

# Role of Exogenous 17- $\beta$ Estradiol in injured Intervertebral Disc

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## ABSTRACT

Herniation of nucleus pulposus occurs in response to injury to the annulus fibrosus of the intervertebral disc. Although Estradiol (E2) has been reported to affect the proteoglycan synthesis and water content of the cartilage, being a steroid hormone its effects on various regions of an intervertebral disc have not been widely reported. An experimental study using rabbit as a model was designed to observe the effects of E2 on stab wounded intervertebral disc (nucleus pulposus and annulus fibrosus), in young male domestic rabbits with emphasis on changes in cell population and matrix. Study was done between an experimental and a control group. Animals in both the groups were operated upon and their intervertebral discs were injured by a surgical blade to a uniform depth. Experimental group was given an injection of E2 after the surgery. Animals were sacrificed after five (120 hrs) and seven days (168 hrs) of injury and intervertebral discs were harvested from 120 rabbits. Slides were stained with hematoxylin and eosin and alcian blue stain. Qualitative and quantitative data was recorded for the two groups. SPSS software was used for applying student's t-test to detect significant difference ( $p \leq 0.05$ ) in the means of cell population between the subgroups. Qualitative data was recorded as percentage of observations between the subgroups. E2 increased the number of cells in the annulus fibrosus and nucleus pulposus in the experimental groups. Results suggested that recruitment of new cells into the injured intervertebral disc can be beneficial in response to injury to intervertebral disc

**Keywords:** Annulus fibrosus. chondrocytes. Estrogen, estradiol dipropionate, intervertebral disc.

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## INTRODUCTION

The intervertebral disc forms the cartilaginous articulation of the spine, enabling it to bend and twist in all directions. It comprises of two main regions; an inner nucleus pulposus and an outer collagenous structure of concentric lamellae encircling the nucleus, the annulus fibrosus<sup>1</sup>. Although no statistics for low back pain are available for Pakistan, a high incidence is expected since a vast majority of our population lives in rural setup, where daily chores include lifting heavy loads and thus involve heavy mechanical loading and bending of spine.

Interest in the intervertebral disc and the results of injury to it can be traced back to the days of great German pathologist, Christian Georg Shmorl (1861-1932)<sup>1</sup>. Whenever the nucleus pulposus bulges out of the annulus fibrosus, it exerts pressure on the spinal cord or impinges upon a nerve root, causing immense pain. Clinically this condition is termed as disc herniation, slipped disc or disc prolapse<sup>1</sup>. Experimental incision of annulus can mimic this condition<sup>2</sup>. Damage to the endplate or annulus typically decompresses the nucleus, concentrates stress within the annulus, and allows ingrowth of nerves and blood vessels<sup>3</sup>.

Platelet-rich plasma (PRP) is the latest technique to be evaluated for promoting intervertebral disc healing<sup>4</sup>. Steroid hormones are administered commonly to treat low back pain due to the injury to the intervertebral disc<sup>5</sup>. but the absolute treatment modalities for efficient healing of intervertebral disc are still being investigated.

Previous studies have found the estrogenic environment to have a significant impact on the glycosaminoglycans and the water content of the intervertebral discs<sup>6</sup>. 17- $\beta$ -Estradiol (E2) is the most potent, primary and naturally occurring placental and ovarian estrogen in mammals<sup>7</sup>. E2 can either be a naturally occurring steroid hormone<sup>8</sup> or a synthetic non-steroidal variant, like diethylstilbestrol<sup>9</sup>.

It has been reported that estrogen stimulates proteoglycan synthesis in rabbit chondrocytes in vitro and has a significant impact on glycosaminoglycans and the water content of the intervertebral discs.<sup>10</sup> In a study involving the measurement of the intervertebral disc height in three different groups of women: menopausal women on hormone treatment, untreated post-menopausal women and pre-menopausal, it was reported that the women with adequate estrogen levels had significantly greater disc heights compared to untreated menopausal women. It was proposed that the estrogenic milieu may have relevance because of its significant impact on the hydrophilic glycosaminoglycans, the water content, collagen and elastin of the intervertebral disc<sup>11</sup>. Extracellular matrix macromolecules (collagens,

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elastin, glycosaminoglycans, proteoglycans and connective tissue glycoproteins) are able to regulate many important cell functions, such as proliferation, migration, protein synthesis or degradation, apoptosis, etc., making them able to play an important role in the wound repair process<sup>12</sup>.

