

# Possible protective role of nutraceuticals in chronic intervertebral disc degeneration – A histological study

Fareeha Mushtaq<sup>1</sup>, Humaira Ali<sup>2</sup>, Shan E. Rauf<sup>3</sup>, Abdullah Qamar<sup>4</sup>, Ayesha Ali<sup>5</sup>, Rabya Khalid<sup>6</sup>, Amna Shoaib<sup>7</sup>

<sup>1</sup> Rawal Institute of Health Sciences, Islamabad, Pakistan

<sup>2</sup> Swat Medical College, Saidu sharif, Swat, Pakistan

<sup>3</sup> Armed Forces Institute of Pathology, Rawalpindi, Pakistan

<sup>4</sup> Army Medical College, Rawalpindi, Pakistan

<sup>5</sup> Army Medical College, Rawalpindi, Pakistan

<sup>6</sup> Army Medical College, Rawalpindi, Pakistan

<sup>7</sup> Al Nafees Medical College, Islamabad, Pakistan

## SUMMARY

The aim of the study was to observe the effects of immobilization on the histomorphology of rats' intervertebral disc (IVD) and observe the protective effects of widely used nutraceuticals, Omega 3 fatty acids and Co-enzyme Q 10 (CoQ10) on it. The animal experimental study was carried out in the National Institute of Health, Islamabad in collaboration with the Anatomy Department, Army Medical College, Rawalpindi. Forty Sprague Dawley rats, weighing 250-300g, were chosen and grouped into 4 equal sets. Control group A was fed normal lab diet. In experimental group B, along with the lab diet, an Ilizarov apparatus was applied to the rats' tails for immobilization. In experimental groups C and D, the rats' tails were immobilized and given Omega 3 fatty acids (260 mg/kg body weight) and CoQ 10 (150 mg/kg body weight) through oral gavage. At completion of the study, IVDs of rats were analysed to see the histologic changes in the nucleus pulposus (NP) and annulus fibrosus (AF).

All the samples showed normal findings for NP in control group A. Degenerative changes were more significant in group B (p-value = 0.001) as compared to group C (p-value=0.005) and D (p-value=0.003). All the samples showed normal findings for AF in control group A. Degenerative changes were more significant in group B (p-value =0.000) as compared to group C (p-value=0.011) and D(p-value=0.003). Chronic immobilization of vertebral column induces degenerative changes in the cellular and matrix content of Intervertebral discs. However, consumption of nutraceuticals mitigates the consequences.

**Key words:** Annulus Fibrosus – Disc degeneration – Intervertebral disc – Nutraceuticals – Nucleus pulposus

## Corresponding author:

Fareeha Mushtaq, Rawal Institute of Health Sciences, Dept. of Anatomy, Ground C, Block 5, Askari towers1, DHA2, 45730 Islamabad. E-mail: dr.fareeha.shan@gmail.com

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## INTRODUCTION

Neutraceuticals have gained significant scientific attention in the past few decades. Omega 3 fatty acids (n-3 FA) have been added to a huge amount of dietary supplements and in a wide range of food products. n-3 FA derived from fish oil are evolving as potent and safe disease-modifying nutrients (Moyad, 2005). Consumption of n-3 FA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in preclinical and clinical research have illustrated beneficial effects in managing the inflammatory diseases including rheumatoid arthritis (Cleland et al., 2006), inflammatory bowel disease (Calder, 2008), psoriasis and neurodegenerative disorders (Dyall and Micheal, 2008). The prostaglandins and leukotrienes produced by EPA are less pro-inflammatory than the ones derived from Arachdonic acid (Stamp et al., 2005). Omega 3FA also inhibits the stimulation and activity of the nuclear factor  $\kappa$ B (NF- $\kappa$ B), the pro-inflammatory transcription factor (Singer et al., 2008). EPA and DHA also produce resolvins and protectins,

which are potent anti-inflammatory lipids (Arita et al., 2005). Long-chain n-3 FA can be used as an important adjunct to NSAID therapy in various inflammatory diseases.

Coenzyme Q 10 is a component of electron transport chain and acts as an electron and proton carrier coupled to ATP production in mitochondria. It also performs functions as an effective antioxidant in its reduced form (ubiquinol) (Saini, 2011). The levels of CoQ10 in tissues are increased under the effects of oxidative stress, e.g., physical exercise, cold adaptation, whereas the levels decrease during aging and degeneration. CoQ10, present in food or taken as a dietary supplement, seems to elevate the ubiquinone level in blood. CoQ10 performs important functions such as it prevents free radical injury triggered by neutrophils in inflammatory diseases, and also offers protection against oxidative injury produced due to ischemia (Lee et al., 2013). So, the term 'vitamin Q' has been also suggested for CoQ10 because of its numerous therapeutic properties when administered as a

