ALGINATE – STRONTIUM HYDROGEL WITH STEM CELLS *IN SITU* FOR THE REPAIR OF NUCLEUS PULPOSUS OF THE INTERVERTEBRAL DISC IN LAPINE MODEL

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INDIA

DECLARATION

I, Mir Mahmoud Mortazavi Roudmiane, hereby certify that I had personally carried out the work depicted in the thesis entitled, "***Alginate – Strontium Hydrogel with stem cells in situ for the repair of nucleus pulposus of the intervertebral disc in Lapine Model***", except where due acknowledgment has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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*Clearance was obtained from the Institutional Animal Ethics Committee & Institutional Committee for Stem Cell Research and Therapy for carrying out the study.

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"Alginate – Strontium Hydrogel with stem cells in situ for the repair of nucleus pulposus of the intervertebral disc in Lapine Model"

Submitted by

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DEDICATED TO MY FAMILY
&
TEACHERS

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ABBREVIATIONS

ADMSCs	Adipose Derives Mesenchymal Stem Cells
AF	Annulus Fibrosus
Alg	Alginate
BMP	Bone morphogenetic protein
cLSM	Confocal laser scanning microscopy
CPCSEA	Committee for the Purpose of Control And Supervision of Experiments on Animals.
DDD	Degenerative Disc Disease
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DMEM-HG	Dulbecco's modified Eagle's medium - high glucose
DMEM-LG	Dulbecco's modified Eagle's medium - Low glucose
MSC	Mesenchymal Stem Cells
FBS	Foetal Bovine Serum
FTIR	Fourier transform infrared spectroscopy
HA	Hydroxyapatite
IGF	Insulin-Like Growth Factor
IVD	Intervertebral Disc
MEM	Minimal essential medium
MRI	Magnetic Resonance Imaging
Mw	Molecular weight
NP	Nucleus Pulposus
PBS	Phosphate Buffered Saline
PDGF	Platelet-derived growth factor
SEM	Scanning Electron Microscope
Sr	Strontium
RADMSCs	Rabbit Adipose Tissue Derived Mesenchymal Stem Cells
TEM	Transmission electron microscopy
TGF	Transforming Growth Factor
µ-CT	Micro CT

ANNOTATION

Cm	Centimeter
ml	Milliliter
µg	Microgram
mg	Miligram
^{0}C	Degree Celsius
mm	Millimeter
h	Hour
M	Molar
min	Minute
g	Gram

"SYNOPSIS"

Title: "Alginate-strontium hydrogel with stem cells *in situ* for repair of the nucleus pulposus of the intervertebral disc in lapine model"

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Changes in the "S" shape spinal column can cause damage on the Intervertebral disc (IVD). The fibrocartilaginous structure of IVD comprising of Nucleus Pulposus (NP), Annulus Fibrosus (AF) and End Plate (EP) that lie in between the vertebrae are responsible for the movement and bending of the vertebral column. IVD prevents the vertebrae from grinding against one another and absorbs shock caused by such movements as running, jumping and even walking.

Degeneration of the Intervertebral disc (IVD) is a major pathological process (age, slip or rupture) implicated in low back pain. A damaged disc may press spinal cord or spinal nerves towards the intervertebral foramen of the vertebral column and cause pain or contribute towards loss of the disc height. It has emerged as the most expensive healthcare problem in the United States ranging $20 to $100 billion per year and also estimated at £12 billion per annum in the United Kingdom.

The most important component inside the disc is collagen, aggrecan, glycosaminoglycans (GAG) and small amount of collagen type IV, VI, IX and XI. Combination with K^+ and Na^+ ions makes the environment negatively charged and in turn suitable for absorbance of water from the surrounding tissue towards the NP which is responsible for the disc height. NP is the inner core of the IVD that usually decreases its height and thereby the Annulus Fibrosis bulges out impinging on the nerves, ultimately causing pain. When NP cells are not able to provide sufficient Extra Cellular Matrix (ECM), NP looses the water content resulting in reduction of the disc height and subsequently low back pain. Besides conservative and surgical treatments (removal of disc and vertebra fusion), cell therapy foresees the possibility of regenerating the damaged NP where cells can proliferate *in vitro* and re-implanted to alleviate the pain and further prolong the damage to sustain quality of life. Hence, the prospect of cell-based Nucleus Pulposus (NP) tissue-engineering strategy has become attractive and relevant in Regenerative Medicine.

Hypothesis

Would Alginate-strontium hydrogel with cells *in situ* assist the repair of injured Nucleus Pulposus (NP) of the Intervertebral Disc?

Objectives:

A. To study the anatomy of the IVD of rat, rabbit, goat, cattle and pig.
B. To study the mechanical properties of the IVD rat, rabbit, goat, cattle and pig.
C. To prepare Alginate/Strontium (Alg/Sr) substitute for NP.
D. To isolate, expand and characterize Rabbit Adipose Mesenchymal Stem Cells followed by their differentiation potential towards the cartilage lineage.
E. To fabricate and characterize Alg-Sr/cell construct *in vitro* using microscopic techniques.
F. To create and develop Lapine (Rabbit) IVD model by NP physical injury.
G. To study the response of Alg-Sr/cell combination product in the injured NP of New Zealand White Rabbits (Lapine model) - *in vivo.*
H. Examining the final data and its application in real clinical condition.

The Thesis is divided into five chapters

Chapter 1 – Introduction – Background of the Study

This chapter describes the structure of the spine, vertebral column and the IVD and its composition. Degenerative Disc Disease and its pathogenesis contributing to low back pain are dealt herein. Current therapeutic options for degenerative disc disease such as non-operative management, surgical management, spinal fusion, total disc arthroplasty, nucleus pulposus replacement, cellular and molecular Therapy, Nucleus Pulposus Tissue Engineering are described in detail. Furthermore, emphasis is laid on Nucleus Pulposus Scaffold substitutes - Preformed Scaffolds, Injectable hydrogels etc that mimic the structure and composition of NP of the disc.

Chapter2 – Literature Review

Different types of natural polymers like Polycaprolactone (PCL)[1], Gelatin/chondroitin-6-sulfate/hyaluronan[2], Agarose/collagen, Collagen/Glycosaminoglycan[3], Collagen, Polyglycolic acid/alginate have been used for repairing the intervertebral disc in different animal models like – Rat, Rabbit, Pig but all of them are far from clinical usage[4]. The major clinical problem of tissue-engineered disc is the insufficient biomechanical behavior and stiffness for long term usage[6]. The attraction of fabricating tissue-engineered disc is by using either the cultured disc cells or by converting stem cells into cartilage cells in combination with the scaffold. This appears to be a better option for NP tissue engineering[5,7].

Chapter 3 – Intervertebral disc of different animals

It was of interest to understand the structure of the IVD before embarking on the synthetic substitute for the damaged NP. Hence, the anatomy, histology and mechanical properties (tensile & compression) of the IVD of different animals like – rat, rabbit, goat, sheep, cattle and pig were studied.

Chapter4 – Material preparation and characterizations

Alginate, a natural polymer has been selected as a good candidate scaffold for NP regeneration. As the NP structure comprises 75% of water content, this hydrogel depicts similar porosity and water content. Combination of alginate (4%) with Strontium chloride ($SrCl_2$ – 1.8%) (via a dual in-house applicator with 22G needle) gave a porous Alginate-strontium (Alg-Sr) hydrogel, that has been characterized by FTIR, DTG, TGA, Micro-CT (porosity) along with mechanical testing for its tensile & compression strengths. Lastly, Alg-Sr proved to be noncytotoxic with L929 mouse fibroblast cell lines.

Chapter 5 – *In vitro*

Rabbit Adipose-derived Stem cells (RADMSCs) were isolated from subcutaneous fat tissue, expanded in culture and differentiated into chondrocytes. Cells grew out into a confluent monolayer of spindle shaped cells in the culture dishes and were tested for their viability encapsulated in Alg-Sr (Confocal microscopy). Cell compatibility and proliferation was favored within Alg-Sr hydrogel.

Chapter 6 - *In vivo*

New Zealand White Rabbit (Lapine) IVD Model was created by using 16 G Needle on L5/L6 Lumbar region under IAEC approval and CPCSEA guidelines. Intervertebral disc of Rabbit has been located from the left dorsolateral side of the animal. Distance of the needle to the lumbar IVD was accessed based on the measurements taken with the Keyence Microscope. Radiographs of the Rabbit vertebral column showed the prolapse of the Disc at L5/L6 in degenerative IVD model after needle injury – post injury 7 days. Thirty animals survived injury. Further, post-surgery one month, a re-surgery was done (from right dorsolateral side) to inject bare Alg-Sr and Alg-Sr/cell combination product *via* a dual in-house applicator after radiographic confirmation of the planned damaged IVD. Post injection one, three and six months, X-ray and MRI were done and retrieved IVD samples were subjected to histology and histomorphometirc analysis.

Conclusion

i. IVD of different animals showed different tensile and compression strength (Rat < Rabbit < Goat < Cattle < Pig)
ii. Alg-Sr hydrogel was prepared and characterized.
iii. TGA and DTA showed the mass loss and enthaplic changes of the Alg-Sr Hydrogel.
iv. FTIR spectra showed no significant difference between Alginate and Alg-Sr.

v. Alg-Sr passed the Cytotoxicity test.

vi. RADMSCs were cytocompatible with Alg-Sr Hydrogel and favored proliferation.

vii. Histomorphometric analysis showed that:

A. Bare Alg-Sr hydrogel improved healing compared to sham at one month.

B. Alg-Sr & cell combination product enhanced healing compared to bare Alg-Sr at one and 3 months.

C. Alg-Sr & cell combination product healing at 3 months is comparable to bare Alg-Sr hydrogel at 6 months.

D. Hence, "Alg-Sr & cell combination product" definitely helped in enhancing the healing of the damaged NP.

Relevance of the study:

- To improve the quality of life of the younger generation affected by low back pain.
- Yet a long way to go before an optimal scaffold for IVD repair is identified.

Future Study:

To validate safety and efficacy 'Alg-Sr & cell combination product' in a larger IVD defect animal model and molecular analysis of implanted tissue samples.

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CHAPTER 1

INTRODUCTION

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INTRODUCTION

Vertebral column is set in the mid-dorsal of the body that connects head to tail. It includes many bony vertebrae and cartilaginous pads. It protects spinal cord from injury and allow the body to fold and unfold. The regions with high flexibility are tail part in quadrupeds. But in human, cervical and lumbar regions are the most flexible part of the body. 25 percent of vertebral column is occupied by 23 cartilaginous pads with thickness of 8 to 10 millimeter. These pads allow vertebral column to shift left and right and move the body trunk (Sylvia S.Mader, n.d.; Greenaway et al. 2001).

1.1 Structure of the vertebral column:

The vertebral column consists of five regions cervical vertebrae, thoracic vertebrae, lumbar vertebrae, sacrum region and coccyx region (Sylvia S.Mader, n.d.).

Vertebrae in vertebral column are separated by cartilaginous intervertebral discs (Singer, n.d.). “S” shape of back bone gives special features and function to the body to support skull and upper extremities, trunk of

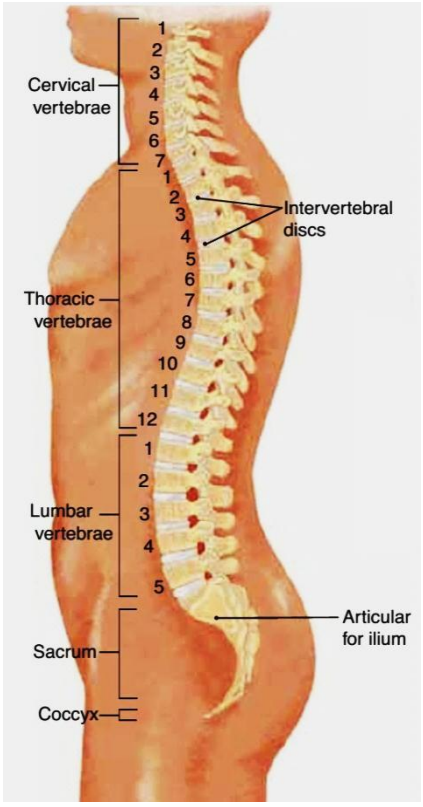

Fig 1-1: Vertebral Column of human body with S-shape. (Adapted from clinical anatomy of spine)

muscles, bipedalism, movement and flexibility (Kent, Graaff, and Ward Rhees, n.d.) (Fig 1.1).

Vertebrae consist of a special shape in the anterior side “Drum Shape”, which is connected to the intervertebral disc above and below. The vertebral arch is connected to the posterior side of the body with two pedicles and two arched laminae. Vertebrae contain special foramen for the passing spinal cord and nerves pass through the intervertebral foramina to the anterior organs (Kent, Graaff, and Ward Rhees, n.d.).

There are distinctive features for the different vertebrae; in cervical vertebrae the transverse foramen is visible; in thoracic vertebrae, facets join for connecting to the ribs and the lumbar region vertebrae contain flat spineous process for the attachment of muscles (Kent, Graaff, and Ward Rhees, n.d.) (Fig 1. 2).

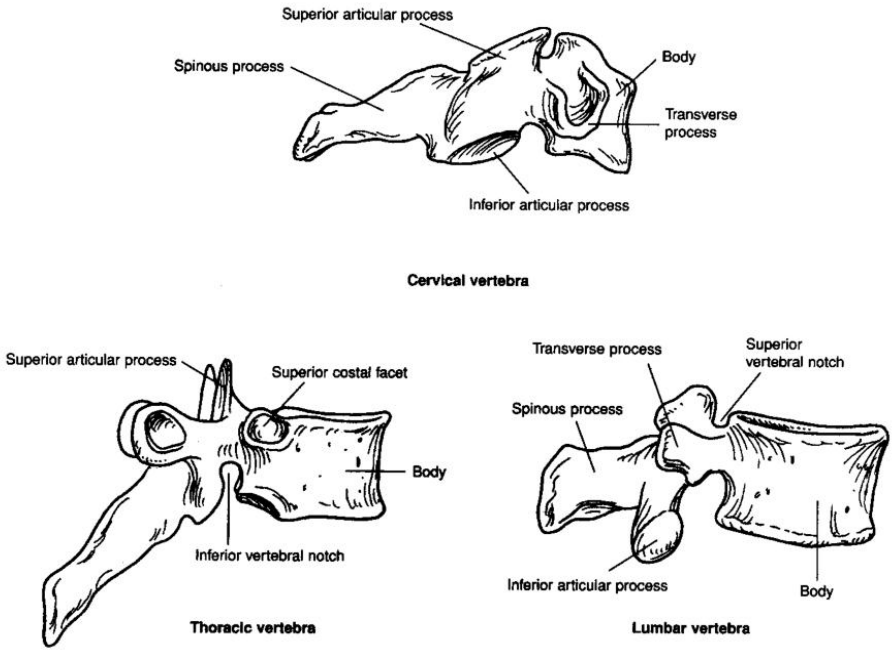

Fig 1-2: Examples of vertebrae from different vertebral regions (adapted from human anatomy and physiology, Kent et al, 2006)

1.2 Intervertebral disc development and composition:

1.2.1 Intervertebral Disc Development:

Notochord with mesodermal origin makes the axial cord at the center of the embryo. Mesenchymal cells which are available in the surrounding notochord provides a prichordal shape (N. A. Scott, P. F. Harris, and K. M. Bagnall 1980). This structure takes the notochordal cells inside a clear notochordal sheath. By pressure of sheath some notochordal cells push towards the vertebra bodies while the segmentation of the notochord to vertebral body is occurring (RJW Hoogendoorn et al., n.d.) (Fig 1. 3 A, B, C).

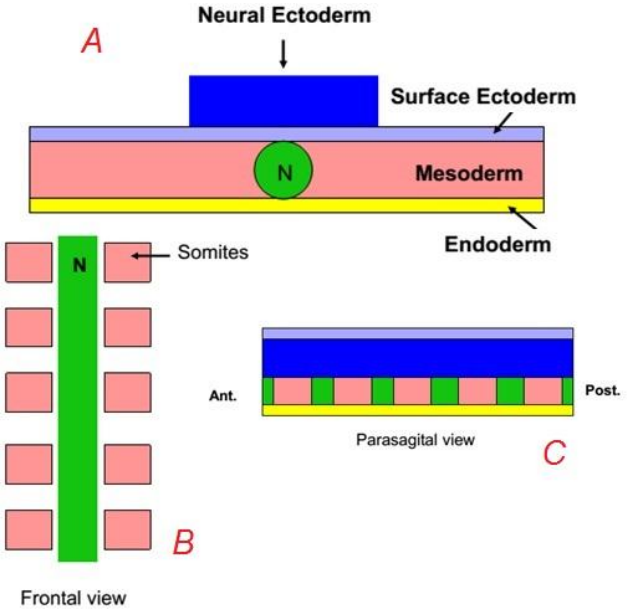

Fig 1-3: Schematic representation of the development of intervertebral discs.

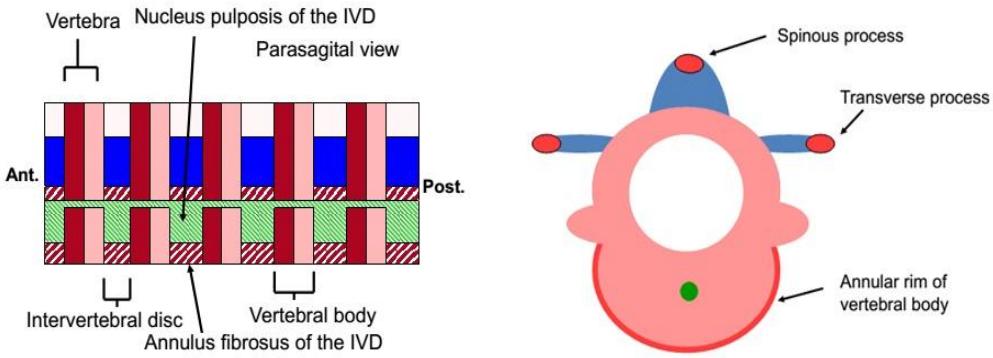

Fig 1-4: Schematic sketch of the construction of the intervertebral disc during embryogenesis.

Cylindrical sheath of embryonic mesenchymal column segmentation occurs towards the dorsal region and converts to the segmented dark and light bands. Dark bands are slow growing and will convert to intervertebral disc while the light bands grow rapidly and it develops into the cartilage mold of the vertebral bodies (Singer, n.d.).

1.2.2 Cartilage:

Cartilage is semisolid tissue with cells trapped in the matrix. Mostly in cartilage there are chondrocyte cells (cartilage cells), notochordal cells and matrix. Cartilage is surrounded by dense connective tissue. It is an avascular tissue and therefore any trauma to this tissue will prolong healing (Kent, Graaff, and Ward Rhees, n.d.).

Cartilage receives nutrients by diffusion from the perichondrium and surrounding tissues. There are three types of cartilage: Hyaline cartilage, fibrocartilage and elastic cartilage based on the different compositions and percentage of fiber in the matrix.

1.2.2.1 Hyaline cartilage:

Contains very fine structure of collagenous fibers visible by electron microscopy. It shows a glossy and clear appearance under the light microscope (Kent Van De Graaff, n.d.).

This tissue is visible mostly in the respiratory system, reinforces nose and joints between ribs and sternum. In embryogenesis process most of the bones are in the hyaline state prior to transformation to bone. This process is called endochondral ossification (Kent Van De Graaff, n.d.).

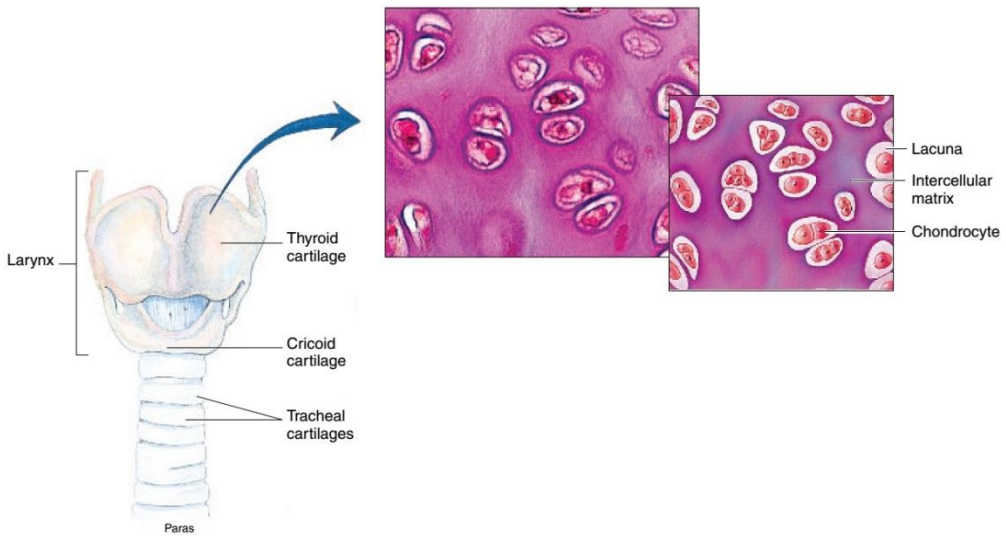

Fig 1-5: Hyaline cartilage in Trachea (adapted from human anatomy, page 96)

1.2.2.2 Elastic cartilage:

This tissue is very similar to hyaline cartilage but there are abundant elastic fibers which give flexibility to the cartilage. Elastic fibers show the yellowish appearance and found in the larynx, outer ear and auditory canal (Sylvia S.Mader, n.d.).

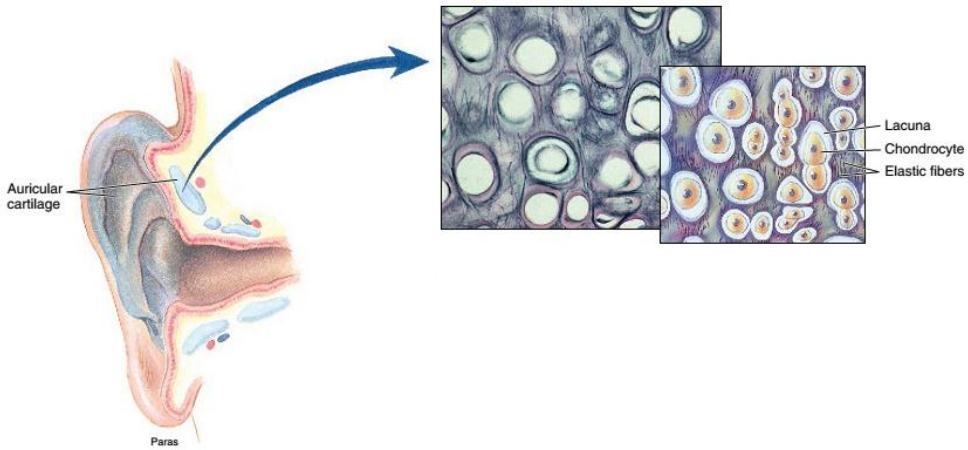

Fig 1-6: Elastic cartilage in the outer ear, auditory canal (adapted from human anatomy, page 97)

1.2.2.3 Fibrocartilage:

This type of cartilage has abundant collagenous fibers durable for compression and extension. Mostly this type of tissue is found in the important part of the skeleton like pelvic bone, knee joint and between vertebrae in the intervertebral discs (Kent Van De Graaff, n.d.).

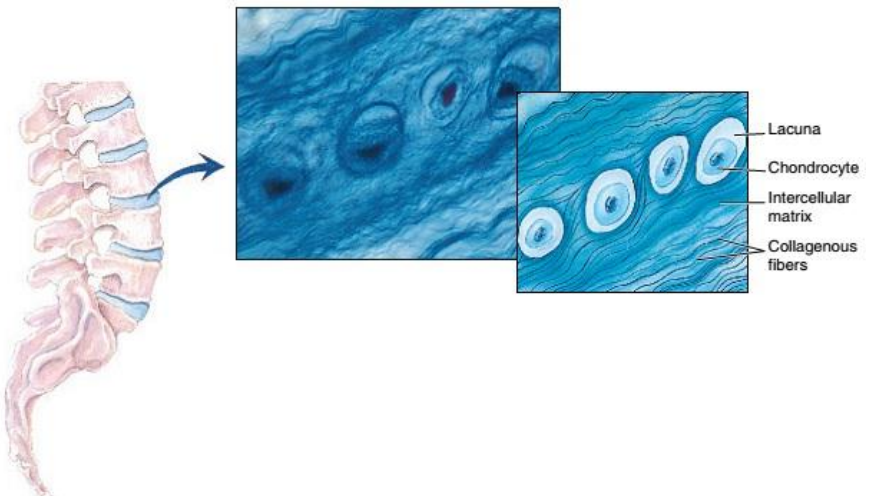

Fig 1-7: Fibrocartilage at the intervertebral disc of vertebral column (adapted from human anatomy, page 96)

1.3 Intervertebral disc structure & composition of IVD

1.3.1 Structure:

In humans and rodents there are different numbers of intervertebral discs in the vertebral column. As we know, in human there are 23 discs in the entire length of the vertebral column which are bigger compared to the rodents. In human, each IVD is around 8 to 10 mm in height and 40 mm in diameter and occupy 25 percent of the vertebral column height (Kent Van De Graaff, n.d.).