The cells of the adult nucleus pulposus more closely resemble articular chondrocytes<sup>13,14</sup>. The annulus fibrosus cells are morphologically undistinguishable from articular chondrocytes. The nucleus pulposus contains mainly large vacuolated cells and a few smaller cells<sup>15</sup> (Fig. 1). Electron microscope studies suggest that chondrocyte like cells in the nucleus and inner annulus have cellular processes as a dominant feature which may serve to sense mechanical strain<sup>16</sup>. Rabbit's IVD resembles the human disc in general structure. It is large enough for an adequate histological study<sup>2</sup> and, therefore, is a suitable model for studying pathophysiology of human intervertebral disc.

## MATERIAL AND METHODS

The study was conducted in two main groups; Control group 'A' and experimental group 'B' with 60 animals in each group (n=120). Young male, 4-6 month old domestic rabbits (*Oryctolagus Cuniculus*) species were selected for the study. Each group was further subdivided into two subgroups (A1, A2 & B1, B2) of 30 animals each. Animals were fed water and food ad libitum and kept in the animal room having a normal fixed 12 hour day and night lighting cycle.

On the day '0', animals were anaesthetized with a combination of ketamine and xylazine and median incision was given on the ventral abdominal wall from the sternum to the umbilicus of the animal. Dissecting in front of the spine between the paravertebral muscles, intervertebral disc was identified as a glistening white band between the two adjacent vertebrae. (Fig. 2) It was stabbed in a direction perpendicular to the direction of the spine to the depth of 4mm. Tip of the blade was secured and restrained to inflict an injury of uniform depth upon all the animals and avoid injury to the spinal cord. Abdomen was then stitched in layers with catgut and silk. Animals in the experimental group (B1 & B2) were additionally given an intramuscular injection of Estradiol dipropionate (E2) 5mg/kg body weight, soon after the skin closure.

Intervertebral discs were harvested from Group 'A1' & 'B1' after 120 hours (5 days), while those in Group 'A2' & 'B2' were sacrificed after 168 hours (7days) of operation for collection of the disc. Harvested intervertebral disc was fixed in 4%

buffered formalin and processed for paraffin embedding. 10µm thick coronal sections were cut (Figure 3). Tissue was stained with hematoxylin and eosin stain for recording quantitative data. Alcian blue staining was done to record intensity of staining for proteoglycan content of the two regions. Intensity was graded as "Absent, Mild, Moderate and Deep" on an ascending scale. Quantitative data was recorded for number of chondrocytes, fibroblasts, macrophages, neutrophils, total number of cells and total number of other cells as the mean cell count per predefined unit area of the annulus fibrosus & nucleus pulposus. "Other Cells" comprised of the cellular population of the observed regions which were not being specifically looked for in the study. Qualitative data was recorded as percentage of observations between the subgroups. SPSS software was used for applying student's t-test to detect significant difference (p≤0.05) in the means of cell population between the subgroups.

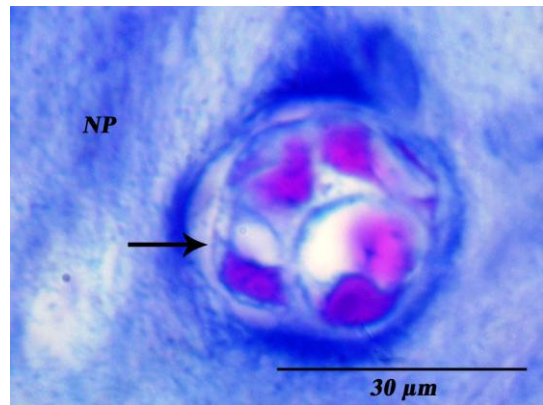


Fig. 1: Peculiar cell nests (arrow) inside the nucleus pulposus.