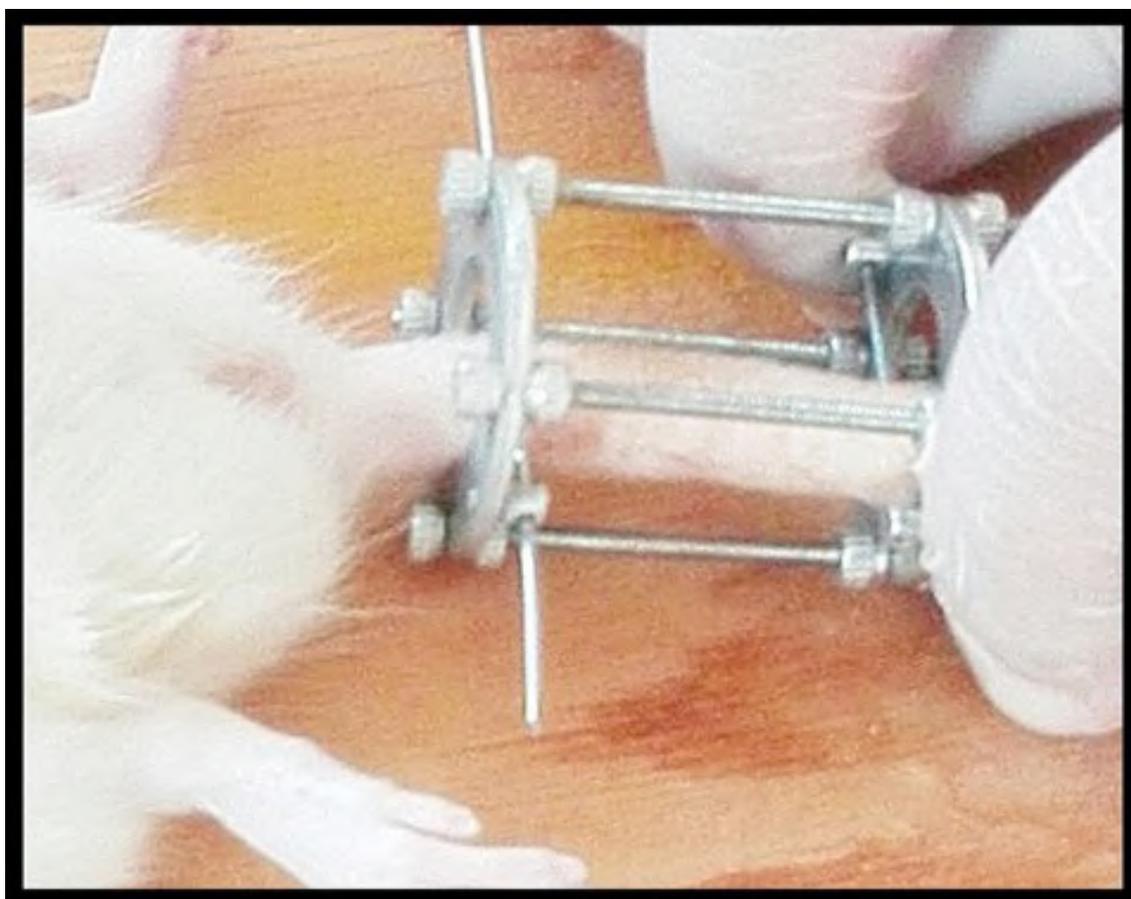


Fig. 1.- Application of an Ilizarov-type apparatus for rat tail segment immobilization.

dietary supplement (Bargossi et al., 1994). Animal studies offer mounting support for beneficial effects of CoQ10 supplements in various diseases, particularly neurodegenerative diseases, ageing and atherosclerosis (Turunen et al., 2004).

The intervertebral disc (IVD) is the avascular structure situated between the vertebrae of the spinal column. The disc is composed of a central soft, jell-like nucleus pulposus (NP) surrounded by the outer fibrous ring of annulus fibrosus (AF), sealed superiorly and inferiorly by the cartilaginous endplates (EP) (Whatley and Wen, 2012). Nutrients and metabolic by-products exchange mainly occurs through diffusion and convection with the surrounding. The NP is a gelatinous structure made up of water, extracellular matrix (ECM), and cellular elements. The AF is composed of 15-25 concentric rings of collagen fibrils arranged in a lamellar pattern parallel to each other. In each lamella layer, parallel running collagen fibre bundles are tilting at about 60° from the vertical axis, and in each successive lamellae the orientation is being reversed. Discrete translamellar bridging fibres radially join the adjacent lamellae (Chan et al., 2011). The morphology of the cellular components differs in AF and NP. The cells of the NP have a rounded appearance, and these chondrocyte-like cells are bounded inside a lacuna. On the other hand, the AF have cells with elongated fibroblastic appearance and are positioned in the same axis as the collagen fibrils, especially in the outer AF. The EP cells are chondrocytic (Wei et al., 2014).

Joint immobilization affects every connective tissue component of an articulation. It leads to degeneration of articular and periarticular connective tissue. Changes occur in biochemical elements of connective tissue, collagen, proteoglycans and hyaluronic acid. This brings about changes in the biomechanical and functional properties of the IVD. There is an increased risk for low back pain and disc degeneration with sedentary inactive lifestyle (Hagiwara et al., 2009).

This study was conducted to verify whether modification of diet with omega 3 FA and Co Q 10 could improve the histological parameters of chronic degenerative IVD disease in rats due to immobilization.

## MATERIALS AND METHODS

### Animals

This animal-based experimental study was completed in one year in the Army Medical College, Rawalpindi, in collaboration with the National Institute of Health (NIH)t, Islamabad, and Armed Force Institute of Pathology (AFIP), Rawalpindi. The necessary approval of the ethical committee of the Army Medical College, Rawalpindi, on animal experiments was taken (No. 02 / CREAM-A / 11 Aug, 2015). 40 mature Sprague-Dawley rats of about 3-4 months old, weighing between 250-300 grams, were taken for this study and divided into 4 equal groups. Animals were fed with standard lab diet and water for two months. Group-A (control): rats in this group were given standard lab diet and water ad libitum. Group-B: the animals' tails were immobilized by applying an Ilizarov-type apparatus (James et al., 1999). Group-C: the rats' tails were subjected to immobilization using an Ilizarov type apparatus. Animals were given standard lab diet, and omega 3FA was administered at a dose of 260 mg/kg body weight (Abdou et al., 2014). Group-D: the rats' tails were immobilized using Ilizarov apparatus and were administered Co-enzyme Q10 through oral gavage at a dose of 150 mg/kg/day (Kwong et al., 2002).

