

Effects of Exogenous Estrogen on Granulation Tissue: A Chronology of First Week in Injured Intervertebral Disc

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ABSTRACT

Work has been done on normal tissue healing but current literature has been found deficient on healing of intervertebral disc (IVD). Although estradiol (E2) has been reported to affect the proteoglycan synthesis and water content of the cartilage, its effects on healing of IVD have not been widely explored. An experimental study using rabbit as a model was designed to observe the effects of E2 on healing of IVD, in stab wounded 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 IVDs were injured by No. 11 blade to a depth of 4mm. Experimental group was given an additional shot of E2 after the surgery. Animals were sacrificed after five (120 hrs) and seven days (168 hrs) of injury and IVDs 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. Neutrophils and macrophages were not found in the annulus fibrosus and nucleus pulposus. Overriding of wound gap by granulation tissue, chondrocyte production and proteoglycan content was also enhanced by E2, suggesting that it can be beneficial for healing of IVD.

Keywords: Annulus Fibrosus. Estrogen. Estradiol dipropionate. Granulation Tissue. Healing.

INTRODUCTION

Interest in the intervertebral disc (IVD) and the results of injury to it can be traced back to the days of great German Pathologist, Christian Georg Shmorl (1861-1932)¹, although sciatica and low back pain were perhaps first mentioned by Hippocrates and later by Galen². Commonly steroid hormones are administered both intramuscularly or epidurally to treat low back pain due to the injury to the intervertebral disc (IVD)³.

The IVD is a highly organized matrix laid down by the relatively few cells in a specific manner. The central gelatinous nucleus pulposus (NP) is contained within the more collagenous annulus fibrosus (AF) laterally and the cartilage end plate inferiorly and superiorly. The strength of the IVD is related to the fluid and proteoglycan (PG) content^{3,4}. Proteoglycans consist of a core protein and one or more covalently attached glycosaminoglycans (GAG) chains. GAGs are hydrophilic, negatively charged, branched chain molecules comprising a protein core and an oligosaccharide. The negative charge on the branched chains and the hydrophilic nature of PG internally pressurize the disc by drawing water via osmosis into the NP⁴. PG content and the amount of hydration within the disc is inversely proportional to

applied stress and age⁵. The majority of disc nutrition is supplied via the capillary beds of the cartilaginous vertebral body endplate.⁶ With increasing age, water of the PG content change and diminish in the IVD.

Previous studies have found the estrogenic environment to have a significant impact on the glycosaminoglycans and the water content of the intervertebral discs.⁷ Topical estrogen administration has resulted in acceleration of cutaneous wound healing without changing plasma estrogen level.⁸ Resveratrol is a phytoestrogen found in various plants including grapes and red wines and found to have a protective effect on articular cartilage in rabbits⁹.

17- β -Estradiol is the primary and most potent naturally occurring placental and ovarian estrogen in mammals.¹⁰ It has been reported that estrogen stimulates proteoglycan synthesis in rabbit chondrocytes in vitro⁷. In one 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 IVD¹¹.

The sequence in dermal wound repair is principally analogous in other anatomical locations

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and starts with the arrest of hemorrhage, followed by an inflammatory response; re-epithelialization of the wound; formation of granulation tissue within the wound space and finally the production of a scar^{12,13}. The normal healing response begins the moment the tissue is injured and proceeds only after the inflammation is controlled¹⁴ (Fig 1)¹².

In first stage of inflammation, platelets elaborate a number of pro-inflammatory substances, such as adenosine diphosphate, tissue growth factor beta, and platelet-derived growth factors. These growth factors stimulate chemotaxis and the orderly migration of neutrophils, monocytes, and fibroblasts into the area of injury. In the second stage, leukocytes replace platelets as the dominant cell type, attracted by chemotaxis^{15,16}. As polymorphonuclear leukocytes begin to wane after 24-36 hours, circulating monocytes enter the wound and mature into tissue macrophages, reaching their peak by five days after injury.¹⁷ Macrophages are perhaps the most essential inflammatory cells involved in the normal healing response and their arrival signals the end of inflammatory and beginning of proliferative phase^{18,19}.