Intervertebral disc consist of three main structures; a spongy component at the center - nucleus pulposus; the surrounding lamellar layer annulus fibrosus and two layers of cartilage on either side of IVD which are called the endplates(Bergknut et al. 2013; Singer, n.d.).

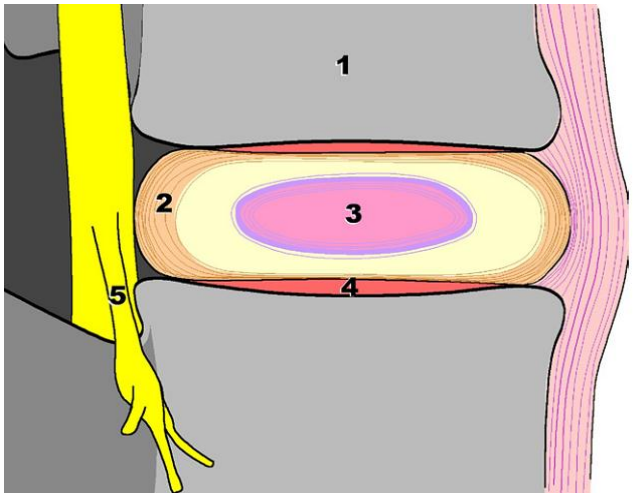

Fig 1-8: Sagittal section of vertebrae (1), annulus fibrosis (2), nucleus pulposus (3), endplate (4) and spinal nerve root (5) (adapted from Anatomy and pathology of intervertebral disc).

1.3.2 Composition:

1.3.2.1 Nucleus pulposus (NP)

NP is a spongy gel-like which consist of Type II collagen organized randomly among proteoglycan molecules (aggrecan), hydrophilic chondroitin, and keratin sulfate. Glycosaminoglycan traps water molecules which give the gel-like properties to the nucleus pulposus. Extra cellular matrix contains other types of proteoglycans like: Versican, biglycan, decorin, fibromodulin and lumican (Shankar, Scarlett, and Abram 2009). Composition of different types of collagen such as Type I, II and Type IV gives more tensile strength to the NP matrix. For more hydration and trapping water molecules aggrecan binds with highly anionic glycosaminoglycans like chondroitin and keratin sulfate which helps to maintain the fluid state at birth is around 90 percent and during aging decreases to 70 percent.

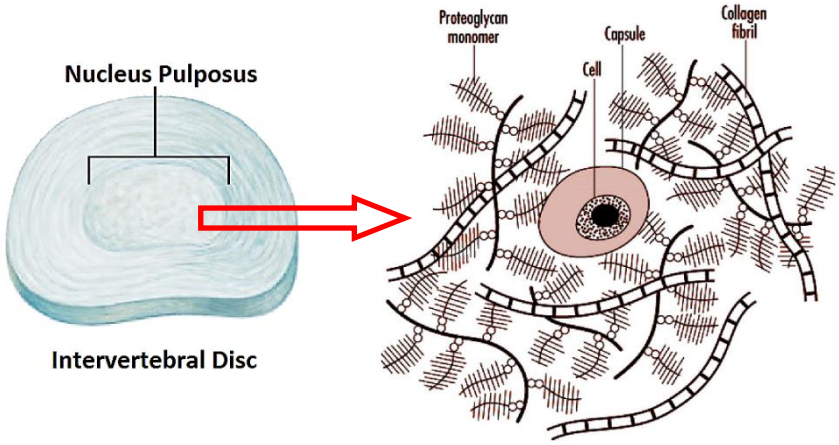

Fig 1-9: The matrix components of Nucleus Pulposus

1.3.2.2 Annulus fibrosus (AF)

Several concentrated layers of Type I collagen and protein construct a special composition called annulus fibrosus.

This structure contains 10 to 20 sheets of fibers in different orientation and angles called lamellae. The lamellae are thicker at the anterior side compared to the posterior. Orientation of fibers in AF is 60 to 70 in vertical aspect. This feature of AF gives flexibility to vertebral column to turn right and left (Singer, n.d.).

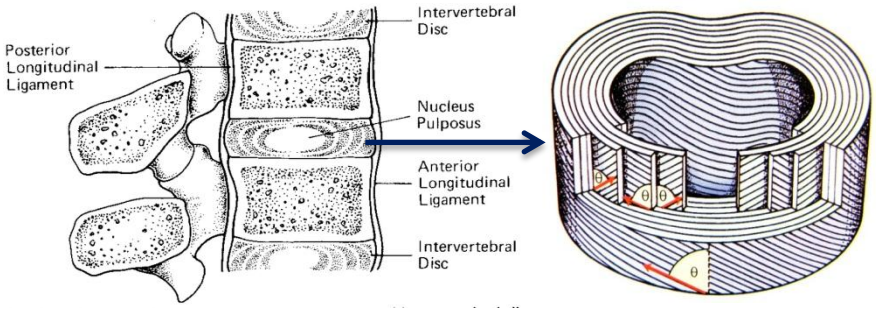

Fig 1-10: Schematic sketch of annulus fibrosus

1.3.2.3 Endplate:

is a layer of hyaline cartilage with thickness of 0.6 mm to 1 mm. Endplate covers two sides of the disc. During the first year of life, endplate is highly vascularized but the measure of vascularity decreases during course of time (Shankar, Scarlett, and Abram 2009).

Endplate consists of Type II collagen which is favorable for compression resistance (Singer, n.d.).

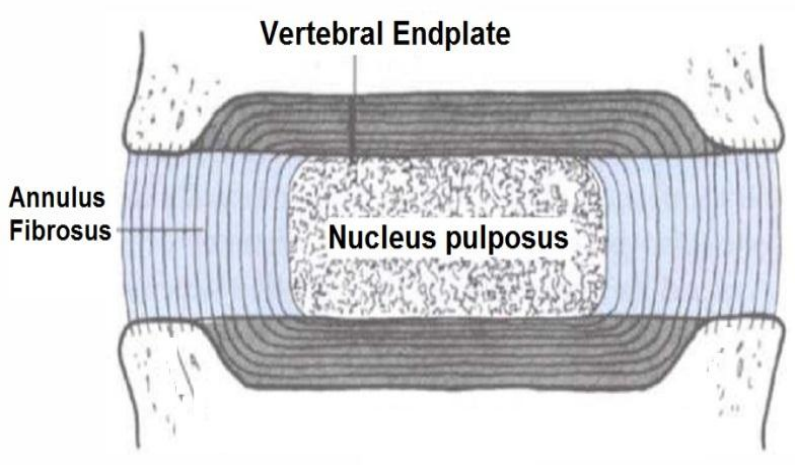

Fig 1-11: Schematic sketch of the endplate positions.

1.4 Extracellular Matrix (ECM):

The functional properties of intervertebral disc depend on 3 factors; Composition, orientation and integrity of ECM. As we know, NP has a heterogeneous structure of water and proteoglycans.

1.4.1 Aggrecan:

is the main component of NP which is responsible for hydration and disc height. It absorbs water through osmotic pressure. Aggrecan decreases with age and NP cells fail to keep disc height and increases the hydrostatic pressure. Cracks therefore appear in the annulus fibrosus and endplates (Shankar, Scarlett, and Abram 2009).

1.4.2 Proteoglycan:

is the core protein with glycosaminoglycans. Decorin, lumican, Biglycan and fibromodulin are small proteoglycans. Proteoglycans like aggrecan withdraw water from the surrounding tissues (Sive et al. 2002).

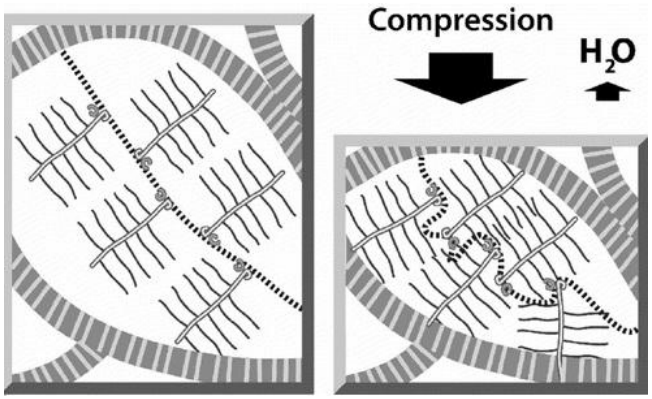

Fig 1-12: Schematic sketch of the compression changes on proteoglycans.

Proteoglycan= Core Protein + Glycosaminoglycan

1.4.2.1 Glycosaminoglycans:

are large molecules with negative charge groups like Sulphate and carboxylic. There are different types of glycosaminoglycans - hyaluronic acid, Keratan sulphate, chondroitin (4-or6-) sulphate, dermatan sulphate (Shankar, Scarlett, and Abram 2009).

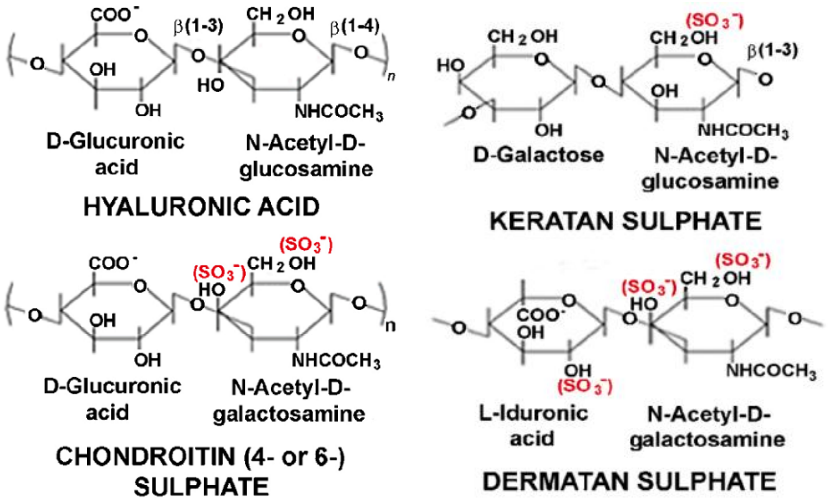

Fig 1-13: Chemical structures of different types of glycosaminoglycans

1.4.2.2 Proteolytic enzymes:

are proteases or proteinases which can degrade long chains of big protein molecules to small fragments (Shankar, Scarlett, and Abram 2009).

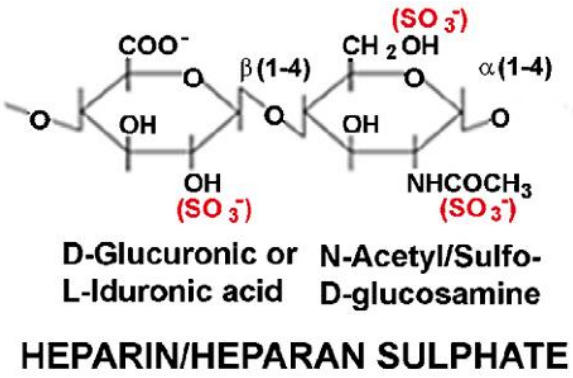

Fig 1-14: Chemical structure of proteolytic enzymes

1.5 Curves of the Spine:

Human vertebral column does a different duty like protecting spinal cord, nerve root and internal organs. Vertebral column provides

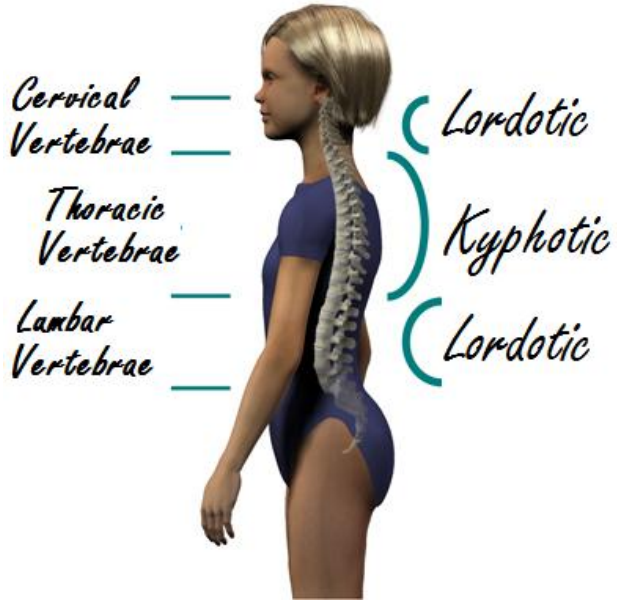

Fig 1-15: Schematic sketch of vertebral column curves.

flexibility as well as balance for upright posture. While vertebral column looses its balance; different types of deformity occurs. Normally 'S' shape of spinal column contains curves and curves towards the front is called lordotic and curves towards the outside is called kyphotic (Kent Van De Graaff, n.d.).

1.5.1 Deformity of vertebral column:

Curvature disorders include: Scoliosis, Spondylolisthesis, Kyphosis and Lordosis.

1.5.1.1 Scoliosis:

is a lateral curvature disorder of vertebral column. This disorder can occur in different ways like: thoracic, thoracolumbar, lumbar and double major curve.

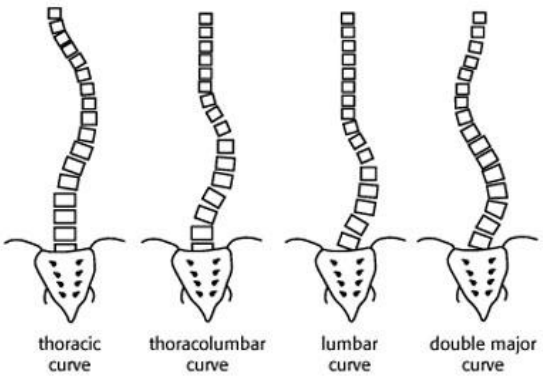

Fig 1-16: Schematic sketch of different types of scoliosis.

1.5.1.2 Spondylolisthesis:

is another abnormality of the vertebral column that one vertebra slips over another vertebra. This type of abnormality happens in children and adolescents. In most cases it happens on the last vertebral level.

Different types of spondylolisthesis include: Dysplastic (congenital), Iisthemic (stress fracture) and degenerative or traumatic.

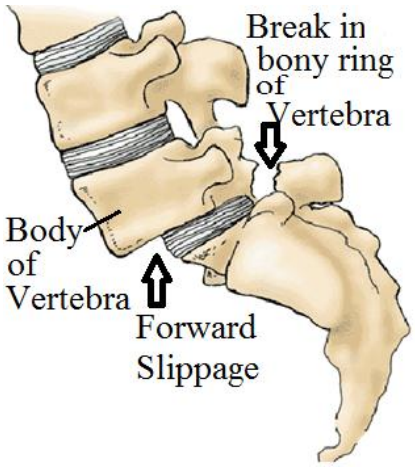

Fig 1-17: Spondylolisthesis at the level of L5/S1.

1.5.1.3 Kyphosis:

is an abnormality that increases posterior thoracic curvature. It is also called hunched back(Sylvia S.Mader, n.d.) - a noticeable round back deformity.

Fig 1-18: Kyphosis or hunched back

1.5.1.4 Lordosis:

is an abnormal increase of the lumbar curvature. It can lead to sway-back appearance (Kent Van De Graaff, n.d.; Gregory D and Darby, n.d.). Females are suitable candidates for lumbar lordosis compared to males. This abnormality is visible during pregnancy.

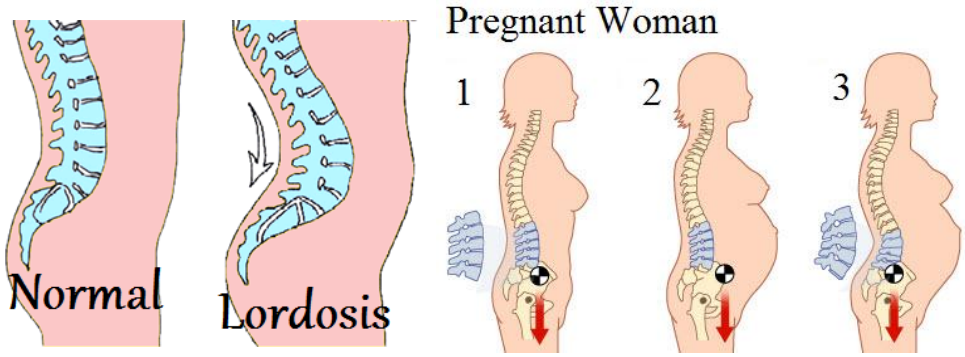

Fig 1-19: Schematic sketch shows that center of gravity moves forward as pregnancy progresses

1.6 Degenerative Disc Disease (DDD):

Whenever there is an imbalance between synthesis and catabolism in the nucleus pulposus, intervertebral disc leads to the degeneration pathway. Increase of catabolism or decrease of anabolism can enhance the degradation of proteoglycans.

In the degradation pathway, long chains of proteoglycans become shorter. As a result, spongy nucleus pulposus looses negative anionic charges and water content which was already trapped in the NP that migrates to the surrounding tissue. So disc looses its height and cell senescence occurs because of impaired nutrient supply (Choi 2009).

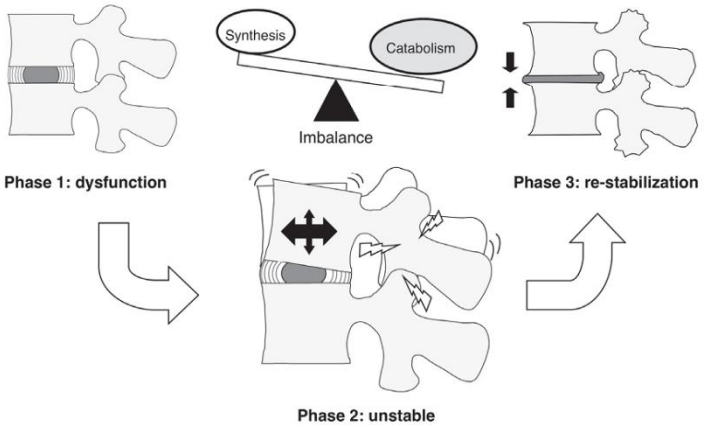

Fig 1-20: Schematic sketch of degeneration of intervertebral disc. Loss of water content by increase of catabolism (Phase 1), movement and load on damaged disc (Phase 2), failure or reconstruction of IVD (Phase 3).

Nutrient exchange and saving height of intervertebral disc is depending on two major natural phenomenon. One is diffusion of the nutrient material from capillaries in the outer annulus fibrosis and the other one is osmotic pressure of the healthy disc. Nutrient exchange occurs by diffusion which is very essential for avascular tissues like NP.

Cells at the center of NP are 8 mm away from the closest capillary. Annulus fibrosus works like a semipermeable membrane. While disc is in the

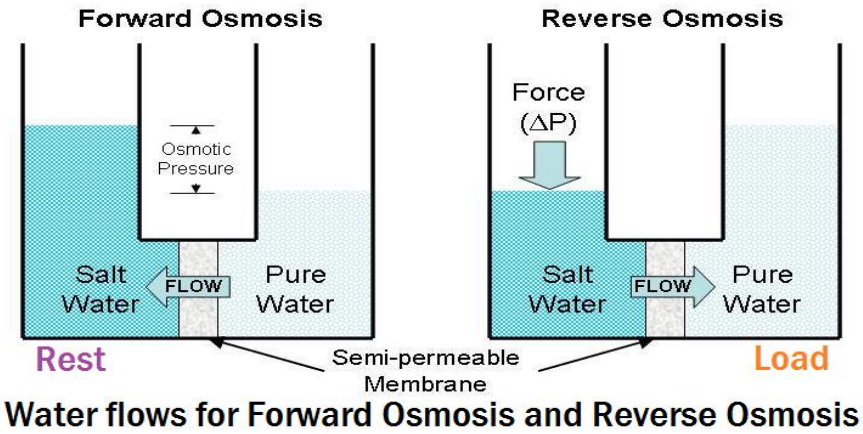

Fig 1-21: Schematic sketch of the role of annulus fibrosus during compression and decompression as Semi-permeable membrane.

rest condition it absorbs water from the surrounding tissue. When intervertebral disc goes under pressure; water content leaks to the outside of IVD (Acosta, Lotz, and Ames 2005).

Disc Degeneration Disease (DDD) is caused by different factors: Genetic inheritance, impaired metabolite transport, altered levels of enzyme activity, cell senescence and death, changes in matrix macromolecules, changes in water content and structural failure. Disc Degeneration Diseases (DDD) usually occurs at the lumbar region due to huge pressure in these discs.

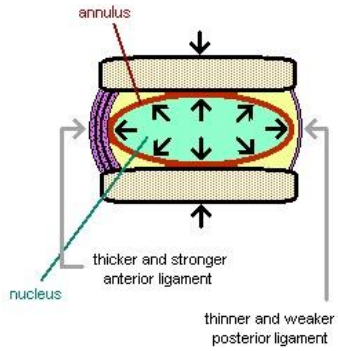

Fig 1-22: Schematic sketch shows distribution of applied forces for on the nucleus pulposus.

Compressive test shows that at the young age tolerance of weight is high compared to middle age. Degeneration happens during course of time and the degenerated disc tolerance of compressive stress is very low (Kotaro et al., n.d.).

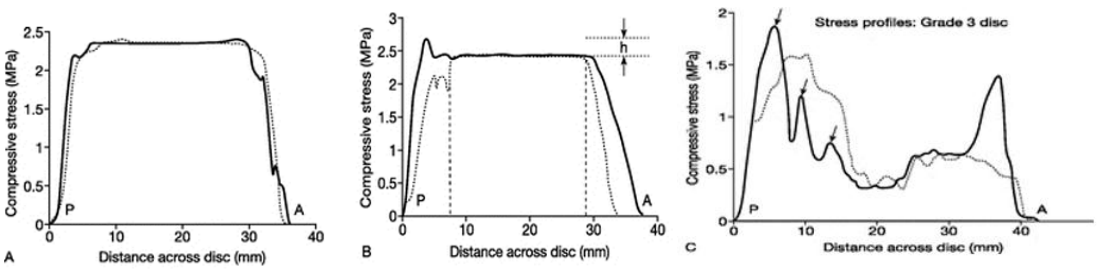

Fig 1-23: Graphical representation of the degeneration of IVD with course of time. Grade 1: young disc (A), Grade 2: Middle aged and Grade 3: Degenerated.

Degradation of collagen and proteoglycans is the first step of disc degeneration. Collagen fibers provide the framework to keep proteoglycan monomers as a 3 dimensional network. Collagen increases from center of nucleus pulposus towards annulus fibrosus.

Swelling pressure depend on ionic concentration in the disc. Space between proteoglycans in a healthy disc is around 0.003 to 0.004 μm. The Fluid Flow through tiny pores is very slow.

While proteolytic enzymes like matrix metalloproteinase (MMPs) are activated by different factors; MMPs starts degradation of proteoglycans. NP framework loosens and space increases between the network and ions like Na^{+}, Ca^{2+} goes out from NP. As a result Na^{+} and Ca^{2+} ions are lost and water content in NP decreases and start to collapse (Choi 2009).

Increase of cell death and cell senescence occurs by intrinsic and extrinsic factors. Cell proliferation is visualized in the degenerative disc by production of cell clusters around nucleus pulposus tear and clefts.

Number of cell clusters increase during course of time. Cell clusters are enhanced by osteogenic protein-1, transforming growth factor- β1 (TGF-β1) and bone morphogenic protein -2(Adams, Stefanakis, and Dolan 2010).

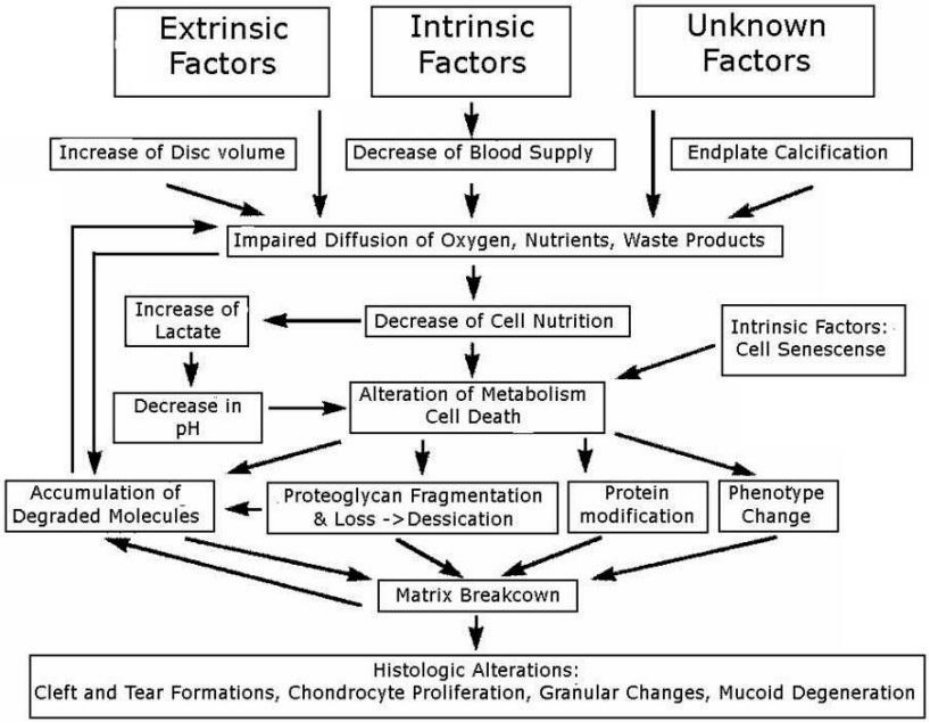

Fig 1-24: A complete chart of Matrix remodeling and disc degeneration.

