph of IVD histology (Group 8) showing presence of chondrocyte like cells within the NP.VB: Vertebral Bone; VEP-Vertebral End Plate; NP- Nucleus pulposus; IAF: Inner Annulus Fibrosus;OAF:Outer Annulus Fibrosus; H and E X100.**

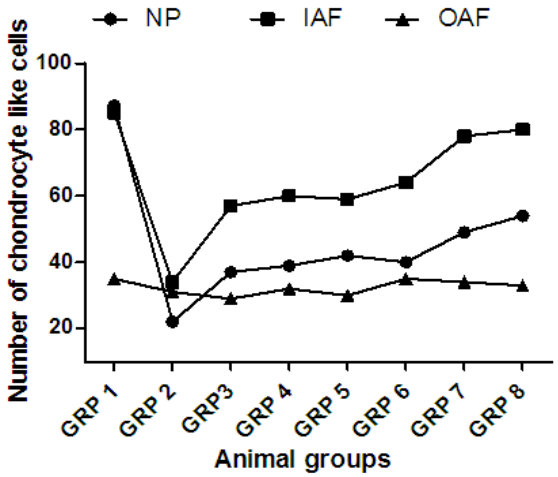


**Figure 11: Showing number of chondrocyte like cells within the NP, IAF and OAF.**

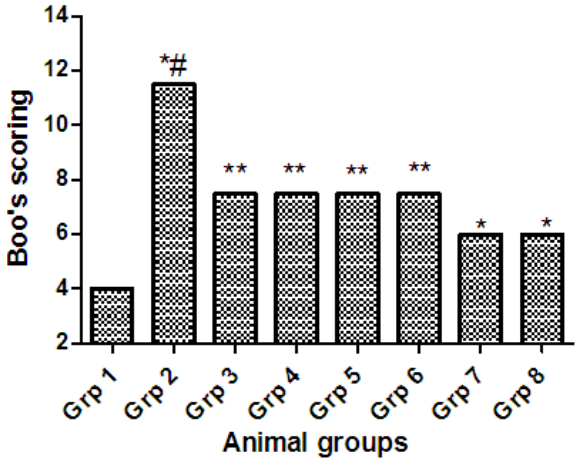


**Figure 12: Histological grading scores for IVD degeneration of rabbit. *: p < 0.05 as compared to group 1;*#: p < 0.05 as compared to group 6; **: p < 0.05 as compared to group 7 and 8**

## DISCUSSION

The age of the rabbit used for this study ranges between 20-24 weeks with average weight of 3.22kg in other to ascertain that no degenerative changes occurs in the animal since no morphological evidence of degenerative changes of the intervertebral disc was observed in previous study till 24 weeks age in rabbit (Kim *et al.*, 2005).[36]

However, the size of the needle used in inducing disc degeneration is based on the animal's body type. For IDD rat a model, a 27-gauge needle is usually used to induce disc degeneration in a rat models with the rate of progression is dependent on the number of punctures.[37] In this present study, multiple punctures with a 21-gauge needle was used to induced IDD leading to greater damage to rabbit discs compared to single puncture.[36]

This study examined the efficacy of the oral administration of nutritional supplementation (sulforaphane and riboceine) into the well-established rabbit annular needle puncture model of disc degeneration.The results of this study demonstrated that the combined administration of sulforaphane and riboceine is effective for 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) (Figure 12) beginning at 4 weeks post-surgery. Thus, this annular puncture model revealed a progressive disc degeneration overtime resembling human IDD.[18,35] The disruption in the cytoarchitecture within the annulus fibrosus in several animal models and in humans has revealed to induce disc degeneration.[38,39,40] The significant correlations between % DHI values and histological scores (Figure 1 and Figure 12) could affirm that DHI can be used as a good indicator in determining the level of disc degeneration.[35]

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 disorganisation and complete obliteration of its cavity. The histological analysis revealed a reparative effect of the combined administration ofsulforaphane and riboceine on the degenerated IVD.

In the sections after 4 weeks of annular puncture, notable progress in degeneration with time courses was observed when compared to the control group in which most of the NP contents have been lost and collapsed, wavy fibrocartilage lamella and associated fibrochondrocyte-like cells of the AF which was in contrast in the sulforaphane and riboceine administered groups.

Research have shown the role of proteoglycan in the water content and swelling pressure in IVD, and a decrease of proteoglycan in the NP triggers IVD degeneration.[41,42,43]

Degeneration is initiated by hydration in the nucleus pulposus due to the disorganization of the IVD. Expression levels of molecules could provide a clue in the onset of IVD degeneration (COL1, COL2, AGC and MMP13).[44,45] The corresponding transcript levels by real-time RTPCR analysis was done to evaluate the expression of the molecules.

It was revealed that there was an increase in the expression of COL1, COL2 and AGC while MMP13 decreased within the administered groups compared to the control. Previous research has revealed the degeneration process has been well described in cultured articular chondrocytes[46] and in osteoarthritic joints.[47] Among the various genes modulated in osteoarthritic chondrocytes,[48] MMP-13 is probably the most trustworthy. MMP-13 is known to degrade collagens and glycosaminoglycans.[19] The increase in MMP13 could therefore be a major contributor of IVD degeneration[45] as it has been extensively reported in cartilage degradation during osteoarthritis.[48]

## CONCLUSION

The present study provides useful insights into the histomorphological, biochemical and radiological mechanisms that govern the use of nutritional supplements of biological origin in response to intervertebral discs degeneration, and suggest the use of nutritional supplements as a potential alternative strategy for treating or preventing disc degeneration diseases.

The administration of sulforaphane and riboceine supplements in the annular puncture model of disc

degeneration was effective in restoring disc height and increasing chondrocytic cells in the IVD of the rabbit.

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