### Method of vertebral disc immobilization

Before procedure, radiographs were taken to confirm the location of the 8<sup>th</sup> and 10<sup>th</sup> coccygeal vertebrae. Intraperitoneal injections of 10 mg/kg xylazine and 50 mg/kg ketamine were used to anesthetize the rats. An Ilizarov-type apparatus (Fig. 1) was applied to the rat's tail in order to immobilize the IVDs. Two K-wires were passed through the tagged locations of the two vertebrae. The wires were bonded to 35 mm diameter aluminium rings. Finally, four steel rods were passed longitudinally through four holes placed around the rings creating an Ilizarov-type apparatus (James et al., 1999).

After 8 weeks, rats were sacrificed and the immobilized segments (Co8-9 and Co10) of the vertebrae and complete IVDs were dissected. The removed part was then fixed in 10% formalin solution. The obtained sections were decalcified

and later infiltrated and embedded in paraffin wax. Five  $\mu\text{m}$  thick cross sections were obtained by using rotatory microtome. Then tissues were stained with Haematoxylin and Eosin (H&E) stain. This processing and staining methods were performed in histology unit of Armed Forces institute of Pathology. The tissue slides were studied under the light microscopic, and observations were documented with the help of two pathologists. 4X and 10X objectives were used to make observations for the qualitative and quantitative parameters. An established grading scale for NP and AF separately by Norcross et al. (2003) was used for histological score and grading (Tables 1 and 2).

## RESULTS

Overall, during the experimental period all 40 animals remained healthy and alive. Changes were observed in NP and AF and were graded separately by using the histological grading scale ranging from 1 (severe disc degeneration) to 5 (normal disc). These scores are based on the cellularity and defects in the central nucleus pulposus and the orientation of collagen fibres and clefts in the annulus fibrosus. Three slides per specimen were observed and recorded.

On examination of NP, 100% specimens of Control Group A revealed normal disc and were placed in histological grade 5 of the Norcross et al.

**Table 1.** Grading Scale for Nucleus Pulposus established by Norcross et al. (2003).

GRADING SCALE FOR NUCLEUS PULPOSUS (NP)	
<b>Score 5</b>	Large, bulging central cavity with abundant NP material > 2/3rd IVD height; smooth borders with minimal disruption.
<b>Score 4</b>	Slightly reduced central cavity size with some NP material present ;> 1/3rd IVD height and < 2/3rd IVD height; minimal border disruption may be present.
<b>Score 3</b>	Markedly reduced and disrupted cavity with minimal NP material and compartmentalization; total cavity > 1/3rd IVD height and < 2/3rd IVD height.
<b>Score 2</b>	Severe disruption of NP with minimal cavity; total cavity < 1/3 <sup>rd</sup> IVD height but > 0; consists only of a few small pockets lined by NP-like cells.
<b>Score 1</b>	Complete obliteration of cavity with no NP-lined pockets.

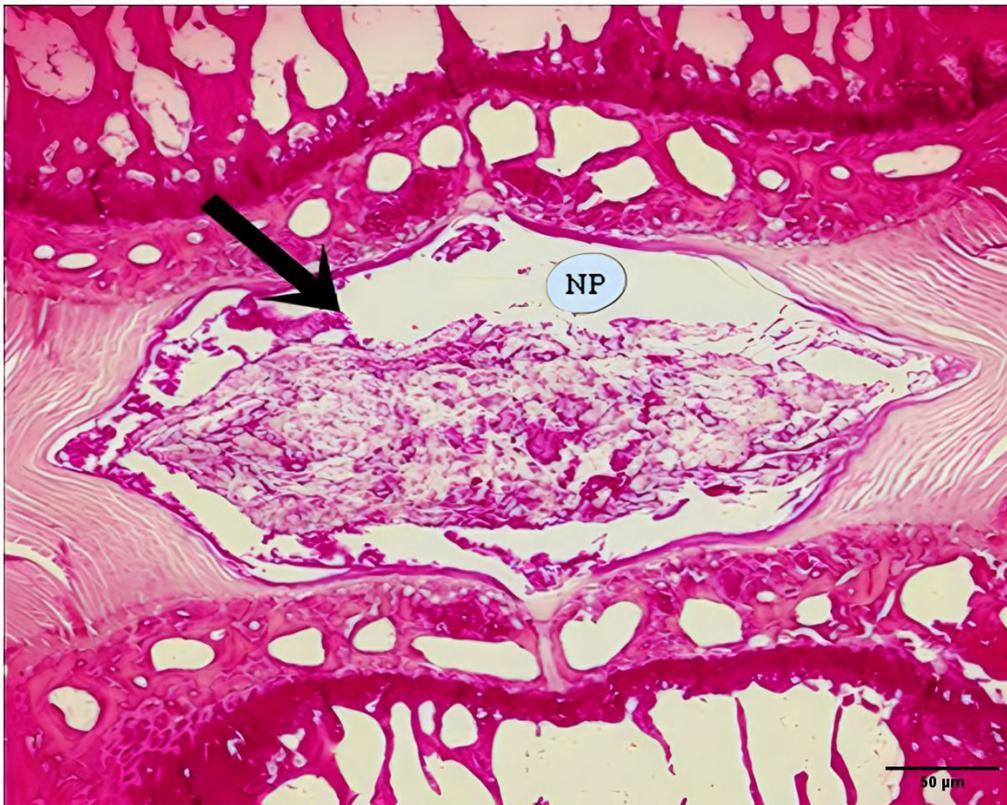
**Table 2.** Grading Scale for Annulus Fibrosus established by Norcross et al. (2003).