Cell senescence increases while normal cell division stops. It is associated with β-galactosidase in NP clustered cells in herniated disc. Proliferation of degenerated NP is lower than non-degenerated. There are two mechanisms of cellular senescence. One is replicative senescence which works by shortening telomerase and cells undergo repeated cell divisions. The other mechanism is stress-induced premature senescence that is a result of various stresses caused by mechanical load or cytokines such as interleukin-1(Adams, Stefanakis, and Dolan 2010).

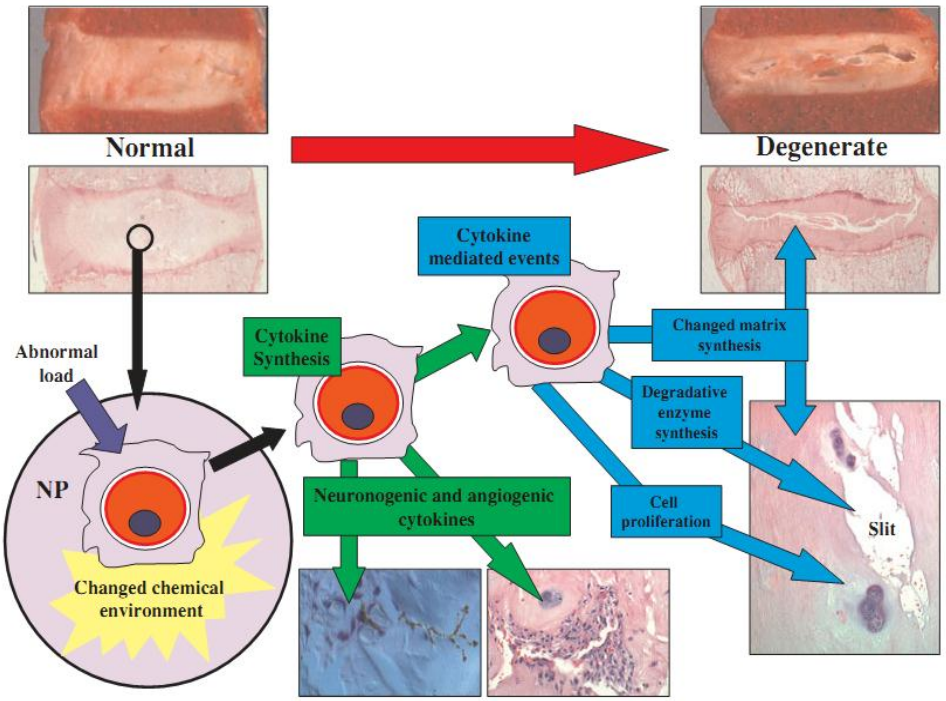

Fig 1-25: A comparison of healthy disc with degenerated disc and schematic sketch of the degenerative pathway.

1.6.1 Herniated Disc:

Any tear of nucleus pulposus due to weakness of surrounding wall toward nerve root provides disc rupture or herniation. There are different types of bulging in the annulus fibrosis area.

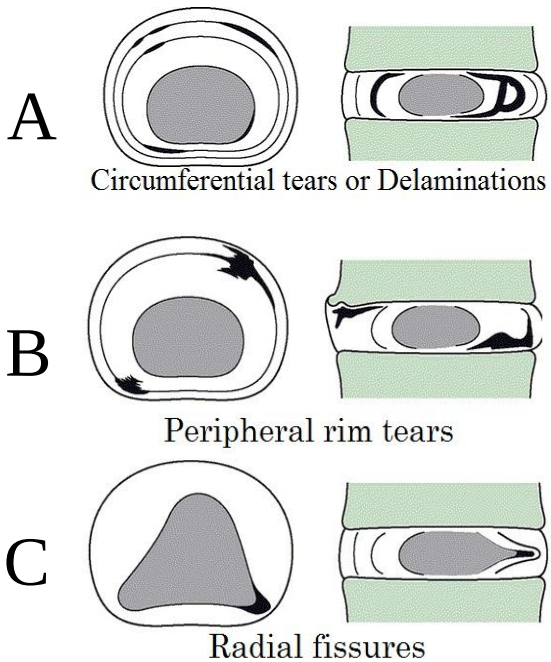

Fig 1-26: Different types of NP bulging

In the first condition circumferential tears or Delamination present in the anterior and posterior

side of IVD occurs at interlaminar shear stresses. The second type of disc herniation appears with bony projection out wards which is called peripheral rim tears. Radial fissures appears with bigger bulge of nucleus pulposus towards the posterior side and again with repetitive loading and bending (Kent Van De Graaff, n.d.).

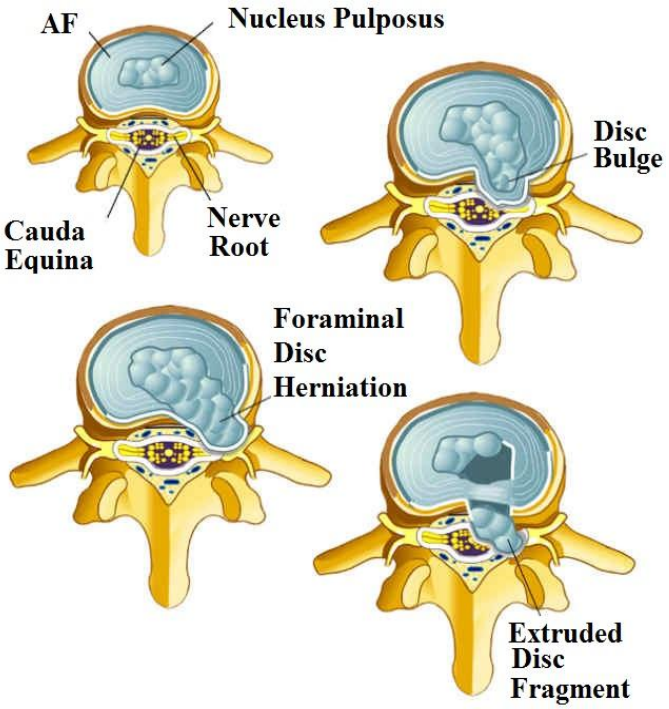

Fig 1-27: Disc prolapse or disc herniation.

Progress of radial fissures bulge leads to disc herniation or disc prolapse. Continuation of disc prolapse leads to press the sciatic nerve and may heal naturally by total rest and by avoiding activities, if damage is not grade four (Adams, Stefanakis, and Dolan 2010).

1.6.2 Current therapeutic option for degenerative disc disease - DDD

Almost 80 percent of people experience low back pain (LBP) throughout their life. Most people of this group recover without any formal treatment. Those people who could recover spontaneously, may need to undergo non-operative management and in severe cases go through surgical management (Nesti et al. 2008).

1.6.2.1 Non-operative management:

In non-surgical management the most important suggestion is total Bed rest for maximum of 2 days. Generally this time should be enough for patient to recover. This suggestion can be combined with anti-inflammatory

Fig 1-28: Low Back Pain (LBP) Exercise.

medication, analgesia and physical therapy (Singer, n.d.). A physical therapy benefits patient for more mobility so at the end it is helping to recover faster.

Ozone chemonucleolysis is another type of non-operative therapy which gained much interest in Europe developed by C.verga in 1983. This method involves applying of 40 to 60 ml of ozone gas (O_2-O_3 combination) by concentration of 30 mg/ml to be applied to paravenrtral musculature and herniation area (Raj 2008).

1.6.2.2 Surgical management

Surgical intervention like total disc replacement and spinal fusion are the most successful treatments (Oehme et al. 2015). But all these treatments have its own advantages and disadvantages.

1.6.2.2.1 Spinal fusion:

It occurs when disc is ruptured (spondylolisthesis). In most cases with curvature of spine which is deformed or fractured, spinal fusion should be performed. Spinal fusion has many limitations like the fast damage of the adjacent intervertebral disc also damage very

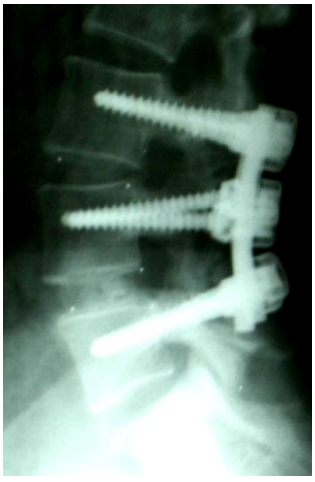

Fig 1-29: Spinal fusion of vertebrae.

fast due to restricted activity of fused vertebrae (Oehme et al. 2015). So bending on the lumbar side provides more pressure to the other IVDs and as a result they undergo fast degeneration.

1.6.2.2.2 Total disc Arthroplasty or Total Disc replacement:

Total disc replacement by artificial disc improves function and reduces pain. Arthroplasty showed significant pain relief compared to other methods. Multi-level arthroplasty is better than multilevel spine fusion methods where discs are able to shift and move and has complete access to L4/L5 and L5/S1.

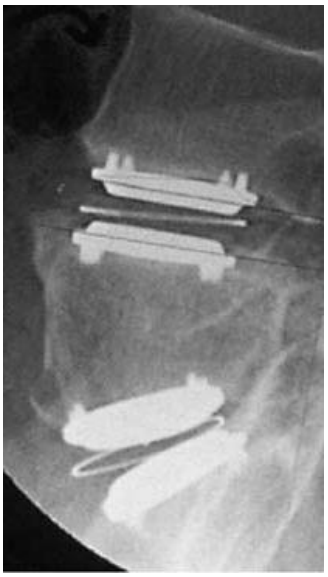

Fig 1-30: Total disc replacement.

1.6.2.2.3 Nucleus pulposus replacement or partial disc replacement (PDR):

It is another type of arthroplasty which removes a portion of the disc especially nucleus pulposus and can be done in early stages of degeneration of the intervertebral disc. Advantage of PDR method is that it is less invasive and compared to other surgical procedures there are multiple ways to approach the target including lateral, posterior and anterior retroperitoneal.

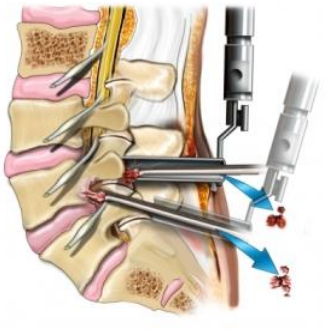

Fig 1-31: Partial disc replacement (PDR) and their approaches.

The most important problem regarding PDR is their migration and expulsion of device because they are not fixed to end plates (Coric and Mummaneni 2008).

1.6.3 Cellular and molecular Therapy:

Most of the current treatments are focused on releasing patient pain rather than solving the main problem**.** Non-operative management like bed rest, analgesia, and usage of relaxant or applying corticosteroids has aimed to reduce pain. Even surgical methods like discectomy (total or partialor partial) or immobilization of intervertebral discs generally gives the same results.

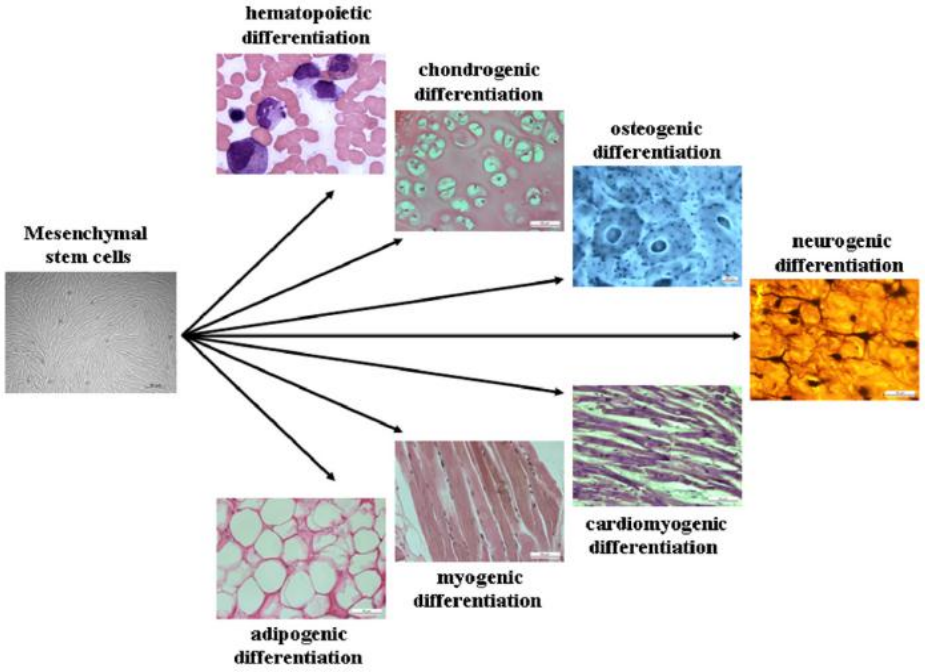

Fig 1-32: Cell based Therapy. Mesenchymal stem cells are able to convert to different cell lineages under proper conditions.

In cellular therapy, there are different ways of using disc cells, cartilaginous chondrocytes or progenitor cells but have limitations for their usage in the clinical aspect like poor expansion and loss of phenotype. The aim of this type of therapy is to repair disc degeneration at the cellular level and increase the extra cellular matrix(Gou et al. 2014). Direct application of growth factors and cytokines to intervertebral disc is not appreciable due to their short-life (Oehme et al. 2015).

At the molecular level of disc degeneration there are different factors interfering in the healing process. Decrease of nutrient and waste exchange with the surrounding tissue results in diminution of cell viability. By reduction of cell number and acceleration of apoptotic process, there will be accumulation of debris in the NP which enhances activation of degradative enzymes to thereby affect proteoglycan synthesis.

Inflammatory mediators in degenerative disc are Nitric oxide (NO), interleukin-6 (IL-6), prostaglandin E2 (PGE2), TNF-alpha, fibronectin, matrix metalloproteinases (MMPs). There are multiple strategies for molecular therapy in degenerative disc diseases. **The first strategy** is anti-catabolics which stop matrix loss by preventing degradative enzymes in the NP. TIMP-1, 2, 3, Anti-TNF-alpha and Anti-MMPs (CPA-926) are example of anti-catabolics mediators. **The second strategy** are mitogens which defined by their potency is to raise the rate

of mitosis and can increase the rate of proteoglycan synthesis. Insulin-like growth factor 1 (IGF-1), Platelet-derived growth factor (PDGF), Epidermal growth factor (EGF) and Fibroblast growth factor (FGF) are effective mitogens. **Another strategy** is the use of morphogens. Transforming growth factor (TGF-beta) and bone morphogenetic proteins (BMP-2, 7, 13) act as anabolic regulators, able to raise the phenotype of the chondrocyte cells. **A further strategy** for molecular therapy is enhancing intracellular regulators. They are not cytokines, because they do not secrete any substance or molecule but their main duty is to control cellular differentiation by intracellular activities (Ciccocioppo et al. 2014; Silva-Correia et al. 2013; Yoon and Patel 2006).

Disc herniation is caused by the degeneration process of the intervertebral disc from the second decade of life (Reitmaier et al. 2014). It is understood that there are different factors leading to low back pain - like nutrition supply, inherent genetic anatomical problems, environmental effects and psychosocial agents. Molecular mechanism of disc degeneration is not fully clear. An important factor is that mechanical stress can increase speed of degeneration process in the intervertebral disc (Melrose et al. 2008; Huang et al. 2013). It is also mentioned that overload to IVDs is sensed by the IVD cells which are converted to biological signals and responses (Rannou et al. 2004).

IVD degeneration hastens by loss of NP cellularity with apoptosis that leads to decrease of extra cellular matrix generation and organization. Imbalance of extra cellular matrix composition is due to loss of proteoglycan synthesis and impaired nutrition to NP cells. Pathway of pathogenesis of the disc degeneration is still unclear. Diagnosis and treatment of Low Back Pain (LBP) and disc degenerative disease (DDD) requires researchers to focus from a different point of view as this remains a controversial subject in Medical Science (Kanna, Shetty, and Rajasekaran 2014; Drazin et al. 2012).

1.6.4 Therapy for disc degenerative disease (DDD)/Low Back Pain in Regenerative Medicine

A very promising alternative therapy to DDD/Low Back pain in Regenerative Medicine which is gaining relevance in Medical Science today is the selection of a suitable scaffold in combination with cells/growth factors underlying the principles of Tissue Engineering to promote regeneration and repair of the injured/damaged tissue(Langer and Vacanti 1993). Scaffold is a temporary structural vehicle for the delivery of cells/growth factors to the tissue defect site to support the building up of the ECM which provides the stiffness/strength/flexibility for cells to attach to the surface and mould the regenerated tissue to their final shape. Simultaneously, ECM also affords mechanical stability for the growing functional tissue on par with scaffold degradation to mend the tissue defect(Khademhosseini and Langer 2016).

In this context, the following study was designed proposing a hydrogel scaffold to introduce cells (NP-IVD Tissue Engineering) into the injured NP of the Lapine (Rabbit) NP-IVD defect Model and to evaluate its performance. In perspective of clinical application, this may be an interim relief therapy for Low Back pain where the primary cause is intervertebral disc (IVD*)* degeneration. Current treatment options are limited with $90 billion spent annually on treatment.

CHAPTER 2

REVIEW OF LITERATURE

CHAPTER 2

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Scaffolds can belong to any biomaterial category depending on the intended clinical application. Suitable biomaterials need to be selected to mimic the properties of the intended tissue to be repaired. Scaffolds require a highly open porous structure for cell attachment, proliferation and differentiation. Porous scaffolds have good interconnectivity and adequate mechanical strength and stiffness for cellular in- or outgrowth (Atala and Lanza 2017).

2.1 Biomaterials

There are several types of biomaterials: metals and alloys, ceramics, polymers, composites, and natural biomaterials. Most of these biomaterials are used for medical devices, prosthetics, biosensors, drug delivery and tissue engineering applications. Metals and ceramics are used for hard tissue application while polymers are known mainly for soft tissue applications.

A **Biomaterial** can be defined as a material which likes to interact with biological systems to treat, boost, repair or replace any tissue, organ or function of the body (Burdick and Mauck 2011). Further, biomaterial is any material, natural or man-made, that contains whole or part of a living

structure or biomedical device which performs, augments, repair or replaces a natural function (Tathe, Ghodke, And Pratima Nikalje 2017).

i. Biocompatibility

Biocompatibilityof biomaterials is simply defined as proper *in-vivo* responses to a material without any irritation and reaction from the host tissue to the implant. It affirms the safety and toxicity of the implant which is not making detrimental affect either locally or immune and inflammatory responses. (Tathe, Ghodke, and Pratima Nikalje 2017).

2.2 Intervertebral disc

IVD is composed of two different parts: jelly center known as the nucleus pulposus (NP) and the surrounding lamella layer called annulus fibrosus (AF). The most important component inside the disc is collagen, aggrecan, glycosaminoglycans (GAG) and small amount of collagen type IV, VI, IX and XI. Combination with K^+ and Na^+ ions makes the environment negatively charged and in turn suitable for absorbance of water from the surrounding tissue towards the NP which is responsible for the disc height. The NP/AF structure acts as a cushion or shock absorber for the intervertebral disc and can carry high load with flexibility. This property of IVD decreases with age as well as by disc degeneration process. NP usually decreases its height and thereby the Annulus Fibrosis bulges out impinging on the nerves, ultimately causing pain. When NP cells are not able to provide sufficient Extra Cellular Matrix (ECM), NP looses the water content resulting

in reduction of the disc height and subsequently low back pain. The proposed biomaterial of choice for NP regeneration/repair of IVD in this study is the hydrogel polymer.

2.3 Polymers as scaffolds for soft tissue regeneration/repair

Polymers are the most versatile of biomaterials. Polymer structure depends on the orientation and organization of the monomers and their related chains. Polymers are classified as natural, synthetic or a combination of both. They can be degradable or non-degradable polymers. There are special structural properties regarding polymers. Longer polymer chain have less mobility compare to shorter polymer chain. By increasing monomer to a polymer chain, rigidity and strength is increasing while shorter chains have less rigidity and strength. Both paraffin and polyethylene are constructed with the same monomer (CH_2-CH_2) but paraffin contains shorter monomer chain length compared to polyethylene. Monomers can form polymers in various ways including linear, branched and Cross linked. Linear monomers are the simplest structure of polymers. If linear chains get some side chain it is branched and the melting temperature and crystallization of polymer will come down. In some polymers, side chain gets linked to each other. In this condition it is called cross link network. According to the structure of polymer which contains just one, two or different types of repeated segments, fall in different classifications as homopolymers (polyethylene, polytetrafluoroethylene (Teflon), poly (vinyl chloride) and polypropylene),

heteropolymers or copolymers poly (glycolide lactide), polyurethane, poly (glycolide trimethylene).

2.3.1 Degradation of Polymers

High molecular weight polymers degrade slower than low molecular weight polymers. Degradation of chain branched polymer is faster than linear polymers and high temperature facilitate polymer for faster degradation. A polymer material should have a degradation rate comparable to the rate of the newly formed native tissue.

2.3.2 Hydrogel

Hydrogels are a special type of polymers which can swell without dissolving in the water or other fluids. Hydrogel has a porous structure and allows maximum water in their structure. Percentage of polymer in a hydrogel varies from 10 to 40 but water percentage varies from 60 to 90. They have special characteristic features like high capacity of water content, mimicking tissue mechanical properties and are capable to be polymerized in real physiological conditions (Madihally 2010).

2.3.3 Composites

Mixture of two or several different components is called composite. Each composite contains two major parts; matrix and reinforcing phase. Matrix phase is weaker than reinforcing phase. Their structure can improve by reconstructing and altering their components for different purposes in the biological, chemical,

mechanical, physical and biocompatibility aspects (Burdick and Mauck 2011; Hubbell 1995).

2.3.4 Biological/Natural Materials

Biological materials are isolated from natural resources like marine organisms, insects, birds, and mammals. There are several products derived from natural resources including alginate, chitosan, coral, silk, fibrin, collagen, elastin and hyaluronic acid (Burdick 2017). Major important biomaterials for IVD applications are collagen, chitosan and alginate.

2.3.4.1 Collagen

Collagen is found in the primary structure of connective and mesenchymal tissue. Almost one third of body proteins are from collagen. Collagen is made of repeated Gly-X–Y residues; proline and hydroxyproline are components residing instead of X-Y. Collagen appears in various types in connective tissue including type I, II, III, IV, V, IX, XI, XIII used for several purposes in scaffold designing specially in gels, meshes, composites with different molecules (Atala and Lanza 2017; Shu Q 2017; Burdick and Mauck 2011).

2.3.4.2 Polysaccharides

Polysaccharides are constructed from repeated segments of monosaccharaides. Monosaccharaides by glycosidic bond attach to each other. The most well-known polysaccharides are cellulose which is made of D-glucose monomers. Cellulose is a linear polysaccharide which is found in

the plant cell walls. Human cannot digest cellulose because of lack of β-Glycosidase.

2.3.4.3 Chitosan

It is another natural polymer with β- 1,4 glycosidic bond between D-glucoseamine monomers by random arrangement of N- acetylglucosamine. Mostly they found in the shell of shrimp and crabs. It is soluble in acidic solution (pH5) and incapable of being dissolved in water. Chitosan is used as a scaffold for gene transfer, drug delivery carrier, cell seeding and transplantation,

2.3.4.4 Alginate

It is another linear polysaccharide which is found in seaweed and bacteria. Alginate is made of two types of monosaccharide (Mannuronic acid, Guluronic acid) and very suitable for different types of hydrogel matrix. Various compositions of alginate give different stiffness and strength to the hydrogel and used for cell encapsulation, seeding, transplantation, drug delivery, gene delivery, wound healing, cartilage, intervertebral disc, liver and bone regeneration. Compared to chitosan which has positive charge, alginate and glycosaminoglycans have negative charges (Shu Q 2017; Burdick 2017; Marijnissen et al. 2002).

2.4 Nucleus Pulposus Tissue Engineering

Tissue engineering evolved from the field of biomaterials development. It involves the practice of combining scaffolds, cells, and biologically active molecules into functional *tissues* to improve or replace

biological tissues (Berthiaume, Maguire, and Yarmush 2011). Tissue engineering provides a promising approach to recover the functionality of the degenerative intervertebral disc. Most studies are directed toward *nucleus pulposus* (NP) *tissue engineering* because disc degeneration is believed to originate in NP region(Boxberger et al. 2008). Emphasis is laid on nucleus pulposus scaffold substitutes preformed scaffolds, injectable hydrogels etc. that mimics the structure and composition of NP of the disc(Leone et al. 2008).