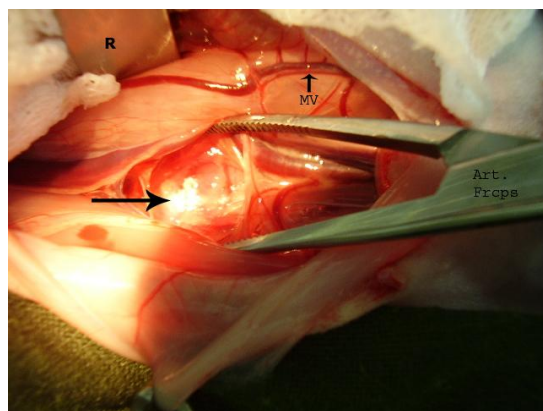


Fig. 2: Perioperative exploration of abdomen for finding the intervertebral disc: delicate dissection with a small artery forceps (Art. Frcps) just in front of the vertebral column showing glistening white intervertebral disc (pointing arrow). Retractor (R) is held by assistant to hold back the gut trapped in wet gauze piece. Mesenteric vessels (MV) can also be seen.

## RESULTS

One hundred and twenty intervertebral disc specimens were obtained from the rabbits that were operated for the study. None developed wound infection during the course of the study. In the individual comparison of control groups an increase in number of fibroblasts was noted in subgroup A2 while the mean cell count of chondrocytes and total number of cells was found to be significantly more ( $p < 0.05$ ) in the subgroup A1 in the nucleus pulposus.

In the comparison of control and experimental subgroups of 5 days, number of other cells were significantly more ( $p < 0.001$ ) in the subgroup A1, in both the annulus fibrosus and nucleus pulposus.

In the comparison of control of 5 days with experimental subgroup of 7 days number of chondrocytes ( $p = 0.001$ ) and other cells ( $p < 0.001$ ) was significantly more in the subgroup B2, while the number of fibroblasts was significantly more ( $p < 0.05$ ) in the subgroup A1 in the annulus fibrosus. In the nucleus pulposus it was found that number of macrophages ( $p < 0.05$ ) and other cells ( $p < 0.001$ ) was significantly more in the subgroup A1.

When the control subgroup A2 & B1 were compared it was found that in the annulus fibrosus number of chondrocytes was significantly more ( $p < 0.05$ ) in the subgroup B1. Number of fibroblasts was significantly more ( $p < 0.01$ ) in subgroup A2. There were no macrophages in the region in the two groups. Number of other cells was also significantly more ( $p < 0.05$ ) in subgroup A2. In the nucleus pulposus, the number of chondrocytes and fibroblasts was more in the subgroup B2 but the difference was not significant ( $p > 0.05$ ). Number of other cells was significantly more ( $p < 0.05$ ) in the subgroup A2.

In the comparison of subgroup A2 with experimental B2, in the AF, number of chondrocytes was significantly more ( $p < 0.001$ ) in subgroup B2. Number of fibroblasts was significantly more ( $p = 0.001$ ) in subgroup A2. Total number of cells was more in subgroup B2 but result was not significant ( $p > 0.05$ ). In the NP, number of chondrocytes was significantly more ( $p < 0.001$ ) in subgroup B2, as was

number of other ( $p < 0.01$ ) and total cells ( $p < 0.01$ ) in subgroup B2. In the comparison of the subgroup B1 and B2 in the NP, number of chondrocytes was significantly more ( $p < 0.05$ ) in subgroup B2.

Proteoglycan content of matrix was assessed and it was noted that more of the matrix stained in the deep category in the nucleus pulposus as compared to the annulus fibrosus. Qualitative data is represented graphically in Figures 4 & 5.