Two to three days after wounding, fibroblasts migrate into wound space in response to macrophage-synthesized growth factors and begin producing glycosaminoglycans and proteoglycans, the ground substance for granulation tissue, as well as collagen^{19,20}. Fibroblasts soon become the dominant cell type, peaking at 1-2 weeks¹⁷. Various phases of healing and only main cells involved in each phase are shown in the following table¹⁴.

Phase of healing	Days post injury	Main cells involved
Hemostasis	Immediate	Platelets
Inflammation	Day 1-4	Neutrophils
Proliferation & Granulation	Day 4-21	Macrophages Lymphocytes Angiocytes Neurocytes Fibroblasts Keratinocytes
Remodelling	Day 21-2yrs	Fibrocytes

The combination of the lack of blood supply and sparsity of cells in a dense extracellular matrix leads to a limited ability to heal in cartilage.²¹ Deeper lesions introduce blood supply from the well-vascularized subchondral bone, bringing fibrocytes that modulate into fibrochondrocytes. These cells produce a disorganized lattice of collagen fibers partially filling the defect with structurally weak tissue^{22,23,24}.

A wide range of doses of glucocorticoids is used for a demanded anti-inflammatory effect in many

diseases.²⁵ Spinal steroid injections, both epidural and intradural, have proved beneficial for patients with advanced degenerative disc disease and chronic low back pain²⁶.

Rabbit's IVD resembles the human disc in general structure and is large enough for an adequate histological study.¹ Stabbing the anterolateral AF of adult rabbit lumbar discs results in a number of histological changes that show a similarity to changes seen in human IVD.²⁷ A rabbit is therefore 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. 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 and 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 newly forming granulation tissue. Intensity was graded as "Absent, Mild, Moderate and Deep" on an ascending scale.

Quantitative data was recorded for number of; fibroblasts with clear nucleus, fibroblasts with condensed nucleus, vessels, macrophages,

neutrophils, total number of other cells and total number of cells as the mean cell count per predefined unit area of the granulation tissue. "Other Cells" comprised of the cellular population of the observed region which were not being specifically looked for in the study. Neutrophils and macrophages were also looked for in the annulus fibrosus and nucleus pulposus.

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 ($p \leq 0.05$) in the means of cell population between the subgroups.

RESULTS

In the individual comparison of control groups, number of fibroblasts with condensed nucleus was significantly more in the subgroup A2. Number of vessels, neutrophils and other cells was significantly more in subgroup A1. It was found in the comparison of control and experimental group of five days, number of fibroblasts with clear nucleus was significantly more in the subgroup B1 (Fig. 2).

Number of vessels and macrophages was significantly more in the subgroup A1 as was number of neutrophils and other cells. When control group of 5 days was compared with experimental group of 7 days, it was found that the number of fibroblasts with clear nucleus and total number of cells was significantly more in the subgroup B2. Number of macrophages (Fig. 3), neutrophils (Fig. 4) and vessels were significantly more in the subgroup A1. In comparison of control of 7 days and experimental group of 5 days, number of fibroblasts with condensed nucleus, macrophages and other cells was significantly more in subgroup A2. In the comparison of control and experimental group of 7 days, Number of fibroblasts with condensed nucleus and macrophages were significantly more in subgroup A2. In the individual comparison of experimental groups number of other cells was significantly more in subgroup B2. Notably, no significant presence of macrophages and neutrophils was found in the annulus fibrosus and nucleus pulposus in any of the subgroups. Qualitative data is given graphically in Fig. 5 and 6.