The following table highlights the various polymer scaffolds that have been used over the past two decades for NP-IVD tissue engineering approaches by several researchers for IVD repair in different animal models.

Cell type	Animal Model	Scaffold	Method	Outcome	Author/ year
NP And **AF cells**	**Rabbit**	**Alginate beads**	1-Cells were seeded within alginate beads at $2*10^6$ cells/ml. 2- Cells were seeded for 14 days in F-12 culture medium	1- Both AF and NP cells kept their natural phenotypes in the scaffold. 2-Histological analysis revealed that AF cells produced extra cellular matrix than 3- NP cells appear as cell cluster and production of extra cellular matrix was less.	(Chiba et al. 1997)
NP cells	**Sheep** lumbar IVDs	**Collagen Type II**	1-Cells were seeded within the scaffolds at $3.3*10^6$ cells/cm^2 2-Cell seeded constructs were cultured in vitro in DMEM supplemented with 10% FBS, which after five days was increased to 20%.	1-DNA content had decreased after four weeks. 2-Cell density was comparable to native NP tissue. 3-NP cells maintained their phenotype, forming a layer of tissue comprising PGs and type II collagen with comparable concentrations to native NP tissue. By week 12 there were signs of deterioration in the in vitro formed tissue.	(Yu 2002)
Autologous MSCs	**Rabbits**	**0.3% type II atello-collagen**	1-MSCs that were infected with Ad-lacZ expressing E. coli lacZ gene, 2-Cells seeded per scaffold: $1*10^6$ cells/ml. 3-cell-seeded scaffolds injected into rabbit NP.	1-The cells survived post-implantation and differentiated into spindle-shaped 2- Cells were synthesized significant amounts of proteoglycans. 3- Histology of eight weeks study revealed that MSCs seeded scaffold reduced speed of degeneration compare to control group.	(Sakai et al. 2003)

MSCs	**Rabbit**	**Atelocollagen gel**	Location of injection is L3/L4-L4/L5 Region Cell count for injection: $2*10^4$	1-MSCs differentiated in to nucleus pulposus cells. 2- there were significant increase of PG content	(Sakai et al. 2005)
IVD cells L3/L4	**Rabbits**	**Nil**	Location: L1-L2, to L4-L5 lumbar intervertebral disc (IVD). Puncturing by 16-gauge needle. Using 1: Digitized radiographs, measurements of IVD for dick height and comparing with Disc Height Index (DHI). 2:Magnetic Resonance Imaging (MRI) Scan of injured discs were compared with Post Surgery MRI	1-Disc degeneration happened in all rabbits by annular puncture. 2-DHIs of injured discs were lower than the control.	(Kong et al. 2008)
Autologous MSCs	**Rabbits**	**Nil**	1-AF puncture; 2-Degeneration time= 1 month. 3- Cell No: $1x10^5$ MSCs. 4- A histological degeneration grading were used (Grade 0 = normal Grade 5 = severe).	1-MSCs could be detected in all IVDs post-surgery. 2- There were no differences in DHI between the sham discs, the medium-injected group 3- MSCs injection has a more effect on severely degenerated discs.	(Ho et al. 2008)
AF and NP Cells	**Ovine model**	**Alginate bead**	1-Ovine AF and NP cells were cultured in monolayer and alginate bead culture in the absence and presence of HA oligos. 2- RNA extracted for RT-PCR to determine relative mRNA expression levels for MMP-2, 9; aggrecan and type I and type II collagen on days 2, 5 and 10 in bead culture.	1-poorly responses of AF cells to HA oligos (0.05-1 g/ml) in monolayer culture + proMMP-2 levels 2-In alginate bead expression of Type I and II collagen were there. 3- Differentially up-regulation and down – regulation by HA oligos. 4- The HA oligos could not ameliorate the healing response	(Melrose et al. 2008)

Human MSCs	**Porcrine**	**Puramatrix hydrogel**	1-Location of injection is L3/L4-L4/L5 Region 2-Cell count for injection: $5*10^5$ 3- Transplantation of xenogenic model	Mesenchymal stem cell differentiated into NP like cells	(Henriksson et al. 2009)
MSCs	**Rabbit**	**Pure fibrinous gel**	1-Location of injection is L3/L4 to L4/L5 Region 2-Cell count for injection: $2*10^6$	Disc height index decreased	(H et al. 2010)
MSCs	**Porcine**	Hydrogel	1-Location of injection is L2/L3 to L5/L6 Region 2-Cell count for injection: $1.25*10^5$	NP regenerated and DHI increased. Function of end-plate was normal	Bendtsen et al (2009)
BM , MSCs, CESCs, AFSCs and NPSCs	**Rabbit**	**Alginate**	Bone Marrow (BM) mesenchymal stem cells (MSCs) Cartilage Endplate-Derived Stem Cells (CESCs) Annulus Fibrosus-Derived Stem Cells (AFSCs) Nucleus Pulposus-Derived Stem Cells (NPSCs) tested with Histology, X-Ray and MRI	1-Cartilage endplate-derived stem cells (CESCs) revealed better regeneration of NP. 2- nucleus pulposus-derived stem cells (NPSCs) and Bone Marrow (BM) mesenchymal stem cells (MSCs) appeared similar result but less than CESCs seeded alginate.	(Wang et al. 2013)
ADMSCs	**Rabbit**	**Type II collagen and Hyaluronan (HA)**	1-Cells were Isolated from rabbit adipose tissue. 2-ADMSCs are Embedded in different concentration of Type II collagen/Hyaluronan hydrogel .	1-Cells were round shape in higher concentration of collagen/HA microgels 2-microgells with higher concentration of COLII/HA are stable 3-ADMSCs appear high level of collagen II, SOX9and aggrecan	(Fontana et al. 2014)
Nil	**Rat**	IVD puncture	1-Rat lumbar region of the L4/L5 and L5/L6 punctured by 27 gauge needle. 2-Determination of rat	1-expression level of COL II and Sox9 mRNA decreases 2-Increasing of COL I	(Li et al. 2014)

			lumbar region asses by MRI, Histological examination and RT-PCR	3-MRI and histological results revealed high pathological process.	
Nil	**Ovine**	**Nucleotomy**	Identify of the most important compartment of motion segment in ovine vertebral column	1-Increased stability after surgery. 2-removing of motion segment increased flexibility	(Reitmaier et al. 2014)
Human NP Cells	**Nil**	**Type II Collagen – Hyaloronic Acid(HA) with 1-ethyl-3(3-dimethyl aminopropyl carbodiimide (EDC)**	1-Cell was isolated from patient IVD tissue by discectomy surgery. 2-Cell Viability assay, Alamar Blue assay and Infrared spectroscopy was done	1-Cell viability test revealed HNP cells are capable to proliferate in the hydrogel 2-morpholy study revealed more cell viability within non cross-linked gel compare to crosslink gel. 3-quantitative PCR demonstrated high phenotypic NP cells within hydrogel.	(Priyadarshani et al. 2016)
NP Cells	**Mouse**	**Perfluorotributylamine (PFTBA) with alginate**	1-Hypoxic condition in IVD diminishes cell growth. 2-Perfluorotributylamine (PFTBA) is as oxygen regulator in alginate scaffold. 3-Tests followed by cell viability and count, Realtime PCR,western blotting, and biochemical evaluation of GAG/DNA	1- PFTBA could increase NP proliferation 2-regulation of ECM to disc like tissue by using 2.5% of PFTBA. 3-Alginate with 2.5% of PFTBA could bring back disc hight.	(Sun et al. 2016)
ADMSCs	**Rat**	**Type II collagen-N,N-(3-dimethylaminopropyl)-N0-ethyl carbodiimide and N-hydroxysuccinimide (EDAC/NHS)**	1-Physical properties like porosity, Biodegradation, cytotoxicity and cell proliferation has been tested.	1-increase of ADMSCs differentiation to NP-Like cells. 2-higher gene expression of COL2 and SOX9. 3-More stability of scaffold.	(Zhou et al. 2016)

Hence different types of natural polymers like Polycaprolactone (PCL), Gelatin/chondroitin-6-sulfate/hyaluronan, Agarose/collagen, Collagen collagen/Glycosaminoglycan, Polyglycolic acid/alginate have been used for repairing the intervertebral disc in different animal models like – Rat, Rabbit, Pig but all of them are far from clinical usage. The major clinical problem of tissue-engineered disc is the insufficient biomechanical behavior and stiffness for long term usage. The attraction of fabricating tissue-engineered disc is by using either the cultured disc cells or by converting stem cells into cartilage cells in combination with the scaffold. This appears to be a better option for NP tissue engineering.

2.5 Alginate as a Scaffold

Alginate is an important family of hydrophilic unbranched polysaccharide. Efficiency of alginate for preparing hydrogel with different compositions and mechanical properties made this polysaccharide a suitable natural biomaterial for cell encapsulation to rein the release of macro molecules, proteins and nucleic acid (Hunt et al. 2014). Different modified hydrogels have been used for IVD Tissue engineering purposes like calcium crosslinked alginate to regenerate NP structure. But the loose structure and infrastructure of the hydrogel could not meet the main purpose. Impaired cross-linking between hydrogel ions, made the hydrogel less stiff and porous (Pawar and Edgar 2012; George and Abraham 2006).

Ionic cross-linking happens in soluble polymer with opposite charges to make divalent ion. Divalent cations interact with anionic monomers in the cross-linked framework. Control of monosaccharide segments is a challenge in polysaccharide chemistry. Ionic bonds are stronger than cross-linked hydrogel as they bear more pressure and mimic NP structure (Chou and Nicoll 2009; Hunt et al. 2014; "Hydrogels in Regenerative Medicine" 2017).

2.6 Role of Strontium

Earth crust contains 0.02–0.03% of strontium (Sr). It is available in the combination of soil, water, plants and animals. Measure of Sr in soil varies from 0.001 to 39mg/l. In the normal diet of drinking water there is 2-4 mg of Sr per day. Sr is mostly available in vegetables (Bruyere et al. 2008). Sr stimulates growth of plants and it can replace Ca. According to the soil ingredients, plants receive the same amount of Sr/Ca.

Less attention has been paid to Sr while other divalent metals like Ca and Mg play a main role in the physiology of the body. Sr is not available as free material and it is always in cooperated with other ions. Sr is an oxidative material and it appears as strontium oxide. Divalent cations like Ca and Mg are in the biological system and they have different roles in serum and cell walls. Sr contains multiple roles in the human body. Sr isotopes are used in physiological diagnostics and it has radiopacity properties for tracing materials. Strontium augments with bone for giving more stiffness and reduce fraction of bone in osteoporotic bone. Sr and Ca have similar behavior in body and both of them give strength to the bone. Measure of Sr is less

compared to Ca in the bone. There were no reports regarding Sr over dosage toxicity (Suguna and Sekar 2011). In muscles Sr can block the intracellular secretion of Ca. When there is loss of Ca; Sr can replace though Ca channel. Sr can pass through capillaries to reach to extra cellular matrix. Sr can be used as a tracer of Ca.

2.7 Alginate strontium (Alg/Sr) as Scaffold of choice in this study

From review of literature, Strontium-Alginate was chosen as the scaffold in combination with mesenchyme stem cells for NP repair in rabbit IVD model (Suguna and Sekar 2011). Alginate is a natural biomaterial which can mimic the structure of nucleus pulposus of intervertebral disc. It can easily encapsulate cells and provide a hydrogel matrix for NP regeneration. It can tolerate different compressive pressures according to different compositions of alginate; compressive forces vary from 1 to 1000 Kpa (Drury, Dennis, and Mooney 2004). Furthermore, Strontium has radiopaque properties with easy bonding with alginate solution. It provides less calcification on the cartilage compared to Ca. Strontium displays antiosteoporotic effect and gives more stiffness and strength to the hydrogel (Pors Nielsen 2004).

Symptomatic intervertebral disc degeneration is associated with several spinal diseases, which cause losses of life quality and money. Besides conservative and surgical treatments (removal of disc and vertebra fusion), cell therapy foresees the possibility of regenerating the damaged NP where cells

can proliferate *in vitro* and re-implanted to alleviate the pain and further prolong the damage to sustain quality of life. Hence, the prospect of cell-based Nucleus Pulposus (NP) tissue-engineering strategy has become attractive and relevant in Regenerative Medicine.

2.8 Hypothesis

Would Alginate-strontium hydrogel with cells *in situ* assist the repair of injured Nucleus Pulposus (NP) of the Intervertebral Disc?

2.9 Objectives:

A. To study the anatomy of the IVD of rat, rabbit, goat, cattle and pig.
B. To study the mechanical properties of the IVD rat, rabbit, goat, cattle and pig.
C. To prepare Alginate/Strontium (Alg/Sr) substitute for NP.
D. To isolate, expand and characterize Rabbit Adipose Mesenchymal Stem Cells followed by their differentiation potential towards the cartilage lineage.
E. To fabricate and characterize Alg-Sr/cell construct *in vitro* using microscopic techniques.
F. To create and develop Lapine (Rabbit) IVD model by NP physical injury.
G. To study the response of Alg-Sr/cell combination product in the injured NP of New Zealand White Rabbits (Lapine model) - *in vivo.*
H. Examining the final data and its application in real clinical condition.

CHAPTER 3

MATERIALS & METHODS

CHAPTER 3

MATERIALS AND METHODS

3.1 Anatomy of the nucleus pulposus and mechanical properties of the intervertebral disc of the lumbar vertebrae

For the selection of the desired animal model and IVD of choice for the study, the anatomy of the different intervertebral disc and its architecture in the lumbar region of small and large animals need to be understood. Each animal has different number of intervertebral discs with respective strength in the lumbar region.

3.1.1 To study the anatomy of the intervertebral disc (IVD) of the lumbar vertebrae of rat, rabbit, goat, cattle and pig

The lumbar vertebrae of the vertebral column of different euthanized/slaughtered small to large animals (rat, rabbit, goat, cattle and pig) were collected in 10% buffered formalin. Subsequently, the Intervertebral discs (IVD) was separated and sectioned to reveal the structure, anatomy, area and size. The gross appearance of each disc of each animal was photographed.

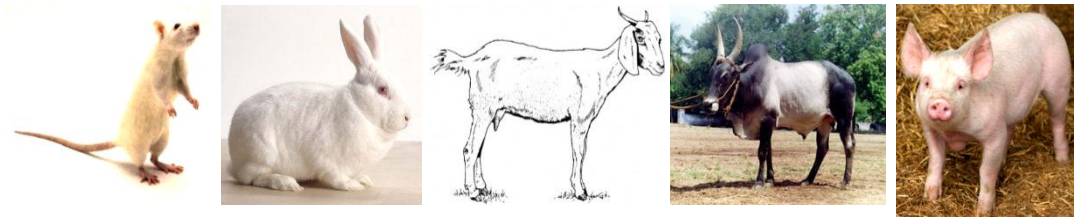


Fig 3- 1: Different animals - rat, rabbit, goat, cattle and pig

3.1.2 Histology of Nucleus Pulposus (NP) of the IVD of small to large animals

The NP of the lumbar IVD disc of every animal was fixed in 10 % buffered formalin, dehydrated in ascending series of alcohol and infiltrated in paraffin wax in a Tissue Processor (Leica TP 102Q) and embedded in paraffin (Tissue Embedder MSP/P1; Cooling Plate MPS/C). Thereafter paraffin blocks were sectioned using stainless steel knives in a Microtome (Microtome Leica RM 2255), stained with H&E, viewed and photographed using the Light Microscope (Leica DM 6000).

3.1.3 The Ultrastructure of the NP of the IVD of small to large animals

The NP of the lumbar IVD disc of animals was fixed in 3 % buffered glutaraldehyde, post fixed in 1 % Osmium tetroxide, dehydrated in ascending series of alcohol, infiltrated and embedded in polyester resin. Thereafter the resin blocks were sectioned using a diamond knife (Diatome) in an Ultra Microtome (Leica UCT Ultracut). Sections taken up on copper grids were stained with Uranyl Acetate and Lead Citrate, viewed and photographed using Transmission Electron Microscope H 7650 (Hitachi) at an accelerating voltage of 80 kV.

3.1.4 To study the mechanical properties of the IVD

3.1.4.1 Compression Study

Rabbit lumbar region was cut by a hack saw to separate into individual intervertebral discs from the vertebral column.

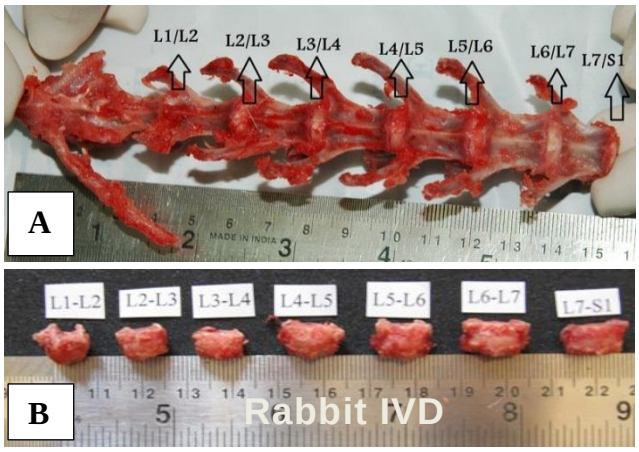


Fig 3- 2: Individual rabbit intervertebral discs

Each intervertebral disc is supported by end plate and surrounding bone tissue. For further study on rabbit IVD, its tolerance has been measured by INSTRON machine. For small and big animals, different types of acrylic molds have been fabricated to facilitate the compression testing.

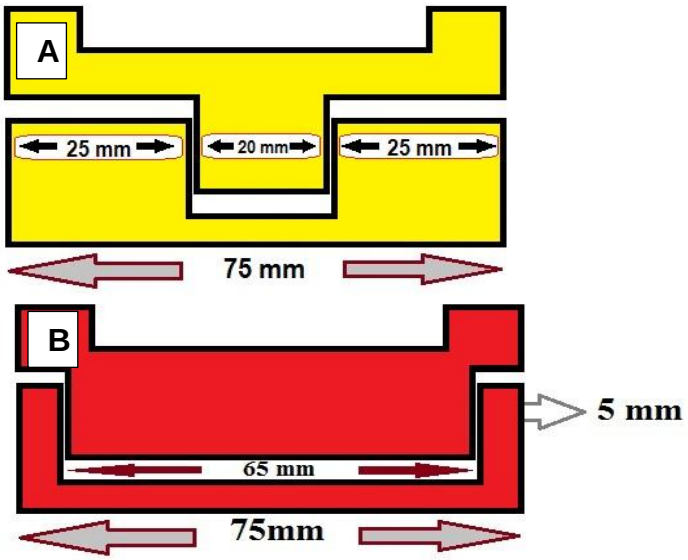


Fig 3- 3: Schematic sketch - designs for acrylic molds for small and big animals for compression testing in the INSTRON machine (A, B).

Two types of acrylic molds have been designed by CNC equipment for small and big animals to fit into the cross head of the INSTRON machine. Cross head speed for compression study was 10 mm/min.

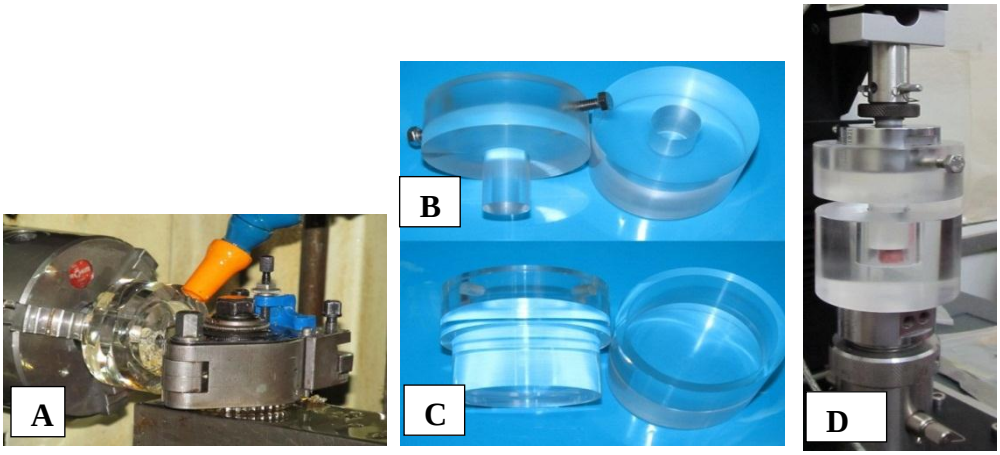


Fig 3- 4: Fabrication of acrylic mold (A), Acrylic mold for small and big animals (B, C), Installation of acrylic mold in the INSTRON machine (D).

3.1.4.2 Tensile Strength Study

Tensile Strength for Rat, Rabbit, Goat, Cattle, and Pig was performed. Each vertebra was drilled to pass the stainless steel suture for tensile strength test. Crosshead speed for Tensile Test was adjusted on 1 mm/min.

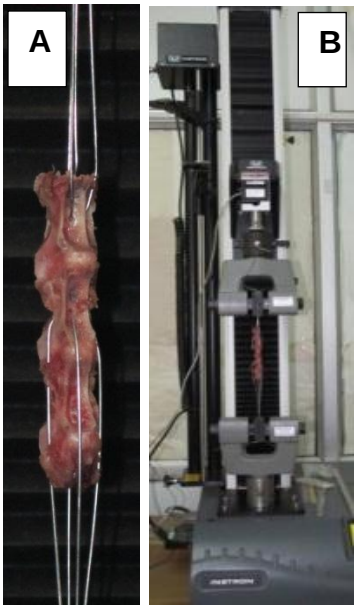


Fig 3- 5: Drilled vertebra bone with stainless steel suture inside ready for tensile test of rabbit IVD (A), INSTRON machine to perform tensile test (B)

3.2 Material

3.2.1 Preparation of porous Alginate/Strontium (Alg/Sr) hydrogel substitute for NP.

Alginate, a natural polymer has been selected as a good candidate scaffold for NP regeneration. Combination of alginate with Strontium chloride ($SrCl_2$) gives a porous hydrogel which can accommodate Mesenchymal stem cells or chondrocyte cells. As the NP structure shows 75% of water content, this type of hydrogel was developed in laboratory by dropping alginate into 1.8% of $SrCl_2$ which showed

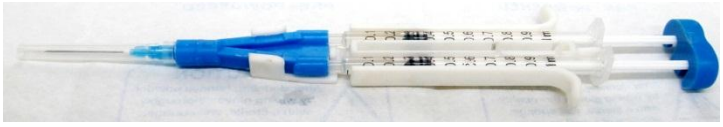


Fig 3- 6: An Applicator (*in-house (SCTIMST) developed fibrin sealant applicator*).

74.28% of porosity mimicking the extra cellular matrix. The hydrogel was fabricated using a dual applicator (in-house (SCTIMST) developed fibrin sealant applicator) as shown in fig 3-6. All solutions such as alginate solution and strontium chloride for the fabrication of Alg/Sr hydrogel were previously prepared using sterile filters. A dual applicator was used to carry the two solutions separately to ultimately combine them in a single outlet channel into Alg/Sr hydrogel as the final product.

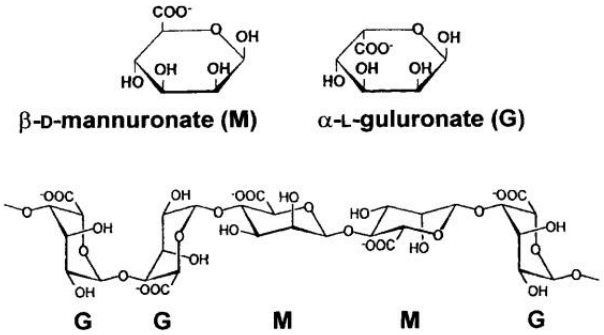


Fig 3- 7: Schematic structure of alginate monomers.

Different concentrations of alginate and strontium tested with several dilutions were tested to obtain the desired strength and size of the desired hydrogel.

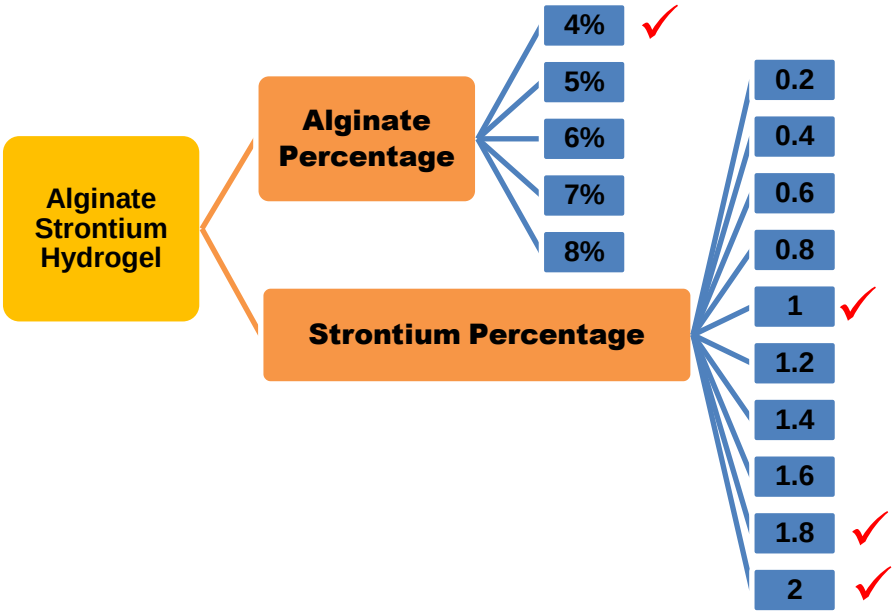


Table 3-1 Flow chart of different concentrations of alginate and strontium solutions to develop the desired alginate strontium hydrogel.