GRADING SCALE FOR ANNULUS FIBROSUS (AF)	
<b>Score 5</b>	Discrete, well-opposed lamellae bulging outward with no infolding; minimal preparation defects with simple radial clefting.
<b>Score 4</b>	Discrete, well-opposed lamellae bulging outward with no infolding; minimal preparation defects with simple radial clefting.
<b>Score 3</b>	Moderate to severe infolding of discrete, relatively well-opposed lamellae; moderate fragmentation of lamellae; AF fibers remain well organized.
<b>Score 2</b>	Severe infolding and distortion of poorly opposed lamellae; severe fragmentation of lamellae; small regions of disorganized fibrous material replacing central lamellae.
<b>Score 1</b>	Severe infolding, distortion, and fragmentation of lamellae; extensive amount of disorganized fibrous material replacing central lamellae.

### Statistical analysis

Data were analysed by SPSS 21.0v. ANOVA was used for intergroup comparison of histological differences followed by Post Hoc Tukey Test. A confidence interval of 95% having p-value <0.05 was considered significant.

(2003) scale (Fig. 2a). In group B, the frequency of nucleus pulposus degeneration was higher when compared to control group A (p-value < 0.001), experimental group C (p-value =0.000) and D (p value=0.000), which was statistically significant (Table 3, Fig. 3a and Bar chart 1). In group C, 60% of the specimens had minimally disrupted cavity in comparison with group B, p =0.000, which was



**Fig. 2a.-** Histological picture of the intervertebral disc of A4, an animal in the control group showing a large central NP (arrow) and smooth borders with AF. H&E staining. Scale bar: 50 µm.



**Fig. 2b.-** Well organized, circumferential lamellae in AF (arrow). H&E staining. Scale bar: 50 µm.

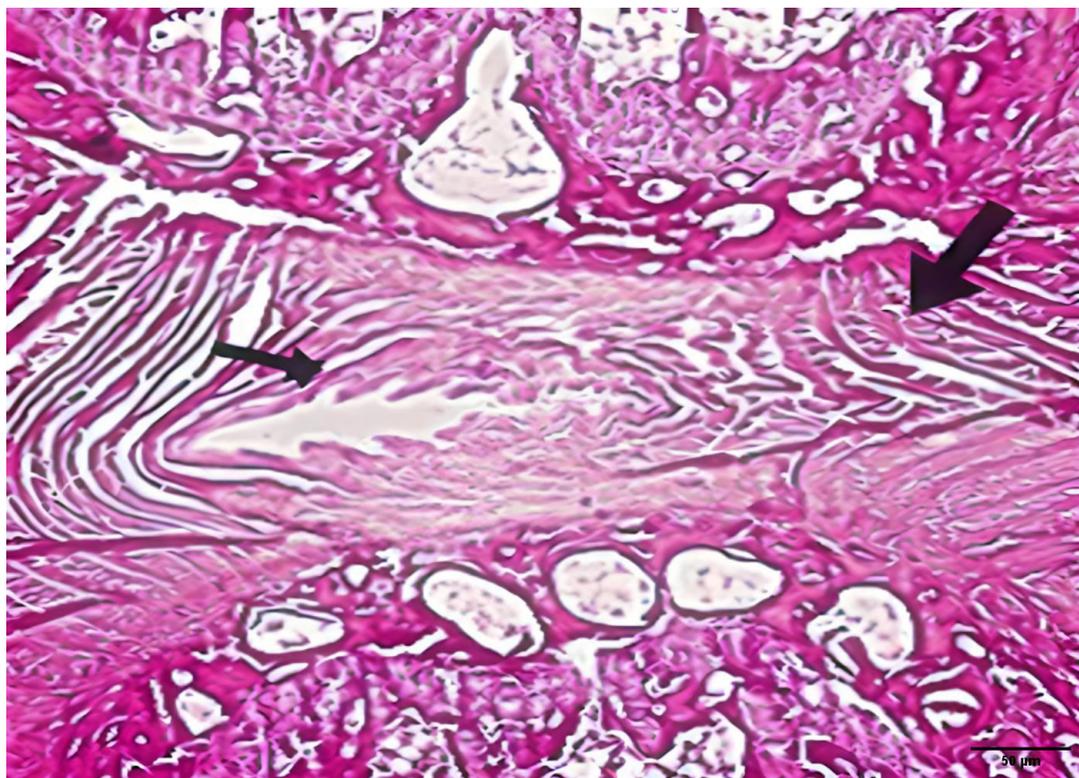
**Table 3.** Comparison of Norcross et al. (2003) scoring for microscopic degeneration of nucleus pulposus (NP) between the control Group A and experimental Groups B, C and D.

Norcross scale for nucleus pulposus	Group A (n=10)	Group B (n=10)	Group C (n=10)	Group D (n=10)	Group A/B	Group A/C	Group A/D	Group B/C	Group B/D	Group C/D
Score 5	10	0	4	3	0.000*	0.005*	0.003*	0.000*	0.001*	0.250
Score 4	0	0	6	4						
Score 3	0	2	0	3						
Score 2	0	3	0	0						
Score 1	0	5	0	0						