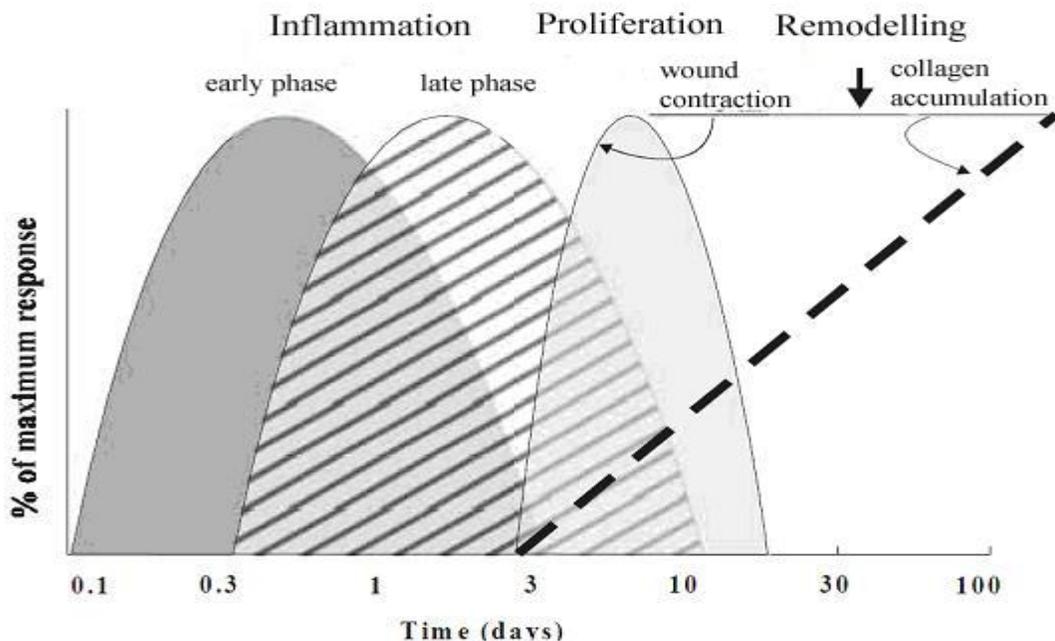


Fig 1: Phases of wound repair. Healing of a wound has been divided into three phases: inflammation (early and late), proliferation, and remodeling. These wound repair processes are plotted along the abscissa as a logarithmic function of time. The phases of wound repair overlap considerably with one another. Inflammation is divided into early and late phases denoting neutrophil-rich and mononuclear cell-rich infiltrates, respectively¹².

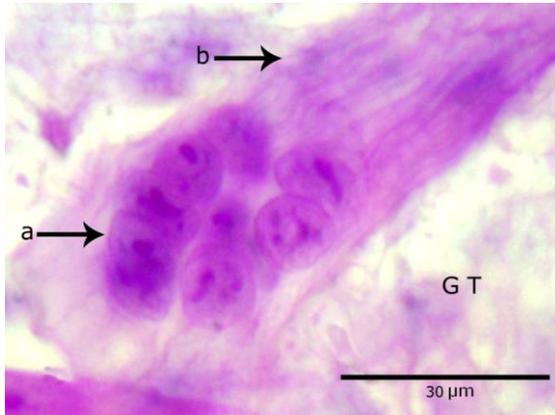


Fig. 2: Fibroblasts with prominent nucleoli (a) in the granulation tissue (GT) leaving behind a trail of freshly laid matrix (b) staining intensely with the stain. (Slide # 107/10, subgroup B1, H&E stain)



Fig. 3: A clear macrophage cell (pointing arrow), seen in the inflammatory exudates (IE). Ruffled border and granular material inside the cytoplasm with a prominent nucleus and nucleoli can be appreciated

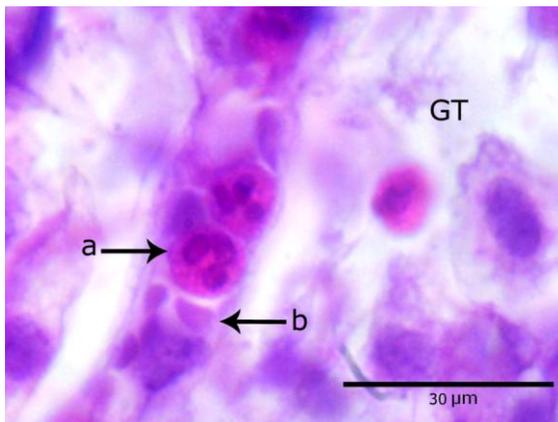


Fig. 4: Photomicrograph showing cells of inflammation in the granulation tissue (GT). Neutrophils with multilobed nucleus (a) and red blood cells (b) can be seen inside a vessel, amongst the fibroblasts. (Slide # 52/10 Subgroup A2, H & E and alcian blue stain).