Alg/Sr Hydrogel was prepared through 2 methods - Alg/Sr Beads as well as Alg/Sr Gel for *in vitro* cytotoxicity test and 3D cell culture test.

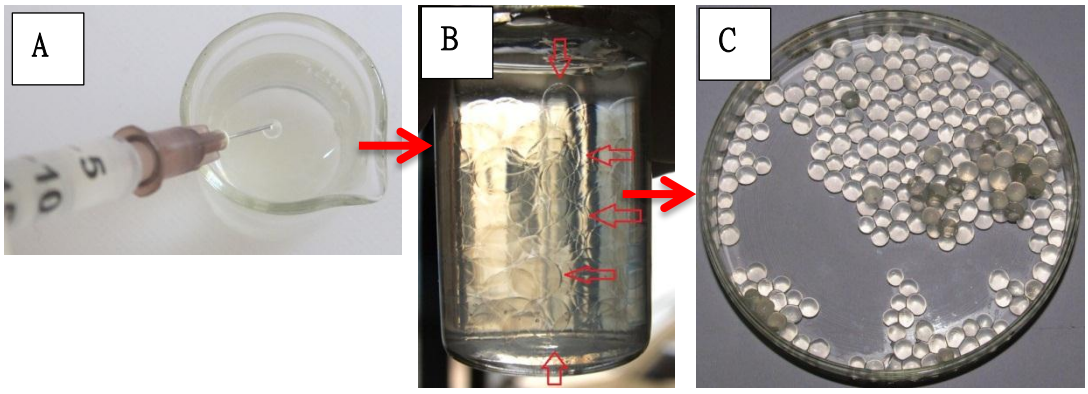


Fig 3-8: Dropping of alginate solution into Strontium Chloride solution (A), Storage of Alginates-Strontium beads (B), visible Alg/Sr Beads in petri dish (C).

Alg/Sr hydrogel has been prepared in 96 wells plate for easy handling and visualization.

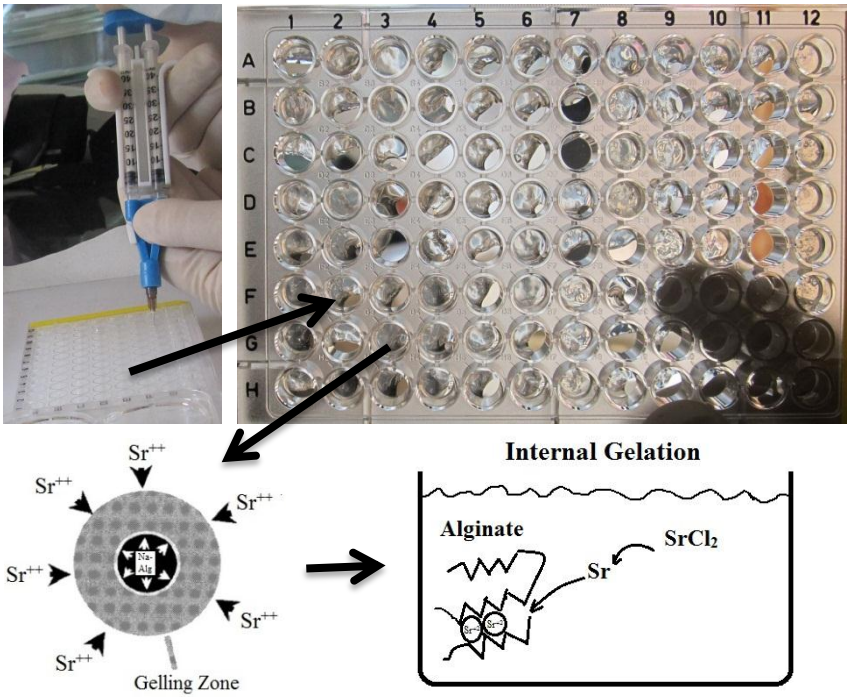


Fig 3-9: Material synthesis of alginate-strontium hydrogel

3.2.2 Mechanical Strength of Alg-Sr

Compression was measured using the INSTRON machine, each disc was separated and detach from vertebral bone. Each IVD were kept in the acrylic mold for compression test. INSTRON machine was setup for speed 0.5 mm per minutes. By this speed resistance of Alg/Sr hydrogel could be track.

3.2.3 Porosity of Alg-Sr

3.2.3.1 Three-dimensional micro-computed tomography analysis to detect Porosity

Alg/Sr hydrogel was analyzed using a desktop µCT (µCT 40, Scanco Medical AG, Brüttisellen, Switzerland) at 45 kVp and 114 µA x-ray tube energy 6 µm voxel size and 0.3 second integration time with approximately 400 slices per specimen. The 3D material part was scanned separately and data assessed by the system software. The region of interest was selected. Hydrogel sample was prepared by lyophilizing for 24 Hours and stained with

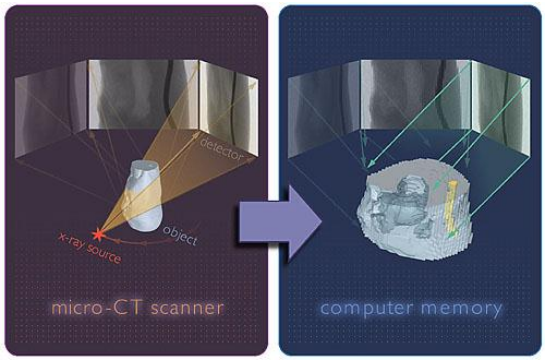


Fig 3-10: Images of micro–CT (x-ray) beam operation.

osmium tetroxide to enhance the contrast. Again sample was lyophilized for 24 hours and scanned with desktop µCT.

3.2.3.2 Scanning Electron Microscopy

Lyophilized Alg/Sr hydrogel stained with osmium tetroxide for enhancing contrast of hydrogel during imaging, was coated (Au-Pd) in an ion sputter (Hitachi E-1010) and viewed under the Scanning Electron Microscope (S2400) to observe the 3D porous structure.

3.2.3.3 Histology

Cryosections of frozen Alg/Sr lyophilized hydrogel samples were taken in the Cryostat (Leica CM 3050S) and stained with H & E, to visualize the internal morphology and porous structure.

3.2.3.4 Radiographic analysis on the opacity of the hydrogel

Prior to implantation, the clinical X ray of the hydrogel scaffold was taken for evaluating radiopacity by using Innova$^{@}$ 3131^{IQ} (ge) X ray system analysis. To assess the radiopacity of Alginate Strontium hydrogel, hydroxyapatite (ceramic) was taken as the reference material.

3.2.4 Thermal analysis:

3.2.4.1 Differential thermal analysis (DTA) to detect enthalpic changes and temperatures at which these events occur.

3.2.4.2 Determination of mass loss of materials by methods of thermogravimetry (TGA).

Thermogravity analysis (TGA) to determine the thermal stability and compositional analysis of the sample was done on a SDT Q600, simultaneous DTA-TGA system (TA Instruments Inc., USA). Periodic calibration of temperature and mass signals of the DSDT Q600 is done using standard reference materials the test method is based on ASTM E 1131-08. Before starting the experiment the mass of the sample and reference pans checked

using the software. 5mg of the sample was taken in a platinum sample cup and heated under nitrogen atmosphere at a heating rate of $10^{o}C\ mon^{-1}$ from room temperature to $800^{o}C$. Calcined alumina is used as the reference material.

3.2.5 Fourier Transform Infrared Spectroscopy (FTIR):

FTIR is a technique which permits to understand structural component of polymeric materials. Another usage of that is to know the intermolecular interaction of polymers. FTIR measurement was done by using Thermo Nicolet (Madison, WI) 5700 spectrometer (USA) and widespread spectra gathered together in the diffuse reflectance (DRIFT) mode. FTIR was done on the powdered lyophilized of Alg/Sr hydrogel as sample and Alginate alone as control.

3.3 *In vitro* studies

3.3.1 Cytotoxicity studies (Direct contact – ISO 10993-5, 2009)

Direct contact method for Cytotoxicity was performed using hydrogel samples as per ISO 10993-5 as test samples together with, negative (polyethylene) and positive (phenol) as controls. Samples in triplicate were placed on sub confluent monolayer of L-929 mouse fibroblasts Cell Line. After incubation of cells at 37 ± 1˚C for 24 ± 1 h, cell monolayer was examined for cellular response around the hydrogel samples using Phase Contrast Microscope (Leica DMI 6000). The reactivity was graded as 0,1,2,3 and 4 based on zone of lysis, vacuolization, detachment and membrane disintegration as per the table given below.

Grade	Reactivity	Description of reactivity zone
0	None	No detectable zone around or under specimen
1	Slight	Some malformed or degenerate cells under specimen
2	Mild	Zone limited to area under specimen
3	Moderate	Zone extending specimen size up to 0.33cm
4	Severe	Zone extending farther than 0.33 cm beyoind specimen

Table 3-2: Reactivity chart of cytotoxicity

3.3.2 Harvesting of rabbit adipose tissue, isolation and culture of Rabbit adipose-derived mesenchymal stem cells (RADMSCs).

Ethical statement and approval:

Studies using MSCs have been approved by the Institutional Committee for Stem Cell Research and Therapy (IC-SCRT: SCT/IC-SCTY/11/JAN 2013). Animal experiments were performed following the guidelines and recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEA) and with the approval from Institutional Animal Ethics Committee (IAEC: B3112010VIII).

ADMSCs were isolated from the subcutaneous site of New Zealand white rabbit weighing around 2–2.5 kg. With the animal under anaesthesia, subcutaneous fat of approximately 5 g was isolated and was collected in PBS with antibiotics.

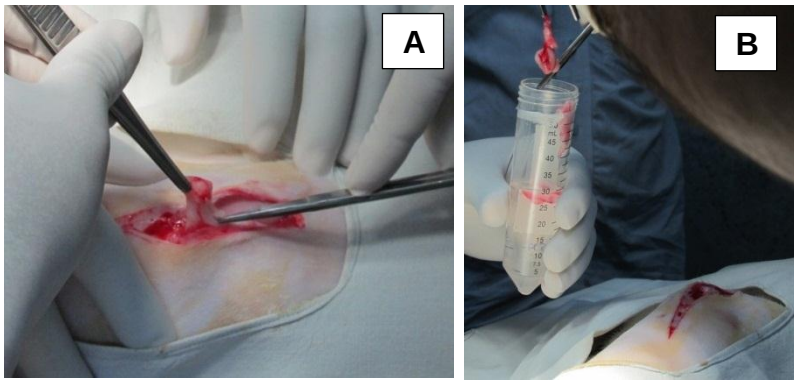


Fig 3-11: New Zealand white rabbit subcutaneous adipose tissue collection in PBS (A,B).

Adipose tissue was washed intensively with phosphate-buffered saline (PBS) thrice, minced thoroughly and treated with 1% type I collagenase (Sigma Aldrich, St. Louis, MO) at 37˚C. Enzyme activity was neutralized with a minimal essential medium (a-MEM) containing 10% fetal bovine serum (FBS), 200 Uml-1 of penicillin and 200 Uml-1 of streptomycin (double dose) (Gibco,India).

The solution was then centrifuged at 1200g for 10min. The pellet was filtered by 180µm nylon mesh to remove cellular debris and was plated in a 25 cm^2 flask (Nunc, India) containing 5 ml of medium with a single dose of antibiotics and incubated at 37˚C in a humid atmosphere and 5% CO_2.

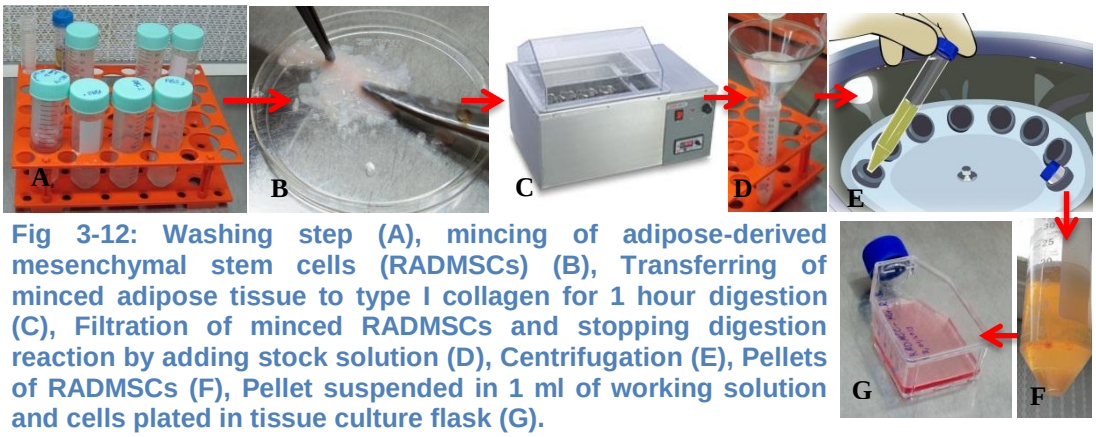


Fig 3-12: Washing step (A), mincing of adipose-derived mesenchymal stem cells (RADMSCs) (B), Transferring of minced adipose tissue to type I collagen for 1 hour digestion (C), Filtration of minced RADMSCs and stopping digestion reaction by adding stock solution (D), Centrifugation (E), Pellets of RADMSCs (F), Pellet suspended in 1 ml of working solution and cells plated in tissue culture flask (G).

Following incubation, the medium was changed after 24 hours to remove residual non adherent red blood cells. The primary cells were cultured for 4-5 days until they reached confluence and were defined as 'Passage 0'. After 5 days in primary culture, the adherent ADMSCs were released with 0.25% trypsin–EDTA (Gibco, India) and centrifuged at 2000rpm for 10 min and subcultured for subsequent passages until

'Passage 3' and cells were characterized prior to experiments. The morphology of the confluent fibroblast-like cells was viewed under the phase contrast microscope.

Compute Relative Centrifugal Force (RCF) or G-force	
Revolutions per minute of rotor (rpm)	1000
Radius of rotor (mm)	100
Relative Centrifugal Force (RCF) or G-force	112
Compute revolutions per minute (rpm)	
Relative Centrifugal Force (RCF) or G-force	112
Radius of rotor (mm)	100
rpm	1000
RCF (G-force) = 1.12 x Radius x (rpm/1000)^2	

Table 3-3: Conversion chart of relative centrifugal force (RCF) to revolutions per minute (rpm).

3.3.2.1 Cell surface characterization

RADMSCs (1 x 10^5 cells - Passage 4) were cultured in 25 cm^2 flask (Nunc) for 48 h at 37˚C in a humid atmosphere and 5% CO_2; washed with PBS; trypsinized with 0.25% trypsin-EDTA for 5 min and centrifuged at 300g for 10 min. The pellet was blocked with 3% BSA (50µl) in PBS for 30 min and further incubated in PBS containing 1µl monoclonal antibodies (FITC labeled, BD Biosciences, USA) to CD45, CD 90 and CD 105 at 4˚C for 1 h. The intensity of fluorescence was recorded under flow cytometry (BD Biosciences, FACS Aria). RADMSCs without staining, but trypsinized, fixed and washed under the same conditions were used as the control.

3.3.2.2 Cell proliferation

Cells were plated in 6 wells plate with RADMSCs (passage 4). RADMSCs were trypsinized with 0.25% trypsin-EDTA for 5 min and centrifuged at 300g for 10 min. The pellet was suspended in 1 ml medium and the cell number was counted manually using hemocytometer. In each well of 6 wells plate, cells were added on the basis of ascending number. Calculated formula is shown as below.

$$\textbf{Total cell per ml } (TC) = \frac{\textbf{Total cells from 9 chamber}}{9} * \textbf{Dilution factor} * 10^4$$

10^4 or 10,000 accounts for the Hemocytometer chamber volume. Parallely, cell counting was 1.2e+05, verified using a Scepter Cell Counting.

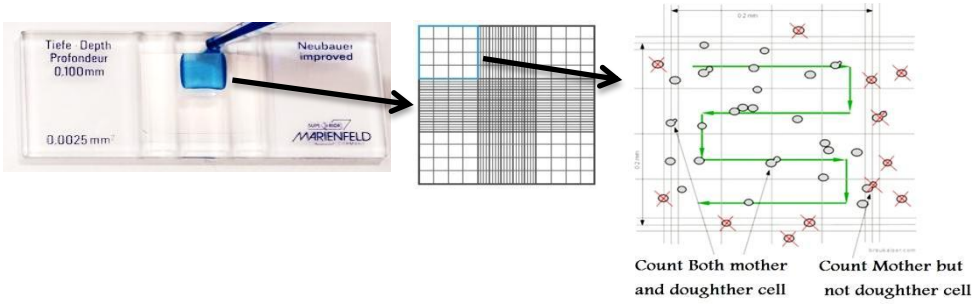


Fig 3-13: Hemocytometer and counting procedure.

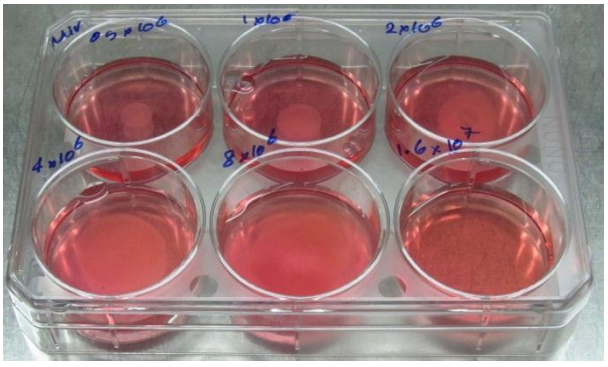


Fig 3-14: Cell proliferation assay, cell numbers from $0.5*10^6$ to $8*10^6$; doubling time 2day.

3.3.3 Fabrication of 3D tissue-engineered construct - Cell titer test

Alg/Sr Hydrogel was plated in 48 wells plate with RADMSCs (passage 4) trypsinized with 0.25% trypsin-EDTA for 5 min and centrifuged at 300g for 10 min. The pellet was resuspended in 1 ml medium and the cell number was counted manually using hemocytometer as well as Scepter Cell Counting. 1×10^6 cells /cm^2 were seeded on Alg/Sr Hydrogel in each well.

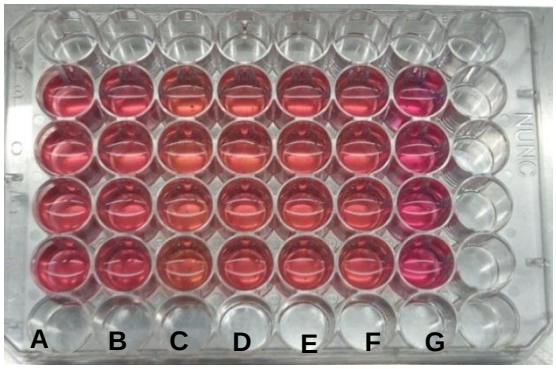


Fig 3-15: Cells alone (A, B); Cell incorporated in Alg/Sr hydrogel (C, D, E and F); Alg/Sr alone (G).

3.3.4 Encapsulation of MSCs in 3D cylindrical gel block

RADMSCs 1.2 *10^5 were encapsulated in 3 D cylindrical Alg/Sr hydrogel blocks 5 mm for mimicking *in vivo* condition. Cells in Hydrogel block were observed and photographed by Light Microscope DM 6000.

Fig 3- 16: Cells within hydrogel cylindrical blocks.

3.3.5 Actin staining

Combination of Alg/Sr with encapsulated RADMSCs (1 x 10^5 cells - Passage 4) were cultured for 48 h at 37˚C in a humid atmosphere and 5% CO_2 on glass cover slips (Blue star India) and thereafter washed with PBS and fixed with 3.7% paraformaldehyde in Sorensen phosphate buffer. The cells washed with PBS were permeabilised using 0.1% Triton X-100 (Sigma) for 5 min in PBS. After washing thrice with PBS, the cells were stained with FITC actin (Sigma) 1: 1000 in PBS) for 30 min and DAPI (D9564 – Sigma) for 1 h (1: 500 in PBS) in dark at room temperature for determining actin filament (green) and nucleus (blue). The cells were then washed thoroughly with PBS and observed under fluorescent microscope (Nikon Eclipse E600).

3.3.6 Chondrogenic potential of RADMSCs

For *in vitro* chondrogenic assays, RADMSCs at a concentration of 1x10^5 cells were plated on glass coverslips placed in 6-well tissue culture plates and maintained in the chondrogenic medium humidified environment at 5% CO_2 and 37^{o}C for up to 14 days, with exchange of culture media every 2 days.

Control culture media comprised Dulbecco's Modified Eagle Media–high glucose (DMEM-hg), 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. The chondrogenic

culture media comprised the control media, 1x insulin-transferrin-selenium supplement (ITS+), 0.15 mm ascorbate 2-phosphate (Sigma), 100 nm dexamethasone (Sigma), and 10 ng/ml rh-TGFb1. In another procedure same cells were kept inunder upright centrifuge tube (15ml) hypoxy condition by closing lid of each tube for differentiation of MSCs towards Chondrogenic cells.

3.3.7 Evaluation of tissue-engineered construct (Live Dead Assay)

Rabbit adipose derived cells (1 x 10^5 cells) were seeded into the Alg/Sr hydrogel and placed in 96 wells TCPS plates (Nunc). After being incubated at 37°C cells adhered were maintained in MEM with 10% FBS, 100units / ml of penicillin and 100 µg / ml streptomycin and incubated at 37°C in humid atmosphere and 5% CO_2. MSCs encapsulated within Alg- Sr hydrogel placed on glass cover slips (Blue star India) were washed with PBS and fixed with 3.7% paraformaldehyde in Sorensen phosphate buffer. The cells washed with PBS were permeabilised using 0.1% Triton X-100 (Sigma) for 5 min in PBS. After washing thrice with PBS, the cells were stained with acridine Orange (1: 1000 in PBS) for 30 min and ethidium bromide 1 h (1: 500 in PBS) in dark at room temperature for determining Acridine Orange (Green- live cell stain) and ethidium bromide (Red- dead cell stain). The cells were then washed thoroughly with PBS and observed under fluorescent microscope (Nikon Eclipse E600).

3.4 *In vivo* Studies – Rabbit

To study the real condition of intervertebral disc degeneration and evaluation of the tissue construct product, an *in vivo* experiment is performed to reveal the effect of treatment on cartilage tissue repair.

3.4.1 Rabbit Implantation experiment

In vivo studies with Rabbit IVD model development and implantation were performed following the guidelines and recommendations of the Committee for the Purpose of Control and Supervision of Experiments on Animals, India (CPCSEA) and with the approval from Institutional Animal Ethics Committee (IAEC). Execution, evaluation and reporting of animal experiments were as ARRIVE Guidelines.

Thirty adult rabbits with an average body weight of 2- 2.5kg were used. Experimental animals were divided into four groups as shown in the table below. All rabbits except positive control animals, passed one month degeneration period to develop IVD rabbit model. Experimental groups were defined for different period of 1, 3 and 6 months. Numbers of defects were one or two per animal.

Table of Animal Experiment						
Rabbit Model	**Development of IVD Model one Month Total Post injury**	**Material -Cell Injection**	**Post Implantation one Month**	**Post Implantation 3 Month**	**Post Implantation 6 Month**	**Total Animals**
Group 1	**9**	**Material L4/L5 or L5/L6**	**3**	**3**	**3**	**9**
Group 2	**6**	**Induced cells+ Material L4/L5or L5/L6**	**3**	**3**	**----**	**6**
Group 3	**9**	**Sham L5-L6**	**3**	**3**	**3**	**9**
Group 4	**6**	**Control L5-L6**	**2**	**2**	**2**	**6**
Total						**30**

Table 3- 4: of Animal Experiment

3.4.2 Development of Rabbit IVD Model and Resurgery

The rabbits were anesthetized with intramuscular injections of ketamine@50mg/Kg and xylazine @5 mg/ Kg. After the animals lost pinna pinching reflex, the hair over the surgical field was shaved. The rabbits were placed in a lateral decubitus position with an approximate 20 degrees inclination provided by a folded cloth towel placed longitudinally under the animal. Aseptic technique was used for all surgical procedures. The surgical field was disinfected with povidone iodine solution and draped. A posterolateral retroperitoneal approach was used to expose the IVD. A longitudinal skin incision

was made from the inferior margin of the rib cage to the pelvic rim, about 2 cm ventral to the paraspinal musculature. The left anterolateral vertebral column from L1-L7 was exposed by sharp and blunt dissection of the overlying subcutaneous tissue, retroperitoneal fat, and musculatures.