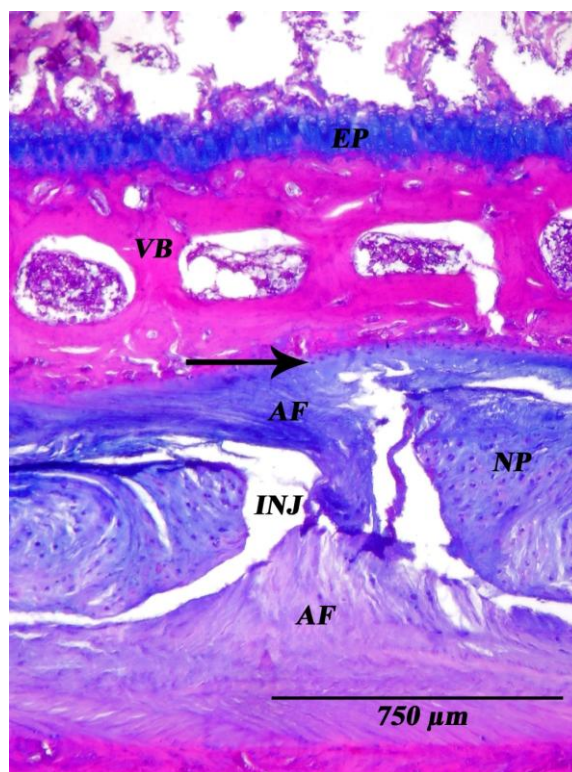


Fig. 3: A low powered photomicrograph of coronal section of intervertebral disc, stained with H & E and alcian blue stain, showing regions of epiphyseal plate (EP), vertebral bone (VB), nucleus pulposus (NP), annulus fibrosus (AF) and injury (In). Arrow points to the very thin region of end plate

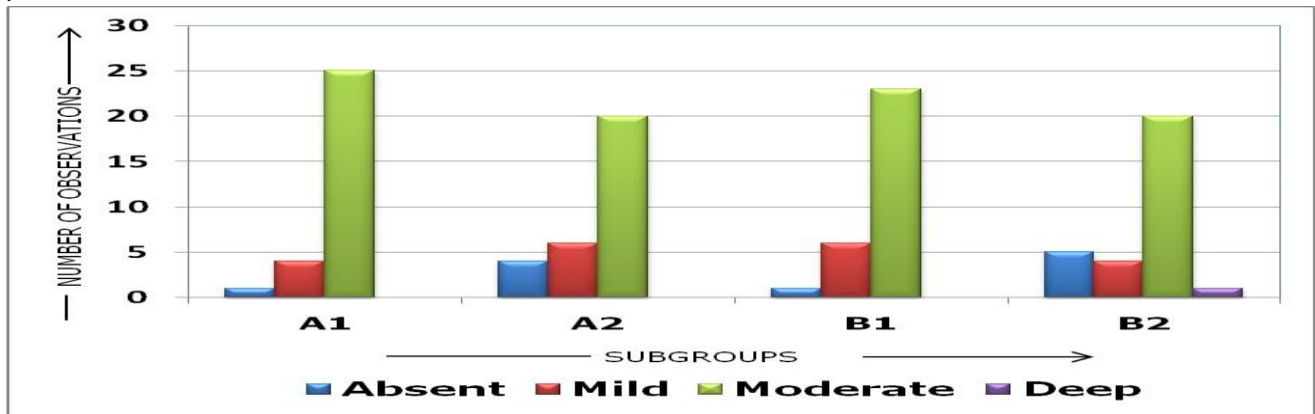


Fig. 4: Graphical representation of observed staining grades of the matrix that has been stained with alcian blue stain to detect the proteoglycan content of the matrix in the region of annulus fibrosus.