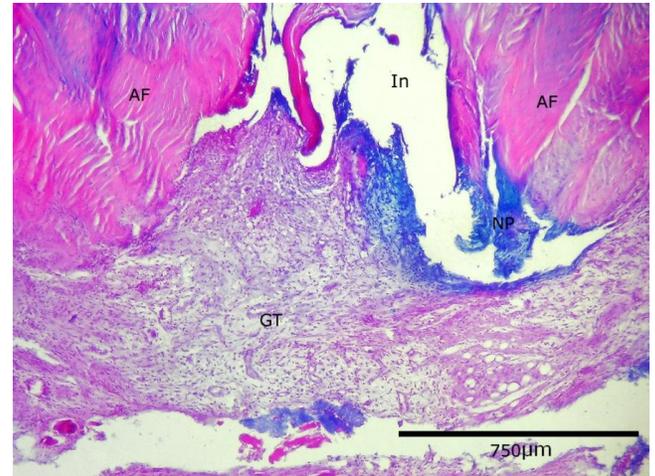


Fig. 5: A low powered photomicrograph showing overriding of wound gap by granulation tissue (GT) at the bottom of the imae. Annulus fibrosus (AF) and region of injury (In) can also be seen. (Slide # 157/6, subgroup B1, H&E and Alcian blue stain).

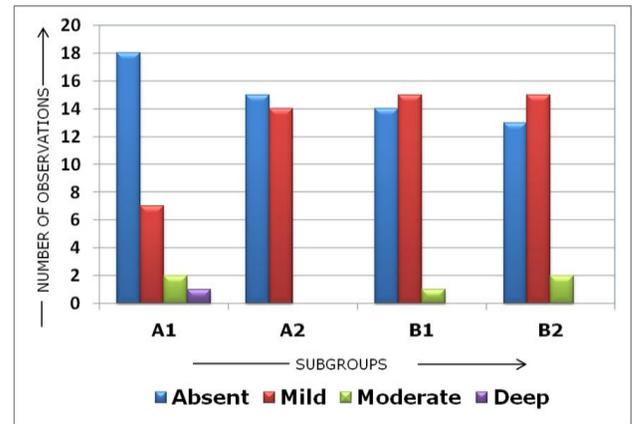


Fig 6: Graphical representation of observed staining grades of the matrix that has been stained with alcian blue stain to detect the proteoglycan content of the matrix in granulation tissue.

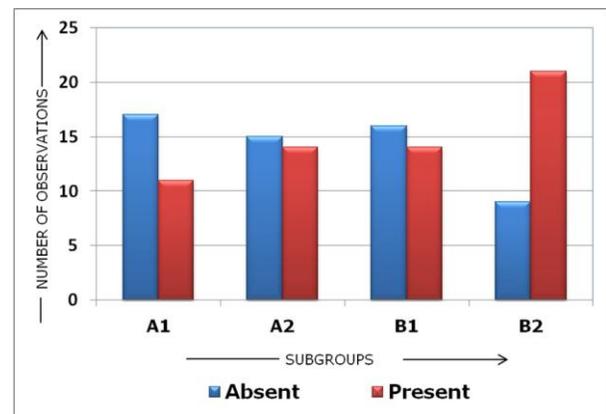


Fig. 7: Graphical representation of presence or absence of overriding of the wound gap by the granulation tissue in all the subgroups

DISCUSSION

The current study was designed to observe the effects of estrogen, beneficial or otherwise, using its parenteral form estradiol dipropionate (E2), on the granulation tissue of injured intervertebral disc (IVD) using rabbit as an experimental animal. It was expected in the current study that number of macrophages would be on the decline in the injured tissue by fifth day of healing and a further decreased number would be observed by seventh day after injury. Similarly, it was expected that cells responsible for laying down matrix would appear earlier in the injured tissue in the experimental subgroups as compared to control to lay down the matrix for the granulation tissue and thus expedite the healing.