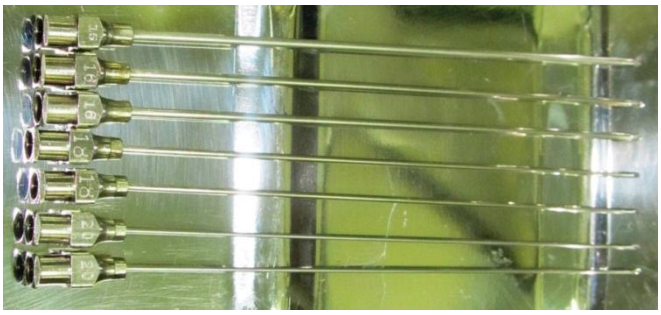


Fig 3-17: Punctuation needles for puncturing of intervertebral discs. There are different gauges (16G, 18G etc) of punctuation needles.

Disc levels were identified using the pelvic rim as an anatomic landmark for the L5-L6 disc level. One of the lumbar IVD L4-L5 or L5/L6 was punctured by a 16-gauge needle to a depth of about 4 mm in the left anterolateral annulus fibrosus. The depth of penetration was controlled by a locking forceps clamped 4 mm from the needle tip. Proper and adequate precaution was ascertained by viewing the out flow of gel like nucleus pulposus. The muscles were opposed with non-absorbable 3-0 braided silk sutures in a continuous lock-stitch pattern, while a non-absorbable 3-0 braided silk suture was used to close the skin incisions in a simple interrupted pattern. All animals were monitored until they regained consciousness. The rabbits were allowed to eat and drink, while their healing status was monitored on

a daily basis. All animals continued to receive pain medication with meloxicam@0.2mg/kg once daily and Ampicillin-Cloxacillin injection@ 10mg/Kg twice daily for seven postoperative days.

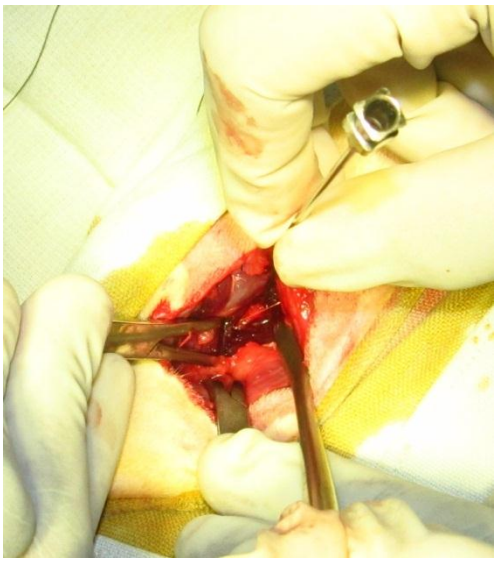


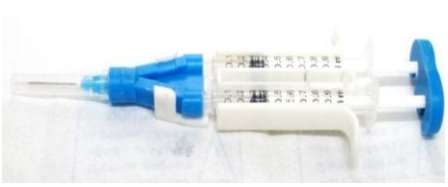
Fig 3-18: Physical injury by 16 gauge needle puncture on the L4/L5 IVD of rabbit lumbar region.

3.4.3 Radiography

Radiographs were taken after administration of Xylazine and ketamine hydrochloride @5 mg/kg and 50 mg/Kg intramuscularly. To obtain similar degrees of muscle relaxation each time, which may affect the disc height, a consistent level of anesthesia was monitored during radiographic imaging of each animal and at each time. Post IVD injury one month is the Rabbit IVD model.

3.4.4 Re-surgery and injection of bare Alg-Sr hydrogel/cell combination

Re-surgery to inject the bare Alg-Sr hydrogel/cell combination using the dual applicator with a 22 gauge needle was carried out after approximately 30 days after the first surgery and radiographic confirmation of the planned IVD damage. Approach and methodology of anesthetic regimen, aseptic precautions, surgical approach as well as post-operative analgesia and care was similar to the initial procedure mentioned above. Radiograph which confirmed the IVD damage was used to locate the damaged IVDs while the approach to the damaged IVD site was through the right side during re-surgery. The other side was chosen since the healing process might alter the tissue anatomy due to adhesions and might not render proper vision and easy access for the healing injections.



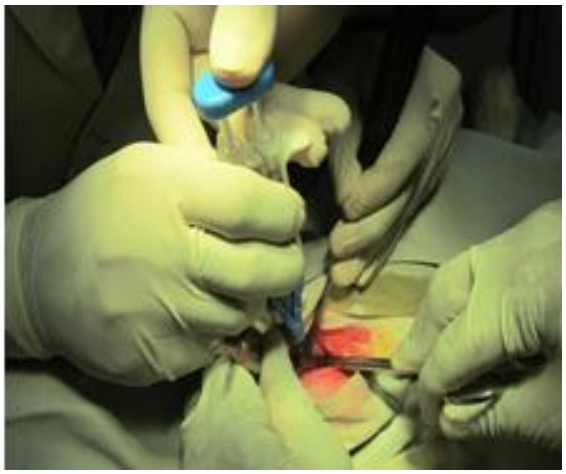


Fig 3-19: A re-surgery to inject bare Alg-Sr and cells combination was carried out one month post injury after the first surgery and after radiographic confirmation of the planned IVD damage. Injection was done with dual applicator.

3.4.5 Rabbit gait and behavior:

Rabbit behavior and movement were checked pre and post-surgery during IVD model as well as before and after re-surgery. IVD degenerative model is created, the rabbit should be able to walk and stand after surgery. This showed that puncturing has just damaged the intervertebral disc and there is no damage to the spinal cord and Rabbit gait and behavior appeared normal.

3.4.6 Post-Surgery/Implantation evaluations

Post-surgery/implantation rabbits after one month degenerative IVD model was evaluated by X-ray and MRI (T2 MRI method) and histology for all study groups by light microscopy. Post implanted IVD was evaluated for disc grading remarks based on histomorphotometry measurement and Disc Height Index.

3.4.7 Histology and Histomorphometry

3.4.7.1 Histological evaluation

The retrieved IVD postimplanted, sham and control tissues after fixing in 10% neutral buffered formalin underwent dehydration in ascending series of isopropyl alcohol followed by infiltration in methyl methacrylate (MMA). Infiltered samples were then embedded in MMA containing 1% Benzoyl peroxide under vacuum. Thin plastic sections (120-140 microns) were sectioned from the PMMA

embedded blocks using high-speed precision saw (Isomet TM 2000, Buehler, USA) and polished down manually to 70–90 microns using variable speed grinder polisher (Eco met 3000, Buehler, USA). PMMA embedded polished sections were stained with Hematoxylin and eosin and viewed under the Light microscope (Leica DM 6000).

3.4.7.2 Histomorphometric evaluation

The Hematoxylin and eosin stained sections were scanned for measuring the percentage of newly regenerated IVD. 1.25x, 5x, 10x and 50x magnification lens were selected randomly and sections were photographed with a CCD camera. These images were suitably calibrated using an inbuilt image configuration. The area of newly regenerated IVD "yellowish colour" was measured using the Quips programme of Q Win software of the microscope (Leica DM 6000). The percentage of newly formed IVD was calculated with respect to the total frame area in the image (μm^2).The percentage of material degraded was calculated by assuming that the total frame area was occupied completely with the newly regenerated NP.

3.4.8 Disc Grading Evaluation

Disc grading remarks were done on the basis of regeneration and disc height of sham, material and cell+material Rabbit Groups compared to the positive control rabbits.

CHAPTER 4

RESULTS

CHAPTER 4

RESULTS

4.1. Anatomy of the nucleus pulposus and mechanical properties of the intervertebral discs of lumbar vertebrae of different animals – rat, rabbit, goat, cattle and pig

Intervertebral discs are located in the lumbar region of the vertebral column. (Fig 4.1)

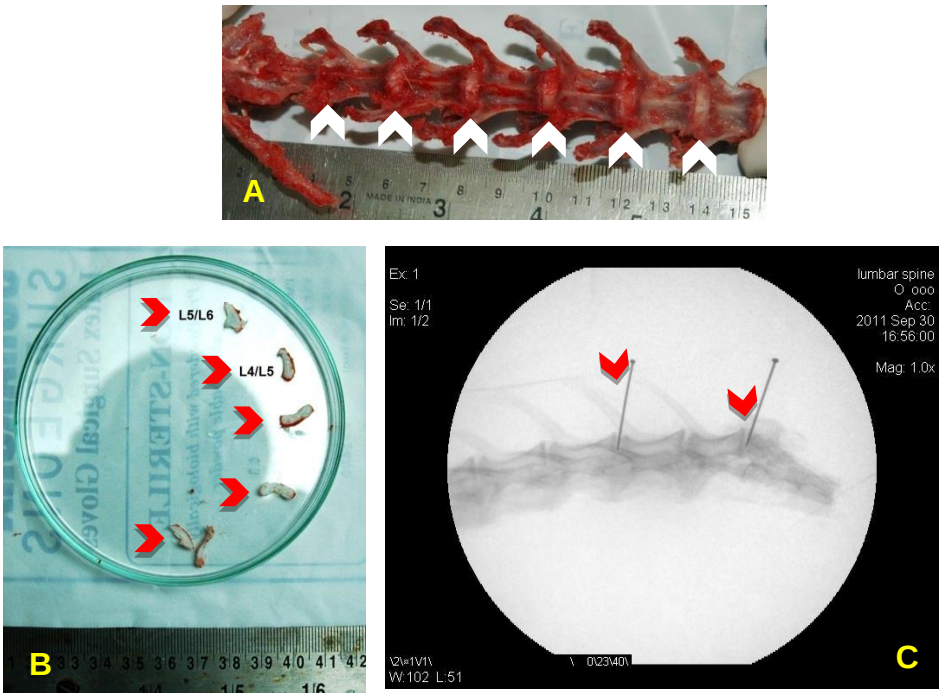


Fig 4-1: Lumbar region of rabbit vertebral column; white arrows are intervertebral discs (A), Red arrows indicates Intervertebral disc of rabbit (B), Location of IVD in the vertebral column, needles are visible in the IVD(C).

4.1.1. Gross images of the anatomy, structure, area and size of different intervertebral discs from small to large animals.

Comparision of appearance and area of nucleus pulposus and annulus fibrosus in different animals. Images indicate bigger size of IVD in cattle compared to goat and pig.

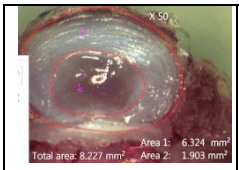
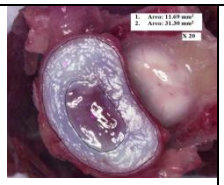
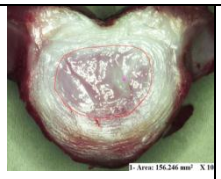
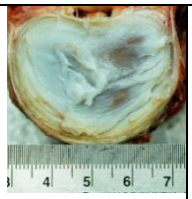
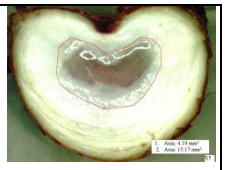
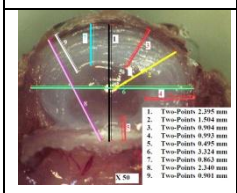
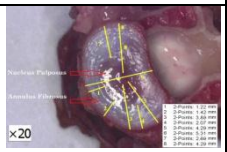
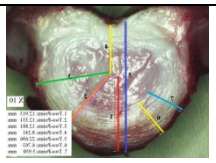
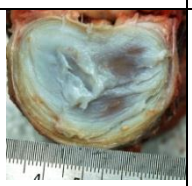
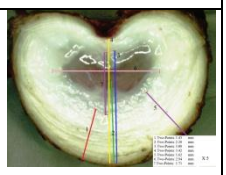
				
				
Gross Image of Rat-IVD	Gross Image of Rabbit-IVD	Gross Image of Goat-IVD	Gross Image of Cattle-IVD	Gross Image of Pig-IVD

Fig 4-1-1-A. Cross sections of intervertebral discs of rat, rabbit, goat, cattle and pig. The soft NP of the IVD in the inner central core is surrounded by a firm elastic annulus fibrosus with a lamellar structure.

Animal	NP area (mm^2)	AF Area (mm^2)	Total IVD Area (mm^2)
1. Rat	1.903	6.324	8.227
2. Rabbit	11.89	31.30	42.99
3. Goat	156.246	368.672	524.919
4. Cattle	457	840	1297
5. Pig	103	350	453

Table 4-1. Area of NP, AF and whole IVD of rat, rabbit, goat, cattle and pig.

As the total area of IVD indicated that big animals have huge area of IVD compared to small animals. Area of cattle IVD is larger than that of other big animals.

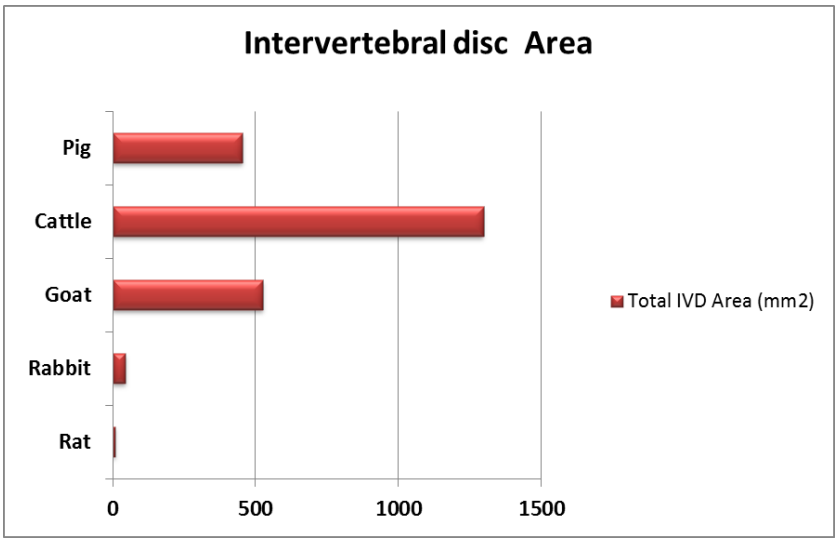


Fig 4-1-1-B: Total area of Intervertebral disc in different animals. Cattle have a bigger area compared to other animals.

4.1.2. Histology of Nucleus Pulposus (NP) of the IVD of small to large animals

Histology pattern revealed notochordal cell in rodents only and not present in larger animals like goat, cattle and pig.

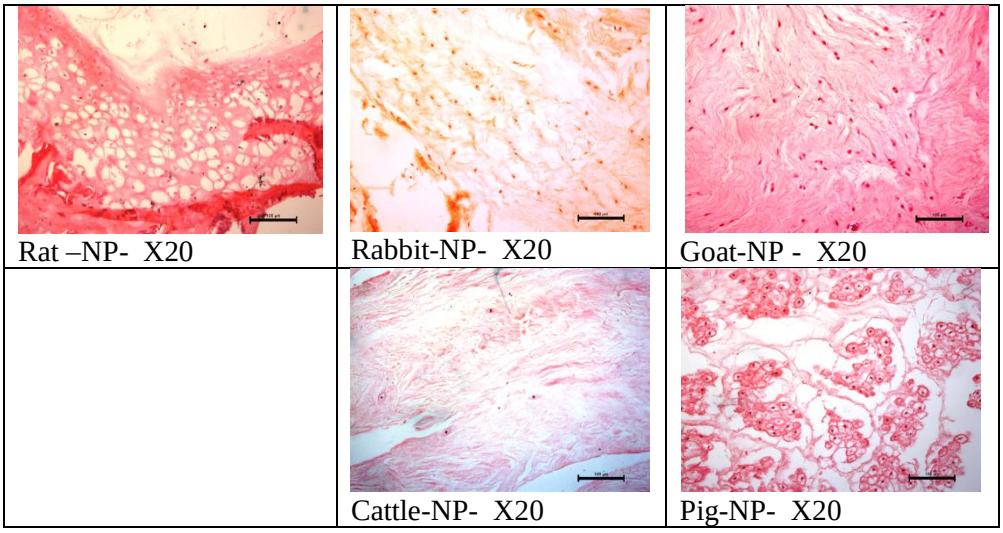


Fig 4-1-2: Light micrographs of NPs of different animals (H & E). In rat and rabbit notochordal cells are visible within big vacuoles while in goat, cattle and pig there are no notochordal cell. But chondrocyte cells are observed in all animals.

4.1.3. The Ultrastructure of NP of IVD of small to large animals.

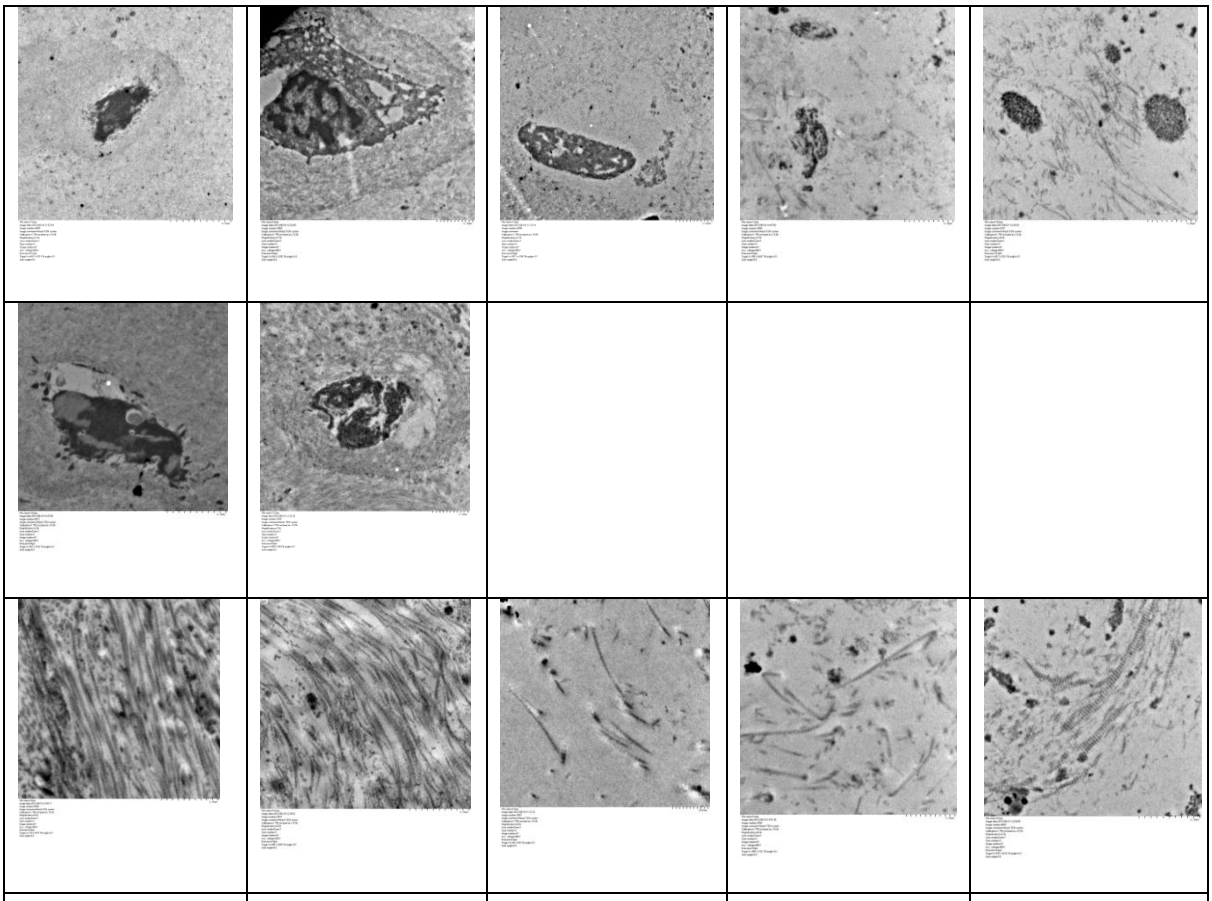


Fig 4-1-3: Transmission Electron Micrographs of NP of different animals.

Chondrocyte - like cells seen in all different animals (top layer) while notochordal cells are observed only in rat and rabbit NP (middle layer). Collagen fibers are abundantly accumulated in rat and rabbit than goat, cattle and pig NP (bottom layer).

4.1.4. Mechanical properties of the IVD different animals rat, rabbit, goat, cattle and pig

4.1.4.1. Tensile Strength Test

Tensile strength increased from small to big animals. It has been observed that tensile strength of pig is higher than cattle while area of cattle IVD is bigger than Pig.

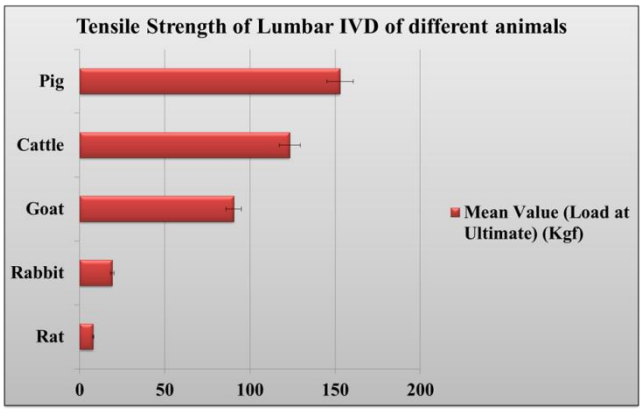


Fig 4-1-4-1: Tensile strength of lumbar region of different animals

Tensile strength in lumbar region of rabbit increased from L1/L2 to L6/L7. This increase is related to the size of the IVD of rabbit from L1 to L7.

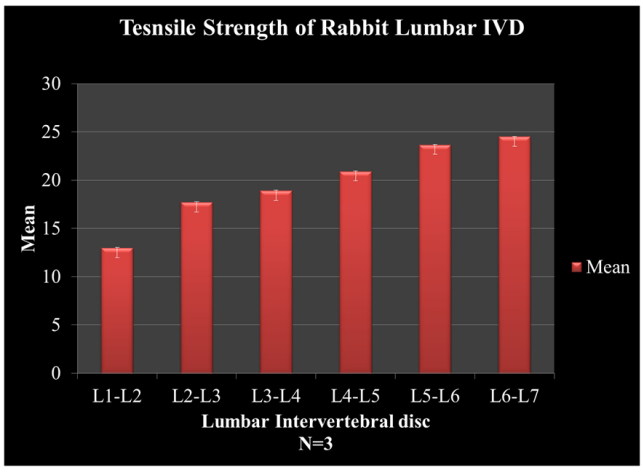


Fig 4-1-4-2: Tensile strength of lumbar region of New Zealand White rabbit

4.1.4.2. Compression Test

Compression Test revealed that by increase of the size of IVD from L1 to L7; tolerance of weight bearing on the each IVD in the vertebral column is more.

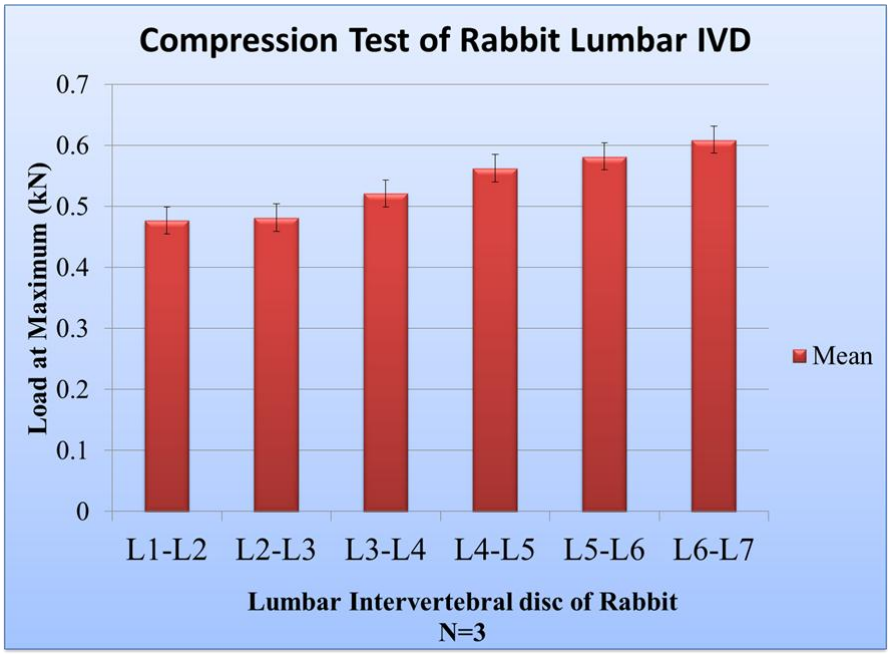


Fig 4-1-4-3: Compression evaluation of lumbar vertebrae of New Zealand White rabbit.