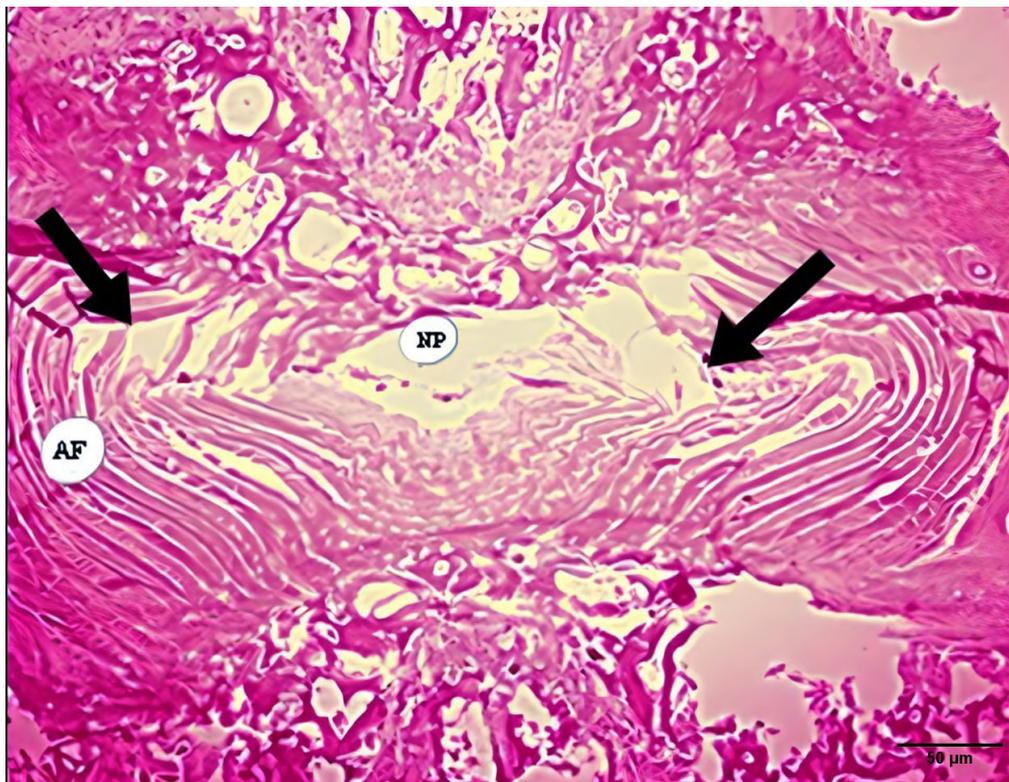
statistically significant. The results were statistically insignificant when compared with group D ( $p = 0.250$ ) (Table 3, Fig. 4 and Bar chart 1). The results were statistically significant when group D was compared with group B ( $p$ -value = 0.001), but in comparison with group C the results were statistically insignificant ( $p$ -value = 0.250) (Table 3, Fig. 5 and Bar chart 1).

When AF was observed, all the specimens in control group A had well-opposed, discrete lamellae (grade 5). No infolding was observed in any of the specimens (Table 4, Fig. 2b and Bar chart 2). The frequency of AF degeneration was higher in group B when compared to control group A ( $p$

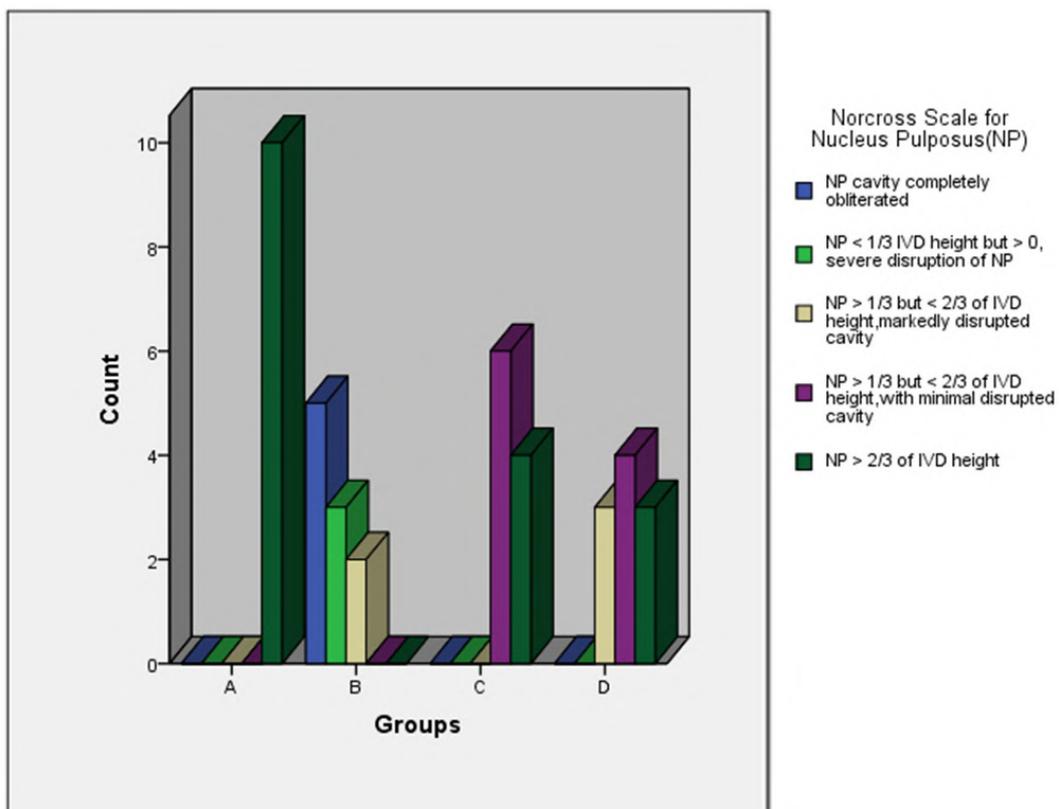
= 0.000), experimental group C ( $p = 0.001$ ) and D ( $p = 0.001$ ), which was statistically significant (Table 4, Fig. 3b and Bar chart 2). In group C, only 20% of the specimens showed a moderate amount of fragmentation and infolding (grade 3). When compared to group B,  $p$  value = 0.001 which was statistically significant. The results were statistically insignificant when compared with group D ( $p$ -value = 0.753) (Table 4, Fig. 4 and Bar chart 2). The results were statistically significant when group D was compared with group B ( $p = 0.001$ ) but on comparison with group C the results were statistically insignificant ( $p = 0.735$ ) (Table 4, Fig. 5 and Bar chart 2).