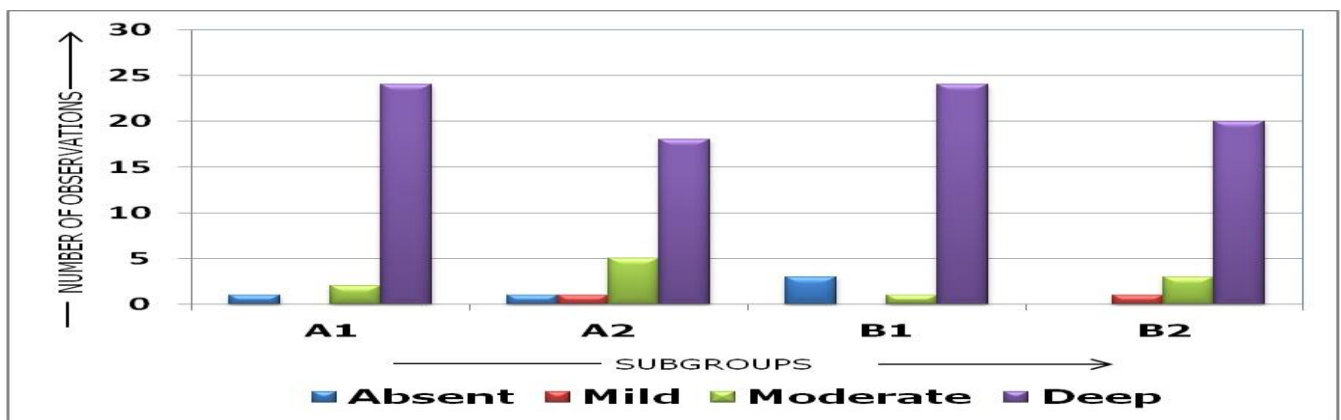


Fig. 5: Graphical representation of observed staining grades of the matrix that has been stained with alcian blue stain to detect the proteoglycan content of the matrix in the region of nucleus pulposus.

## DISCUSSION

The current study was designed to observe the effects of estrogen, beneficial or otherwise, using its parenteral form estradiol dipropionate (E2), on the injured intervertebral disc (IVD) using rabbit as an experimental animal.

The nucleus pulposus and inner annulus fibrosus depend upon diffusion for nutrition so avascular tissue was encountered in the study.

Although previous works showed that intervertebral disc is an active tissue capable of self-maintenance, repair and having considerable regenerative properties,<sup>17</sup> a gap of knowledge regarding detailed account of healing in intervertebral disc has been found.

In the region of annulus fibrosus, estradiol promoted the mitogenesis of the chondrocytes but had no effect on the fibroblast population. Fibroblasts in the AF may be lacking the estrogen receptor beta.

Macrophages and neutrophils are important cells of inflammatory response, so they were also looked for in the injured tissue but they failed to show

up in the annulus fibrosus and nucleus pulposus. Avascularity of the annulus fibrosus might be a contributory factor for this finding. It can also be suggestive of the fact that inflammatory reaction of injury does not spread to annulus fibrosus. This avascularity of the annulus fibrosus, therefore, might be its protective feature. Observation of the staining grades of matrix for the proteoglycan content was also suggestive of a lack luster response to estradiol.

A trend of chondrocytes and fibroblasts similar to annulus fibrosus was found in the nucleus pulposus. There was a little infiltration of macrophages into nucleus pulposus. However, as the population of these cells was almost negligible, no definite conclusion shall be drawn about their presence in the nucleus pulposus, except that they wandered by diffusion by chance into the nucleus pulposus, as the physiological barrier had been broken by the injury.

Study of staining grades of the matrix for the proteoglycan content suggests that there is always a high proteoglycan content in normal and protruded



nucleus pulposus. As proteoglycan is a water imbibing tissue due to its structure and charge, responsible for the shock absorbing capacity of nucleus pulposus, the proteoglycan content can also be an indicator of the hydration of the tissue, in this case, nucleus pulposus.

In experimental subgroups the number of other cells was low as compared to control after five days of injury and increased after seven days in experimental subgroups. Total cell population increased in experimental subgroups as compared to control subgroups and this was expected as the E2 had been having a growth promoting effect on the cells in general.

Observed staining grades for the content of proteoglycan did not suggest a massive lay down of matrix and it is expected as PG was not excessively being laid down within a week of healing response.

**Key Message:** Steroids are used as treatment for relief of inflammation and pain in herniated disc. Normally glucocorticoids are used. Estrogen hormone shall also be given clinical trials since it improves the hydration of the disc also.

## CONCLUSION

This study provided insight into effects of estrogen on the injured intervertebral disc. Estradiol dipropionate (E2) had effects on the epiphyseal plate recruiting more cells into the intervertebral disc. In the annulus fibrosus, E2 promoted the replication of chondrocytes.

In the light of above observations it is deduced that improving mitosis and lay down of the proteoglycans might be helpful in expediting the growth of the injured tissue.

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