4.2.**Material - Alg/Sr hydrogel**

4.2.1. Mechanical test of Alg/Sr hydrogel scaffold.

Compression test of Alg/Sr Hydrogel indicated that 1.8% of Sr in cooperated with Alginate gave good strength compared to 1.2% of Sr. Material 3 (2% of Sr) gave a strength which is very high compared to material 1 and 2 and not suitable for injecting application due to the blocking of 22G needle.

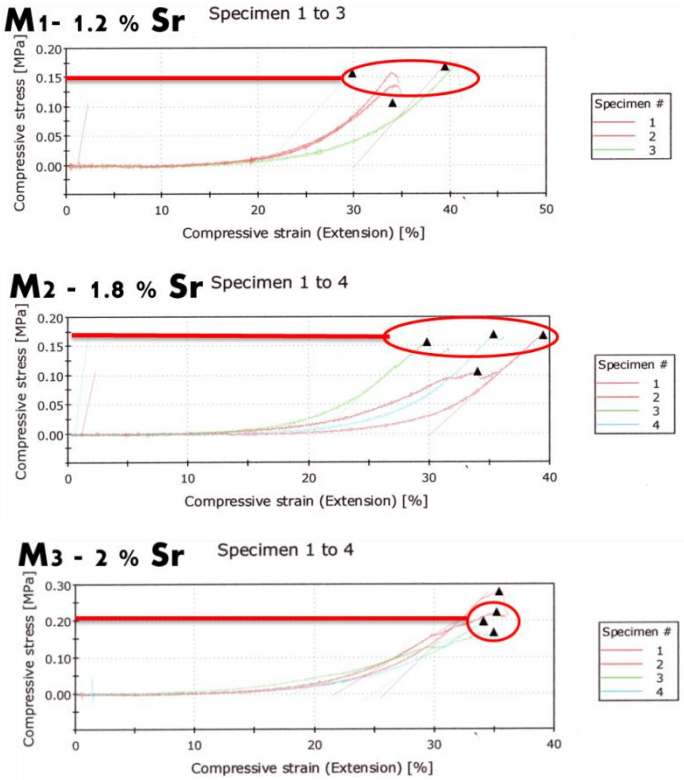


Fig. 4-2-1-A: Compressive stress on the Alg/Sr Hydrogel at different compositions of 1.2 % (M1), 1.8 % (M2) and 2 % (M3) of Sr.

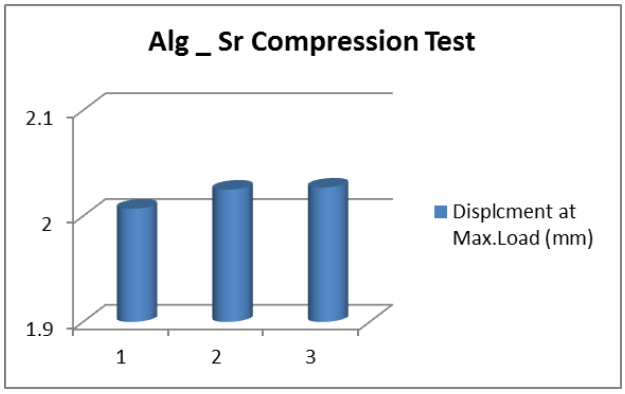


Fig. 4-2-1-B: Compression test of Alg/Sr hydrogel estimated by INSTRON machine.

4.2.2. Porosity of 1.8% Alg-Sr Hydrogel

4.2.2.1. Three-dimensional micro-computed tomography analysis (Porosity)

μCT revealed a 3D structure of 1.8% Alg/Sr hydrogel block with a porosity of 74.28%. This hydrogel mimicked the structure of nucleus pulposus.

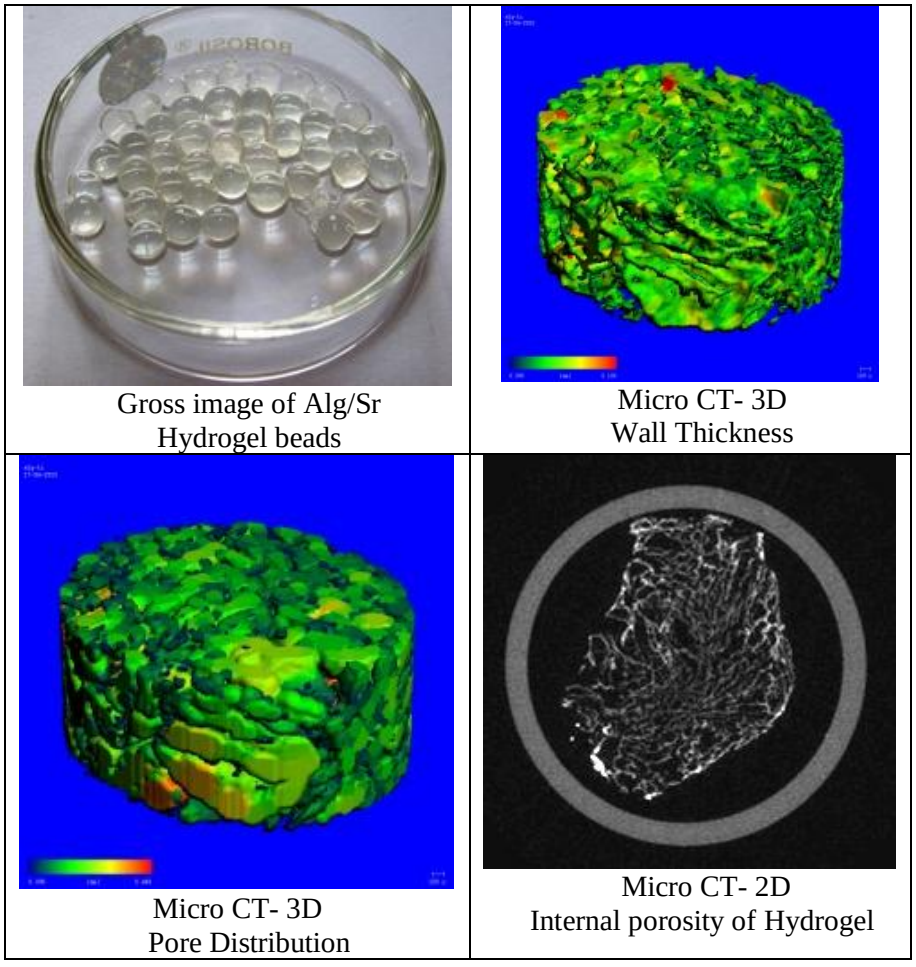


Fig. 4-2-2-1: μCT of Alg/Sr Hydrogel Block.

4.2.2.2. Scanning Electron Microscopy (SEM)

SEM analysis revealed that 22G needle gave more porosity compared to 16G needle due to pressure and thickness of the needle exhibiting a 3D porous structure of lyophilized 1.8% Alg/Sr hydrogel.

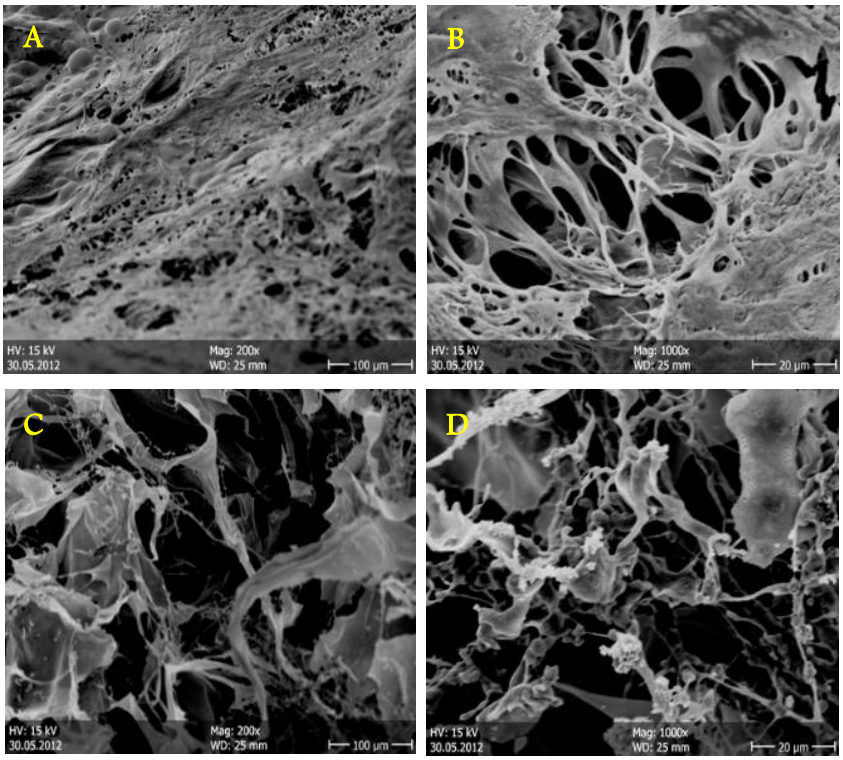


Fig 4-2-2-2: Scanning electron micrographs depicting porosity of 1.8 % Alg/Sr hydrogel prepared with 16G Needle (Fig A & B) and 22G Needle (Fig C & D).

4.2.2.3. Histology

1.8% Alg/Sr Hydrogel beads revealed an elaborate interconnected porous network structure.

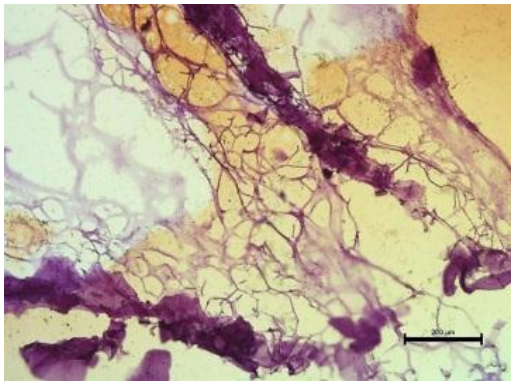


Fig 4-2-2-3: Light Micrograph of cryosectioned toluidine blue stained Alg-Sr hydrogel beads depicting the porous network (Mag. 10X).

4.2.3. Radiographic analysis on the opacity of the hydrogel

Radiograph enabled tracking of hydrogel due to the presence of strontium in the hydrogel beads. Hydroxyapatite has been radiographed as the reference material.

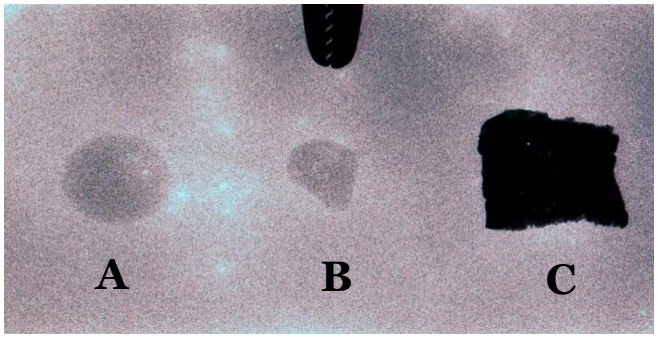


Fig 4-2-3: Radiopacity of Alg/Sr beads in X-ray Radiograph (A, B). Hydroxyapatite material (C).

4.2.4. Thermal analysis:

4.2.4.1. Differential thermal analysis (DTA)

The graph indicated that hydrogel is endothermic as it goes under different thermo cycle. The peak revealed enthalpic changes which are not affected by heat capacity of sample.

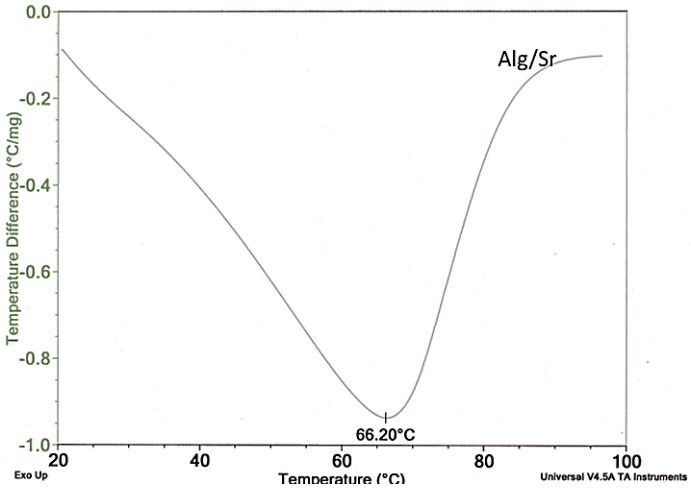


Fig 4-2-4-1: Enthalpic changes and temperatures at which these events occur.

4.2.4.2. Thermogravimetry (TGA)

Thermogravimetric analysis about alginate strontium hydrogel indicated that 87% of the material remained at 37^0C. By increasing the temperature to 55^0C, 50% of the material was left behind. At 79^0C mass loss is 1.4% and 87% of the material was left behind at 37^0C.

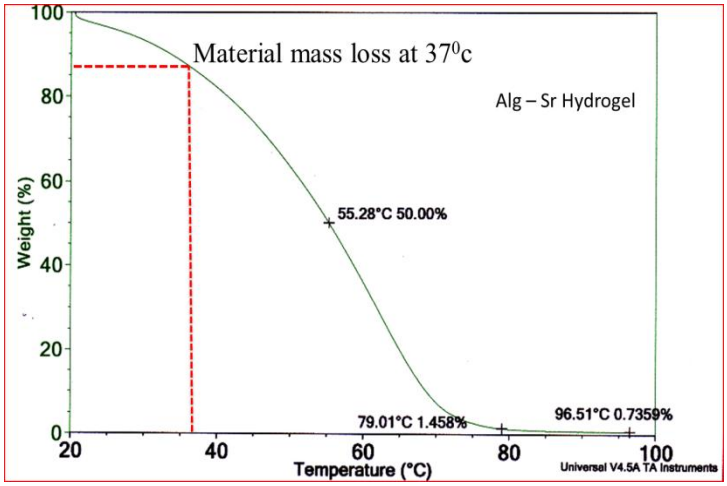


Fig 4-2-4-2: Mass loss of total weight of Alg/Sr Hydrogel.

4.2.5. Fourier Transform Infrared Spectroscopy (FTIR):

FTIR graph did not reveal much difference between alginate powder and alginate incorporated with strontium.

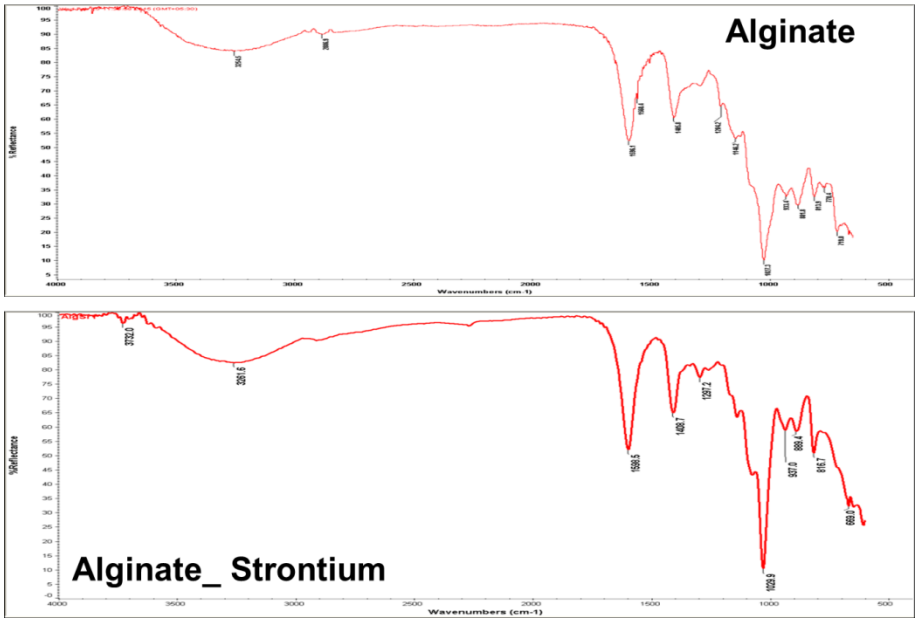


Fig 4.2.5: FTIR Spectra of Alginate powder and Alg/Sr Hydrogel.

4.3.*In vitro*

4.3.1. Cytotoxicity (Direct contact – ISO 10993-5, 2009)

Cytotoxicity evaluation indicated that material is non-toxic to fibroblast cells.

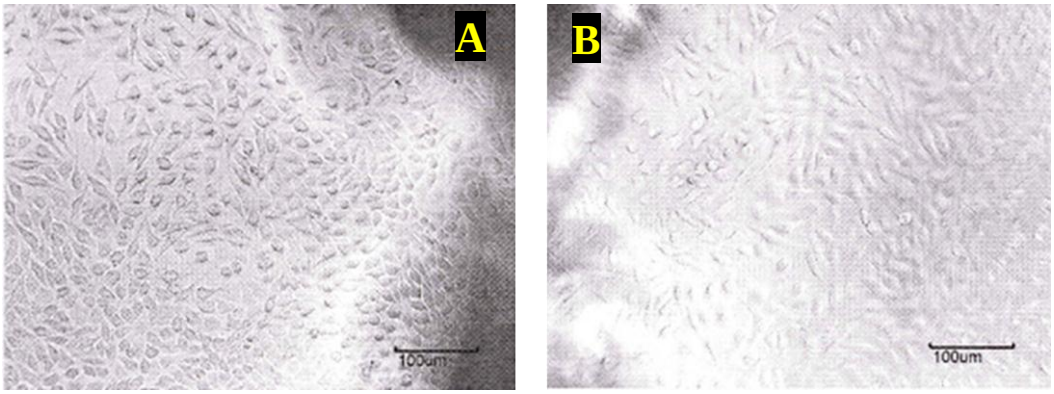


Fig 4-3-1: Al/Sr hydrogel showed no cytotoxic reactivity to fibroblast cells (L-929) after 24 Hours of contact. Control (A), Al/Sr hydrogel (Test) (B).

4.3.2. Harvesting of rabbit adipose tissue, isolation and culture of Rabbit adipose-derived mesenchymal stem cells (RADMSCs).

Isolation of mesenchymal stem cells was done from rabbit adipose tissue. Spindle shaped cells were confluent after 6 days in tissue culture flask.

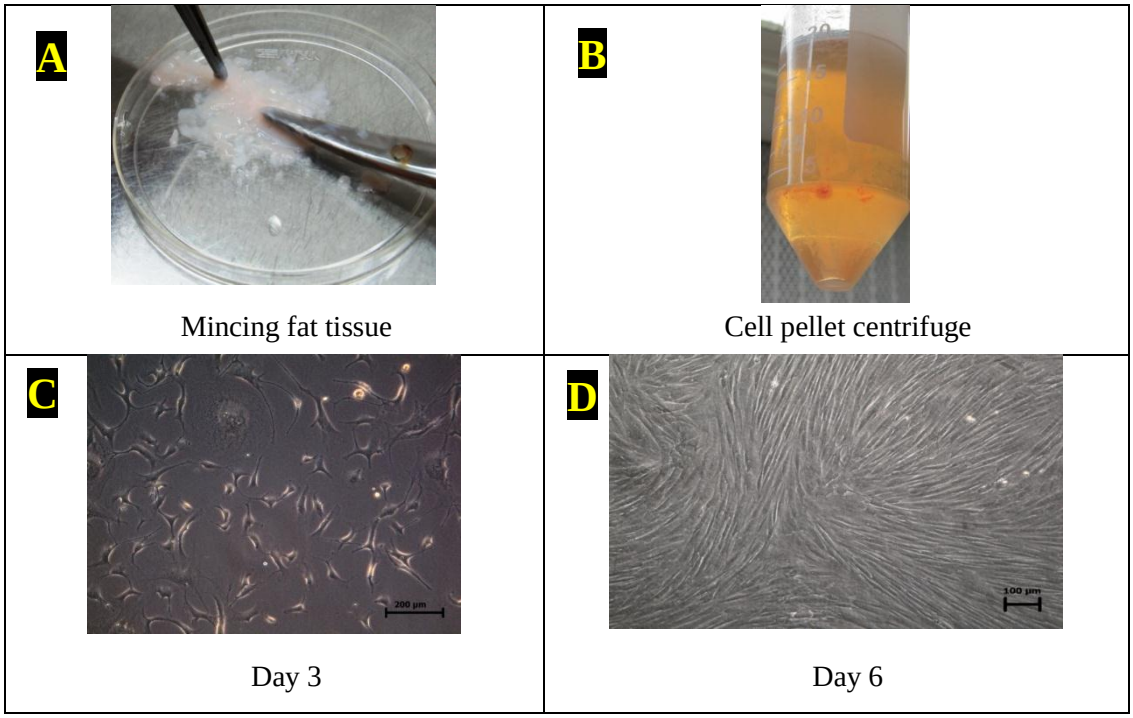
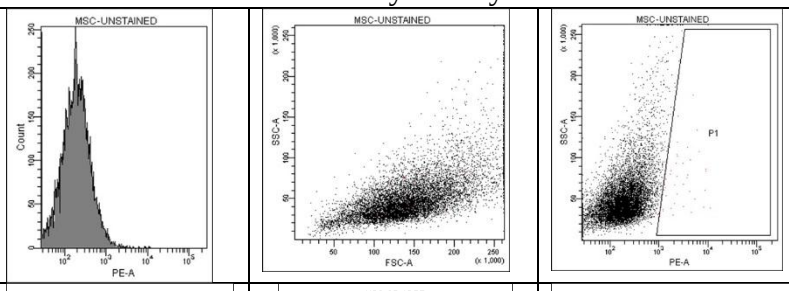
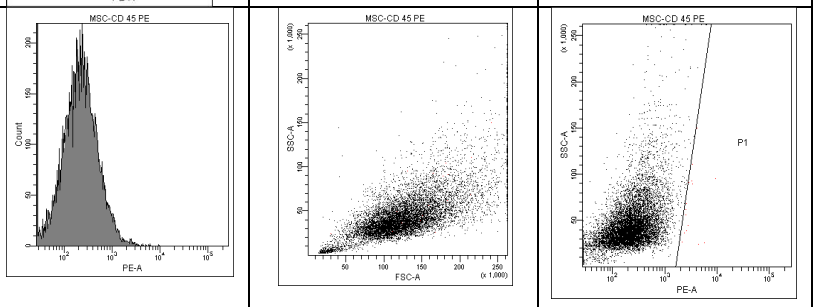
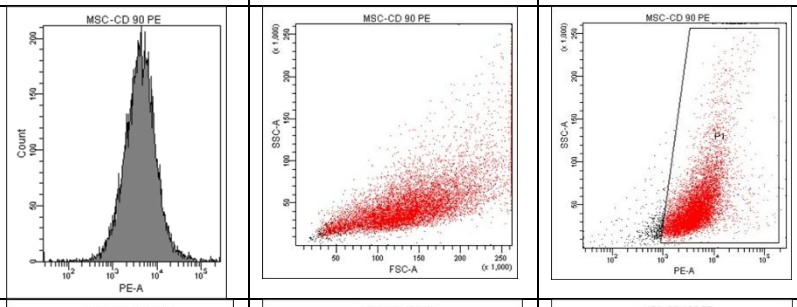
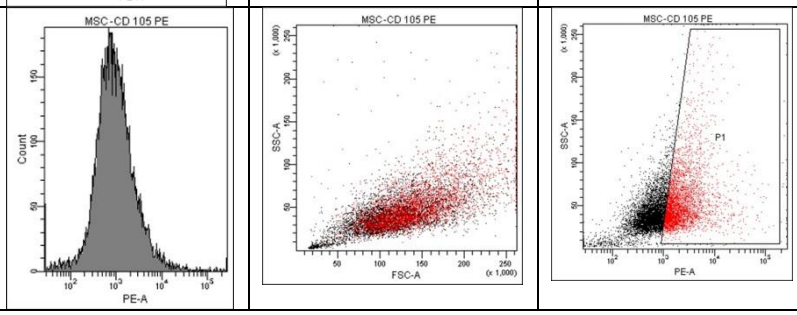


Fig 4-3-2: Harvest of rabbit adipose tissue, minced and centrifuged in preparation of cell pellet of RADMSCs (A, B); phase contrast micrographs of cells in culture - day 3 (C); and confluent layer of cells - day 6 (D).

4.3.3. Cell surface characterization by Flow Cytometry

Flow cytometry was done to ensure cells are RADMSCs.

RADMSCs	Flow-cytometry
1. Unstained	
2. CD45	
3. CD90	
4. CD105	

Sample	Percent	Result
Unstained	%0.4	Negative
CD45	%0.6	Negative
CD90	%94	Positive
CD105	%39	Positive

Fig 4-3-3: MSCs were positive for CD90 and CD105 and negative for CD45.