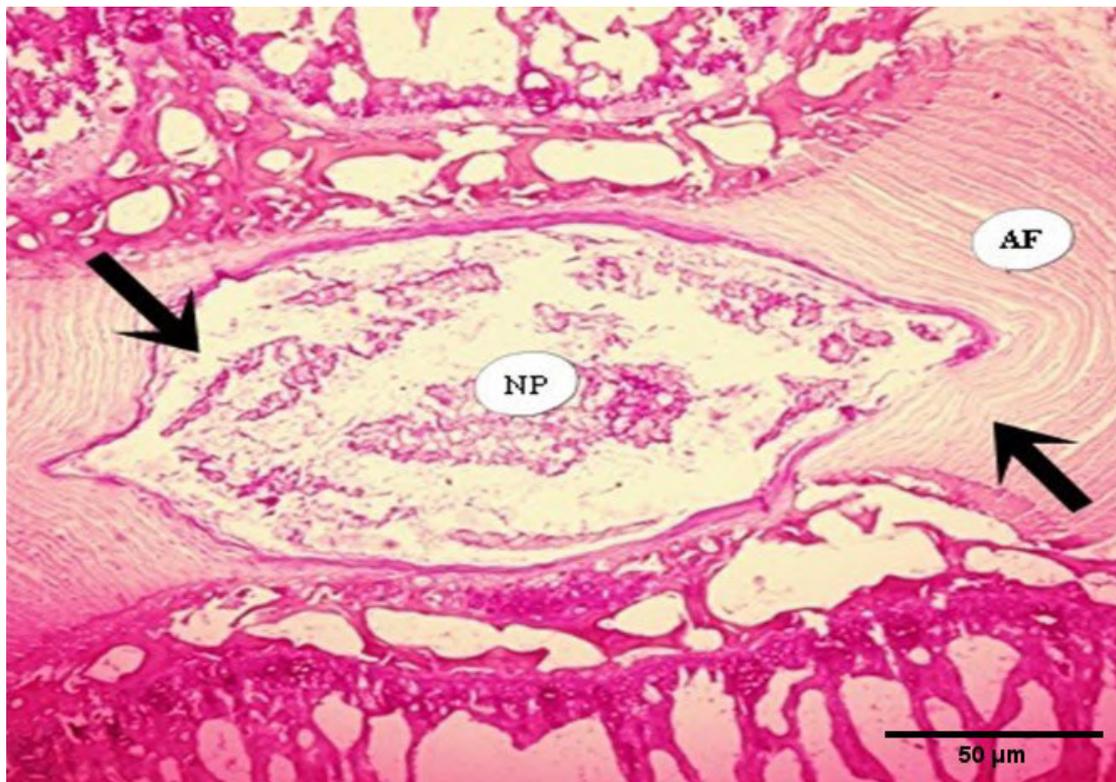
**Fig. 3a.-** Intervertebral disc of rat number B5 in the disc immobilized group B showing highly disrupted and reduced NP cavity (arrows). H&E staining. Scale bar: 50 µm.



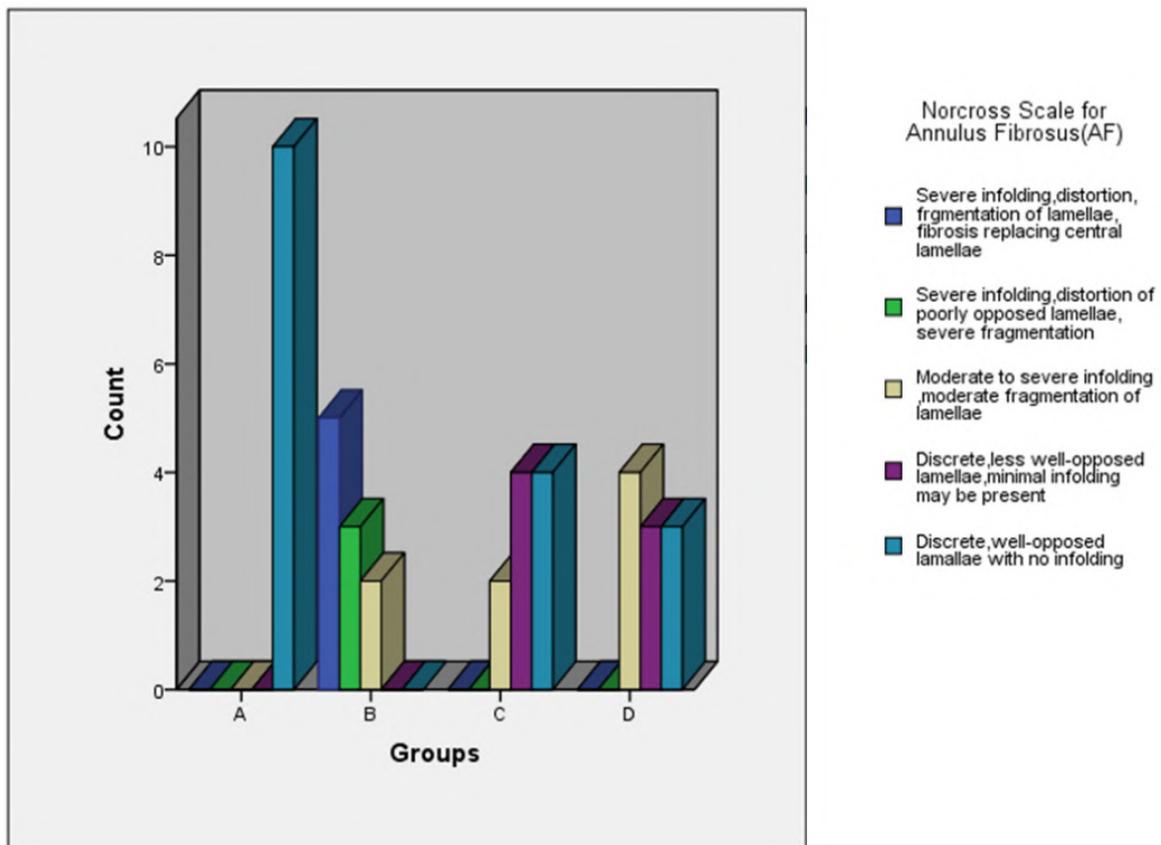
**Fig. 3b.**- Intervertebral disc showing, distortion, disruption and clefts in AF (left arrow) and highly reduced to obliterated NP cavity in B3 and B7 rats of group B (right arrow). H&E staining. Scale bar: 50 μm.