Intervertebral disc has various regions; one is avascular which comprises of epiphyseal plate, end plate, annulus fibrosus and nucleus pulposus, while the vascular portion comprises of the outer most rim of annulus fibrosus. It is this vascular tissue which is thought to have provided base for the formation of granulation tissue in the current study.

Previous works showed that intervertebral disc is an active tissue capable of self-maintenance, repair and having considerable regenerative properties,²⁸ still a gap of knowledge regarding detailed account of healing in intervertebral disc was found.

In the region of Annulus fibrosus (AF), cells of inflammation failed to show up in the AF. Avascularity of the AF might be a reason for this finding, although area of AF being observed was very close to vascularised tissue. It is also suggestive of the fact that inflammatory reaction of injury does not spread to AF. Although inflammation is a vital component of process of healing, it can be damaging to the tissue as well, if it stays for a longer duration. This avascularity of the AF, therefore, might be it's protective feature. Another proposed possible mechanism for the failure of these cells to show up in AF can be the intricate lamellar structure of the AF, forming a physiological barrier that is not allowing the cells inside. Also these cells might be lacking the required enzymes to breakdown into AF or the chemotactic factors are not produced by the cells of AF for these cells.

There was a little infiltration of macrophages into NP and timing of the appearance corresponded to the hypothesized picture, However, as the population of these cells was so negligible that no statistical analysis could be done, no definite conclusion shall be drawn about their presence in the NP, except that they wandered by diffusion by chance into the NP, as

the physiological barrier had been broken by the injury.

It was also observed during the study that granulation tissue (GT), would never form if the NP had protruded out of the disc space. Protruded nucleus might be hindering the formation of fibrin plug by some chemical or physical mechanism. This fact also suggests that NP shall be reinserted into the IVD as soon as possible, probably by some laparoscopic procedure, for initiation of efficient healing. Fibroblasts with clear nucleus i.e. fibroblasts which were synthetically active were a good representative of progress of healing. Fibroblasts with condensed nucleus i.e., fibroblasts which were metabolically inactive, decreased in experimental subgroups. It suggests that E2 has a beneficial role on fibroblasts in granulation tissue where it not only increases the number of metabolically active fibroblasts, but also decreases the number of metabolically inactive fibroblasts. A rapid turnover of cells can also be a possible mechanism for this picture.

Absence of inflammatory cells from the granulation tissue in the experimental group was suggestive of early end of the inflammatory phase and also that E2 was acting as an anti-inflammatory agent.

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. Cleaning up by macrophages may be possible for this reduced population. E2 might be a mitogenic factor for other cells of inflammation also.

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.

Analysis of overriding of wound gap by the granulation tissue showed that in a large majority of cases wound gap was 'bridged' by the granulation tissue in the E2 treated subgroups (Fig 7).

CONCLUSION

This study provided insight into healing of the intervertebral disc. Estradiol dipropionate (E2), a potent form of estrogen, had effects on the epiphyseal plate recruiting more cells into the intervertebral disc. In the granulation tissue, the fibroblasts with clear nucleus increased significantly after administering E2. More cases of overriding of wound gap by granulation tissue were found in the experimental subgroups. A decrease in the number of inflammatory cells in the E2 treated group shows that the inflammatory response was over earlier in

the experimental subgroups. Inflammation is necessary for healing but lesser inflammation would lead to reduced tissue damage.

In the light of above observations it is deduced that improving mitosis, continuous lay down of the proteoglycans and earlier overriding of wound gap by the granulation tissue may actually 'seal off' the injury, effectively expediting the healing of IVD.

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