**Bar chart 1.** Clustered bar chart showing frequency of nucleus pulposus (NP) with scoring of degeneration (Norcross scale) among the control group A, disc immobilized group B, disc immobilized+Omg3 administered group C and disc immobilized+CoQ10 administered group D.



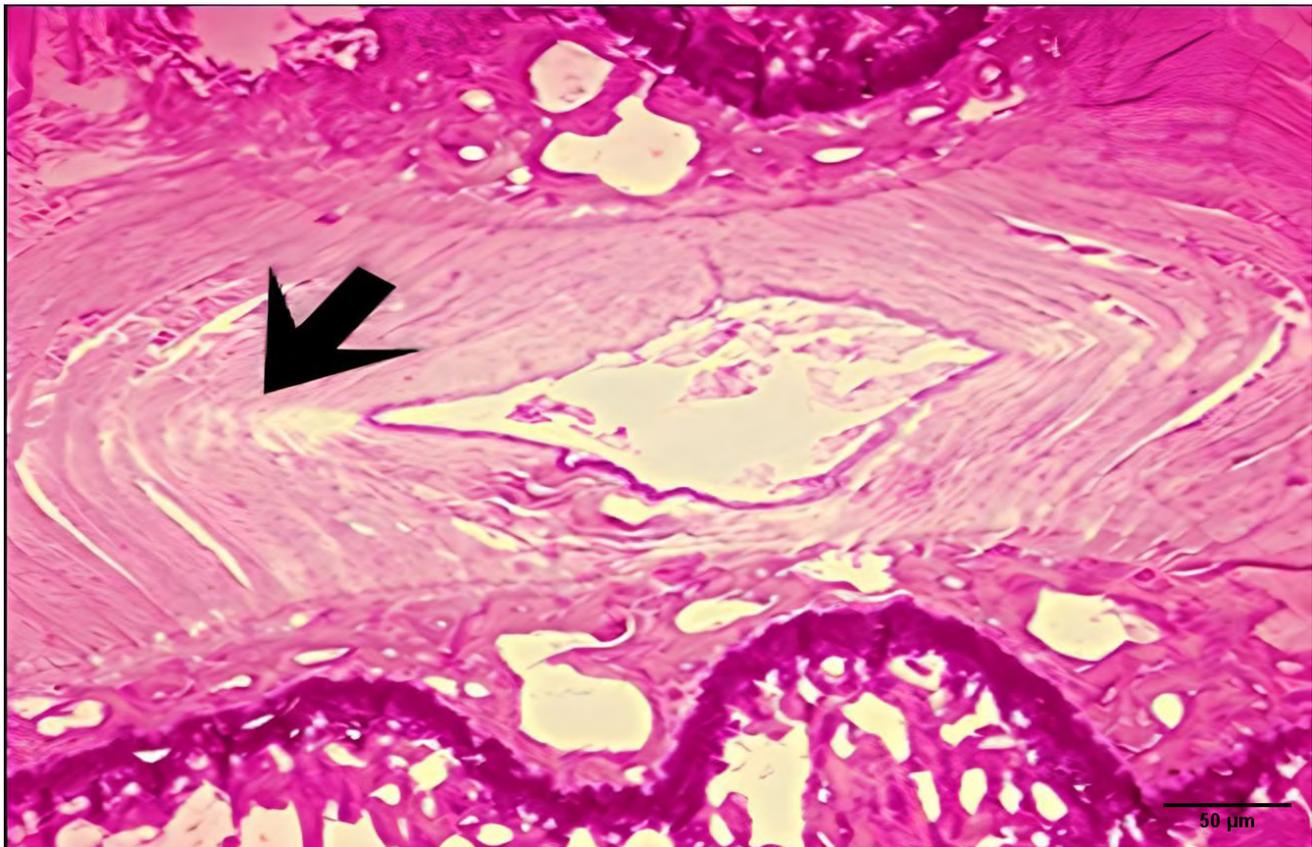
**Fig. 4.-** Histological section of the intervertebral disc of animal number C8 in the disc immobilized + omega 3 administered group C showing large NP cavity (left arrow) and discrete lamellae in AF (right arrow). H&E staining. Scale bar: 50 μm.



**Bar chart 2.** Clustered bar chart showing frequency of annulus fibrosus (AF) with scoring of degeneration (Norcross scale) among the control group A, disc immobilized group B, disc immobilized+Omg3 administered group C and disc immobilized+CoQ10 administered group D.

**Table 4.** Comparison of Norcross et al. (2003) scoring for microscopic degeneration of annulus fibrosus (AF) between the control Group A and experimental Groups B, C and D.

Norcross scale for annulus fibrosus	Group A (n=10)	Group B (n=10)	Group C (n=10)	Group D (n=10)	Group A/B	Group A/C	Group A/D	Group B/C	Group B/D	Group C/D
Score 5	10	0	4	4	0.000*	0.011*	0.003*	0.001*	0.001*	0.735
Score 4	0	0	4	3						
Score 3	0	2	2	3						
Score 2	0	3	0	0						
Score 1	0	5	0	0						

**Fig. 5.-** Intervertebral disc of animal number D3 in the disc immobilized+COQ10 administered group D showing slightly reduced NP cavity and minor fissures in inner lamellae of AF (arrow). H&E staining. Scale bar: 50  $\mu$ m.

## DISCUSSION

The histomorphological examination of nucleus pulposus of the intervertebral disc in experimental group B showed complete obliteration of nucleus pulposus (grade 1) in 50% and grade 2 in 30% of the specimens. The frequency of grade 1 and grade 2 degeneration was higher in disc-immobilized group B as compared to control group A, where all discs had normal histological features and showed no signs of degeneration (grade 5). Degeneration in nucleus pulposus was observed

in the form of reduced height of NP as compared to intervertebral disc height and severity of disruption of NP cavity. These findings were consistent with a study performed on the rat intervertebral disc where disc degeneration caused damage to NP cavity and NP showed severe disruption and a slow on-going decline in cell count. This leads to decrease in the NP cavity until it is completely obliterated (Silveira et al., 2014). In group C, nucleus pulposus had mild degeneration (grade 4) in 40% specimens while 60% specimens showed no

signs of NP degeneration (grade 5). The decreased frequency of severity of degeneration of nucleus pulposus in group C was in consistency with a study performed by Berbert et al. (2005), where administration of omega 3 fatty acids reduced inflammation and resulted in decreased production of PGE<sub>2</sub>, thromboxane B<sub>2</sub>, LTB<sub>4</sub> and LTE<sub>4</sub> by inflammatory cells and consequently reduced degenerative changes in joints. Degeneration in nucleus pulposus was also found to be higher in the experimental group D as compared to control group A, however, the frequency of severity of degeneration in NP was greater in experimental group B as compared to group D. The degenerative changes in NP were less severe in group D as compared to group B, because CoQ10 has anti-oxidant action. It counters the free radical injury and the subsequent degeneration in the NP due to the oxidative stress caused by disc degeneration. This was in agreement with the study conducted on progressive neurodegenerative diseases where CoQ10 had a preventive role (Shults et al., 2004).

The histological examination of annulus fibrosus (based on Norcross scale) in intervertebral disc specimens in disc-immobilized group B showed highly disorganized, fragmented and in-folded lamellae (grade 1) of the specimens. Fibrosis was also present in the central lamellae. The frequency of annulus disruption was higher in the experimental group B as compared to control group A. Studies have shown that degenerated IVD is in a chronic inflammatory state with increased expression of various inflammatory cytokines like interleukin 1 (IL-1), matrix metalloproteinase (MMP)-10, IL-8, tumour necrosis factor (TNF- $\alpha$ ), IL-10, and prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). This leads to significant inflammation causing the annular damage (Wang et al., 2006). In group C, annulus fibrosus showed moderately fragmented and in-folded lamellae (grade 3) in 20% specimens, mild changes were present in 40% (grade 2) while 40% specimens showed no signs of AF degeneration (grade 5). IVD degeneration is linked with apoptosis of cells in AF and ECM degeneration due to activation of the mitochondria-dependent apoptosis (Rannou et al., 2004). The ingestion of omega 3 fatty acids had a protective effect on the annulus fibrosus in group C. The results are in

agreement with the study performed by Goldberg and Katz (2007). They proposed that consumption of Omega 3-rich fish oil reduced the number of painful and swollen joints. There was a marked reduction in production of pro-inflammatory factors like PG and leukotriene B<sub>4</sub>. According to Calder (2002, 2008), n-3 PUFAs can influence transcription factors such as nuclear factor kappa B (NF $\kappa$ B) and peroxisome proliferator-activated receptors (PPARs) and alter inflammatory gene expression. The down-regulation of transcription factors plays a significant part in decreasing activity of a number of inflammatory signalling pathways (Calder and Grimble, 2002).

Degenerative changes in annulus fibrosus were also found to be higher in the experimental group D as compared to control group. However, the frequency of severity of degeneration in AF was lower in Co Q 10-treated group as compared to disc immobilized group B. Coenzyme Q10 is known as an effective anti-oxidant and proven to have outstanding capability to scavenge ROS. The CoQ10 had protective effect on the cells and matrix of AF in the experimental group D. This is supported by the fact that studies have shown that CoQ10 exert beneficial effect on osteoarthritis and suppresses pain and cartilage degeneration (Lee et al., 2013). Bauerova et al. (2010) proved that when treatment with CoQ 10 was provided in arthritis model, the high expression of pro-inflammatory cytokines, IL-1 $\beta$ , IL-6, and IL-15, decreased.

Owing to the therapeutic effects of n-3 PUFAs and CoQ10, in wide variety of chronic inflammatory and neurodegenerative diseases, they have become a focus of interest nowadays. However, further research is required to address important aspects such as how lifetime intake of n-3 PUFAs and CoQ10 can influence the progress of age-linked and chronic degenerative disorders for instance DDDD. More research is needed to claim them as preventive agents and establish their clinical efficacy in chronic IVD degeneration.

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