4.3.4. Cell Counting

Cell number for *in vitro* and *in vivo* experiments was measured by hemocytometer and ensured by scepter cell **counter.**

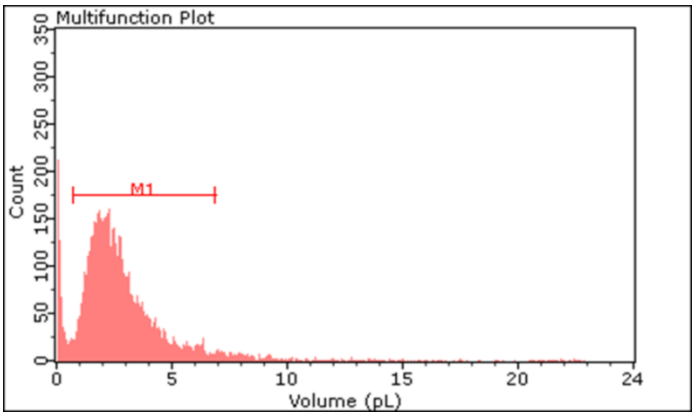


Fig 4-3-4: Stained with toluidine blue and cells were counted by hemocytometer. Cell counting was 1.2e^{+05}; verified by scepter cell counter.

4.3.5. Fabrication of 3D tissue-engineered construct - Cell titer test

RADMSCs were cultured in the hydrogel for proliferation assay and compared with growth of cells in the normal condition in 48 wells plate. The same numbers of cells used for in *vivo* condition has been used in the cell proliferation assay too.

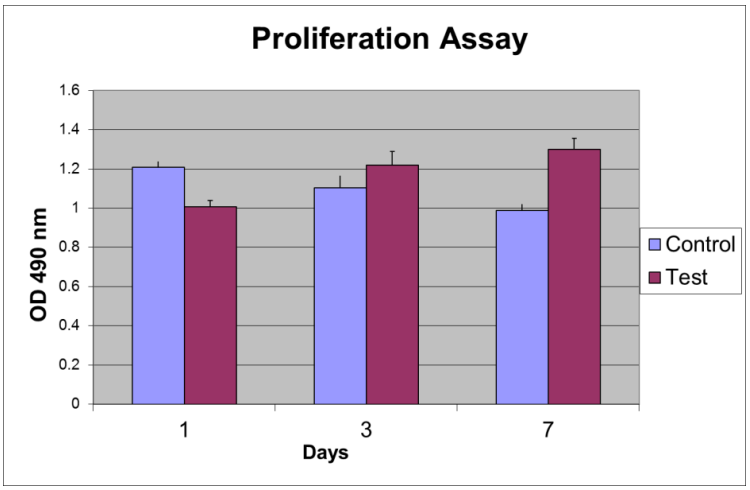


Fig 4-3-5: Cell number increased with 7 days of 3D culture. Number of cells decreased in the control (cells alone).

4.3.6. Encapsulation of MSCs in 3D cylindrical blocks

RADMSCs were cultured in 3D cylindrical Alg/Sr hydrogel blocks for its viability test. Spindle shaped cells ADMSCs proliferated and expanded in 3D culture visible by light microscopy.

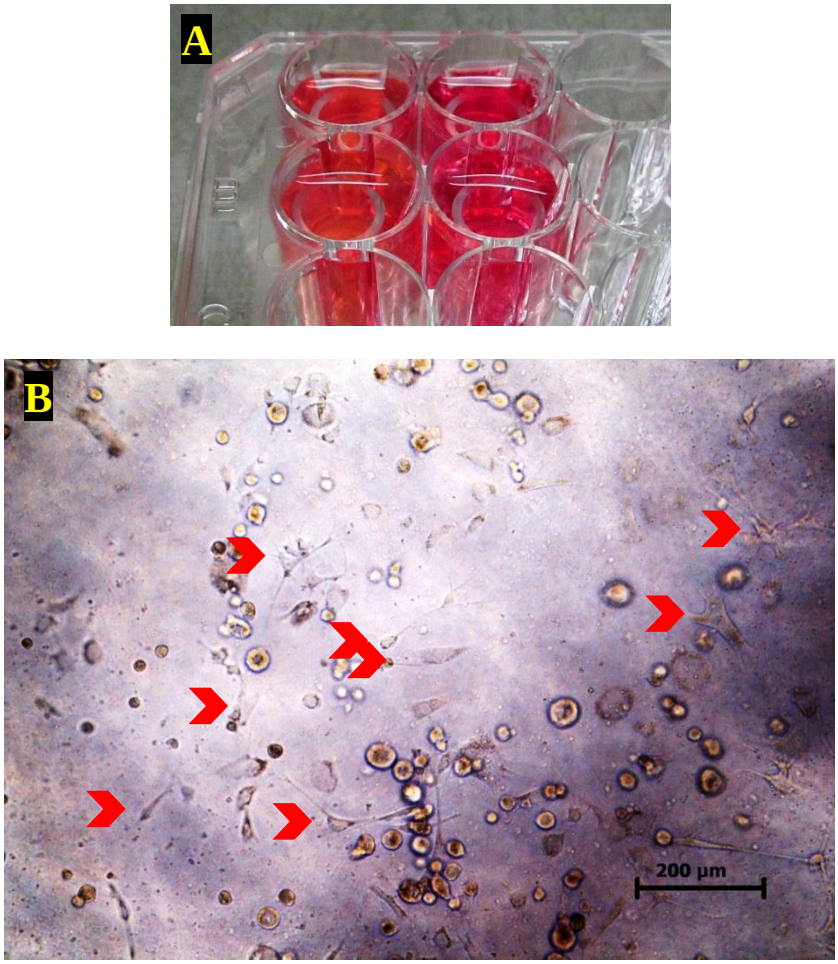


Fig 4-3-6: Light micrograph of RADMSCs encapsulated in 3 D cylindrical Alg/Sr hydrogel block. Arrows indicates RADMSCs in side Alg/Sr hydrogel.

4.3.7. Actin staining of RADMSCs within Alg/Sr hydrogel

Actin/DAPI staining was done to depict the presence of cells within the depth of hydrogel which was revealed by confocal microscopy by the blue stained nucleus of clustered cells.

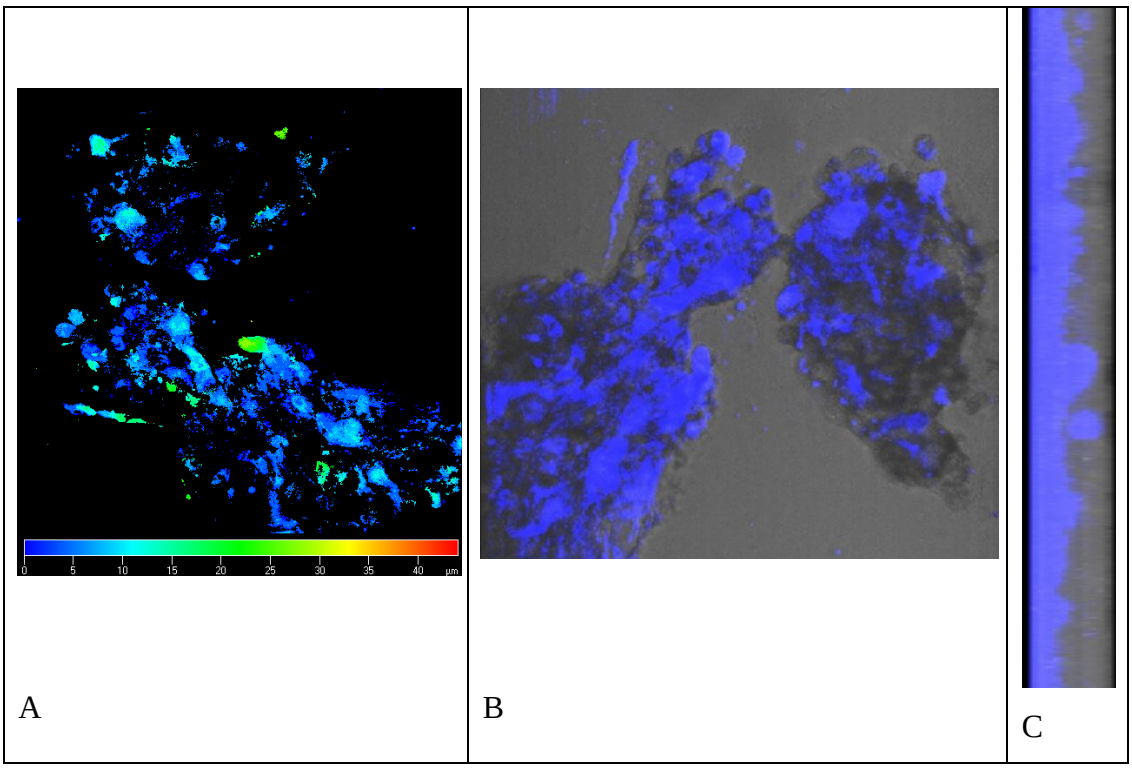


Fig 4-3-7: Confocal Micrographs of cluster of RADMSCs encapsulated in Alg/Sr hydrogel (A, B) – DAPI Staining. Actin filament (green) and nucleus (blue). Depth of Alg/Sr Hydrogel - 3D image (C).

4.3.8. Evaluation of tissue-engineered construct - RADMSCs within Alg/Sr hydrogel (Live Dead Assay)

Live – dead assay was done due to understand the viability of cells within the depth of hydrogel. Green cluster of cells shows viable cells in the depth of hydrogel. In 3D micrograph thickness and cluster of cell is visible.

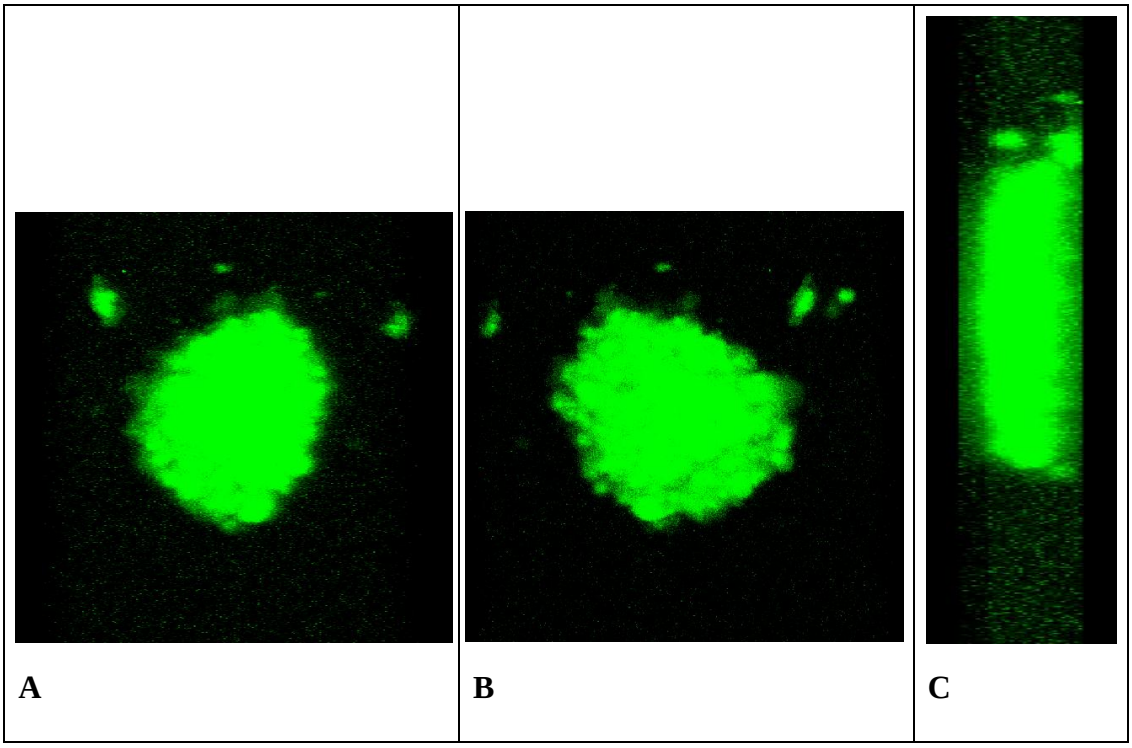


Fig 4-3-8: Confocal Micrographs of cluster of RADMSCs encapsulated in Alg/Sr hydrogel, (A, B) - Acridine Orange (Green- live cell stain) and ethidium bromide (Red- dead cell stain). Depth of section and orientation of cells within the hydrogel (C).

4.4.*In vivo* Studies – Rabbit NP-IVD defect model

Anatomy of Intervertebral disc was studied by the surgical section of IVD. Structure and location of the center of nucleus pulposus was essential to enable easy and direct insertion of the 16G needle for puncturing the NP and thereafter the injection of the material and cells. Nucleus pulposus and annulus fibrosus are visible in the rabbit lumbar IVD. There is a distance of 3.89 mm from the edge of intervertebral disc to the center of the nucleus pulposus.

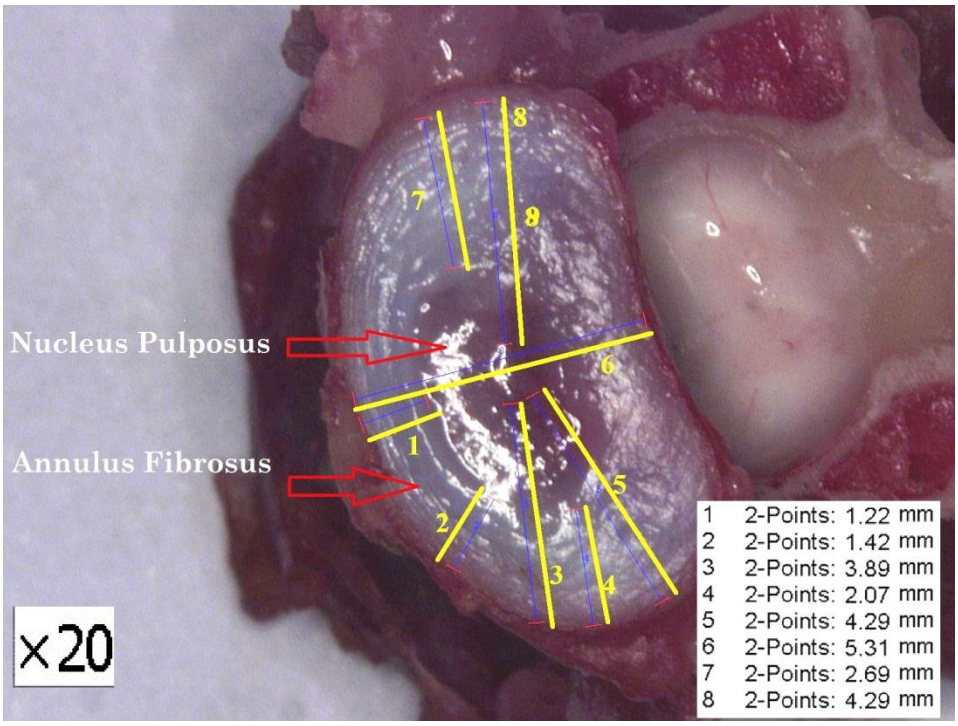


Fig 4-4: Light micrograph of intervertebral disc of lumbar region of New Zealand white rabbit.

4.4.1. Rabbit gait and behavior – post IVD degeneration

Post-surgery of NP degeneration and again re-surgery thereafter for introducing the hydrogel with and without cells, experimental rabbits showed normal gait and behavior where they are able to walk and feed and drink *ad libitum*. This indicated that damage was restricted to the NP area of the IVD alone and the spinal cord is uninjured. There was neither inflammation nor necrosis and healing was uneventful.

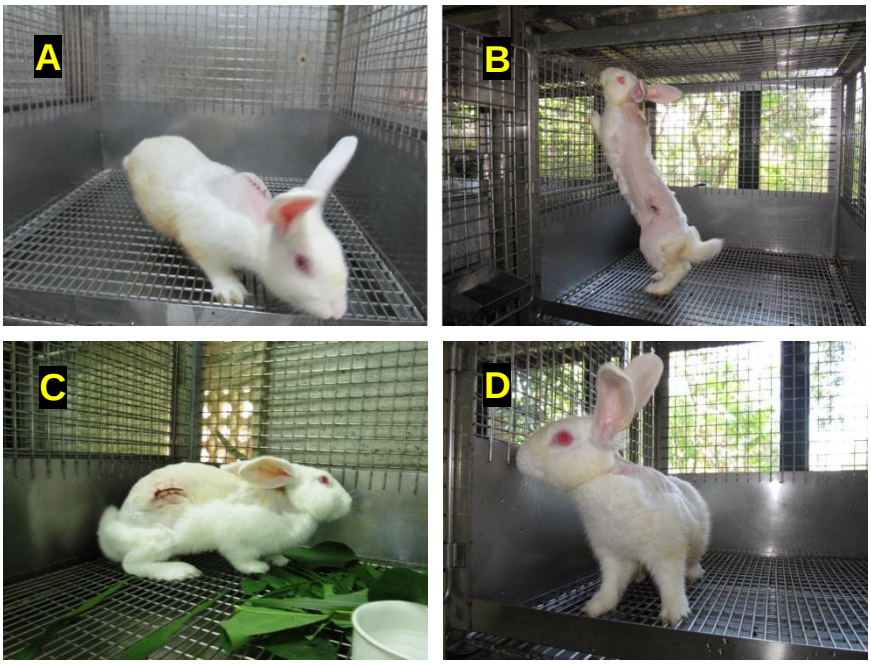


Fig 4-4-1: Rabbit behavior – gait and movement (1) post-physical injury of NP degeneration in developing the IVD model (A, B); (2) injection of Alg/Sr with cells (C, D), one month post NP injury.

4.4.2. Radiography – post degeneration of NP to create Rabbit IVD model:

After inserting the punctuation needle for ensuring the NP degeneration of disc, radiography is essential. Radiography of the vertebra - sham of rabbit IVD model. The degeneration of lumbar NP-IVD is visible by the needle puncture in the L5/L6 intervertebral disc.

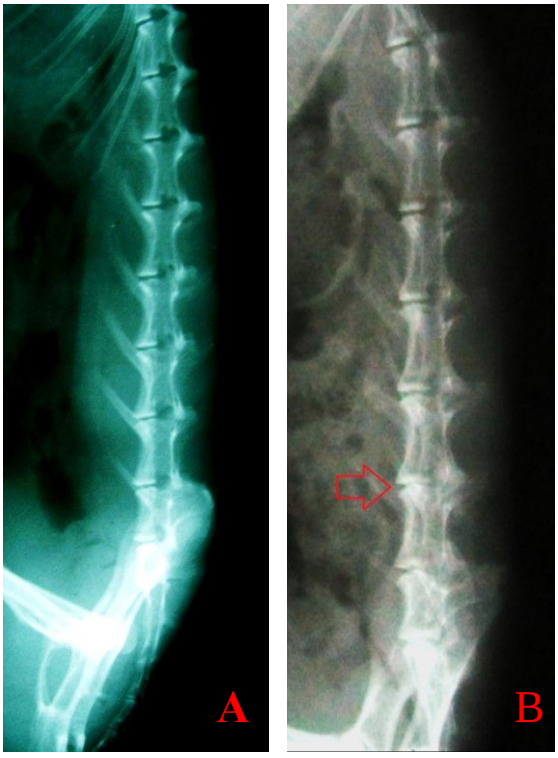


Fig 4-4-2: Radiographs of the lumbar region of vertebral column of rabbit NP-IVD defect model. Control (A). Degenerated disc - arrows shows degeneration (B).

4.4.3. Rabbit NP-IVD model - Injection of Alg/Sr hydrogel with and without cells using the applicator, post NP injury one month

4.4.3.1. Radiography

X-Ray radiographs revealed degeneration in the L5/L6 in the Rabbit NP-IVD model (sham), Interestingly, regeneration of the injured NP of the intervertebral disc of Alg/Sr-cells Group and Alg/Sr Group was evident from the radiograph after a period of three and six months respectively.

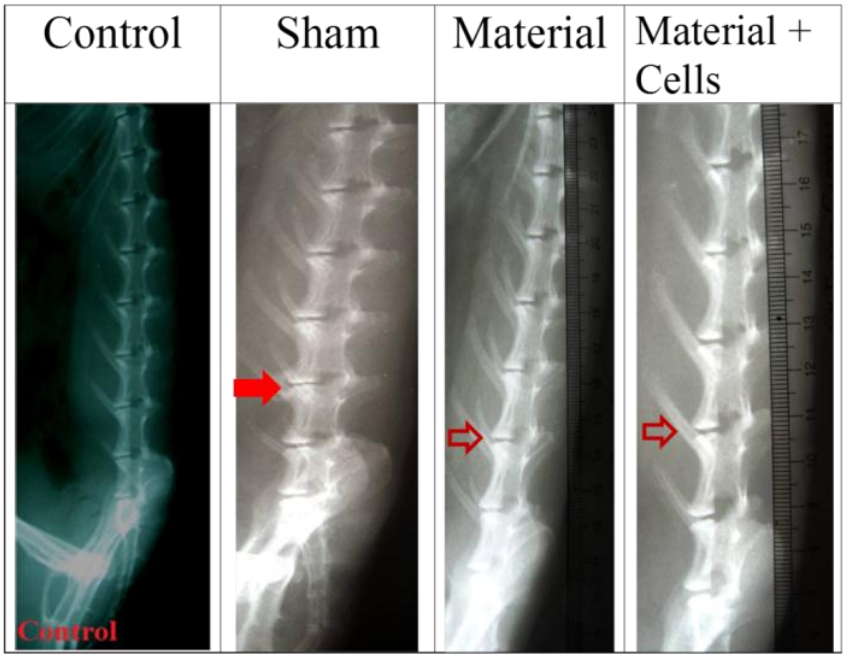


Fig 4-4-3-1: Radiographs of Rabbit lumbar region gap between 2 vertebrae in Material cells Group is more than sham after a period of 3 months.

4.4.3.2. Magnetic Resonance Imaging (MRI) of rabbit NP-IVD defect site

Magnetic resonance imaging (MRI - used T2) gave better resolution of NP-IVD images. Regeneration was very prominent in the Alg/Sr-cells Group after a period of 3 months and with Material alone Group at 6 months.

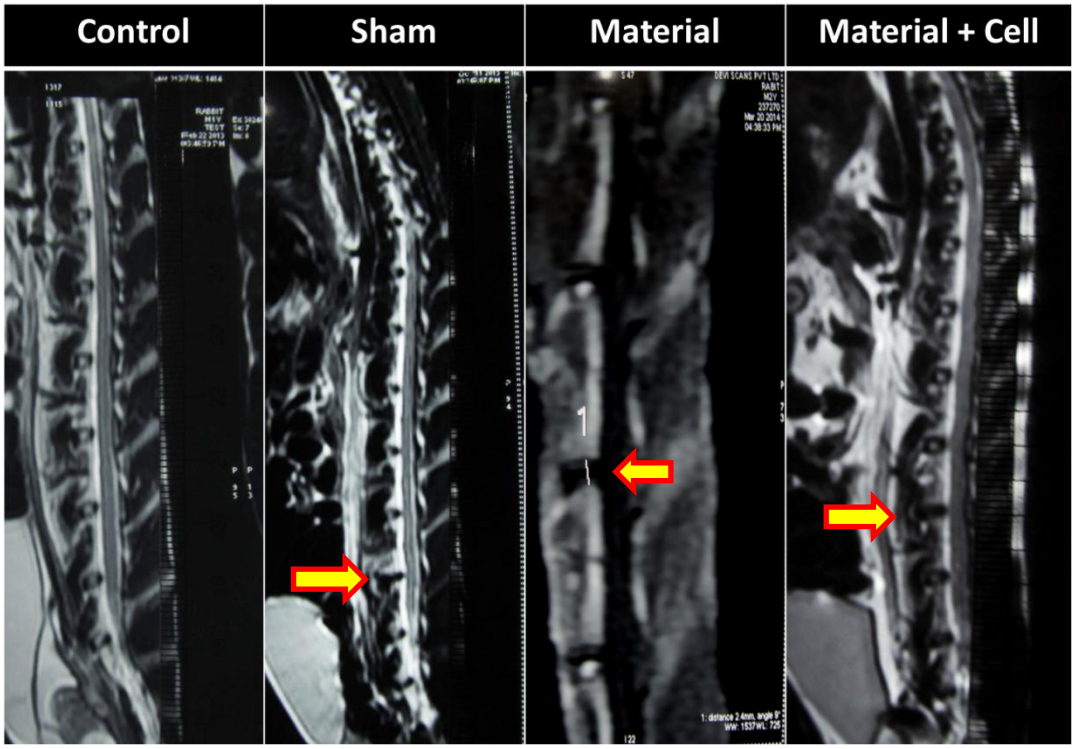


Fig 4-4-3-2; MRI images showed good regeneration at NP-IVD defect site in Material+ cells and Material Groups compared to Sham.

4.4.4. Histology and Histomorphometry of regenerated Rabbit NP-IVD defect model

Light micrographs indicated that in sham group total degeneration was observed after one month. After 3 months, regeneration was observed in the material Group. By 6 months, regeneration was observed in the damaged NP-IVD. Regeneration in material-cell Group was high in 3 months comparable with the control Group. Histomorphometry was done according to the area of regeneration at the NP-IVD defect site.

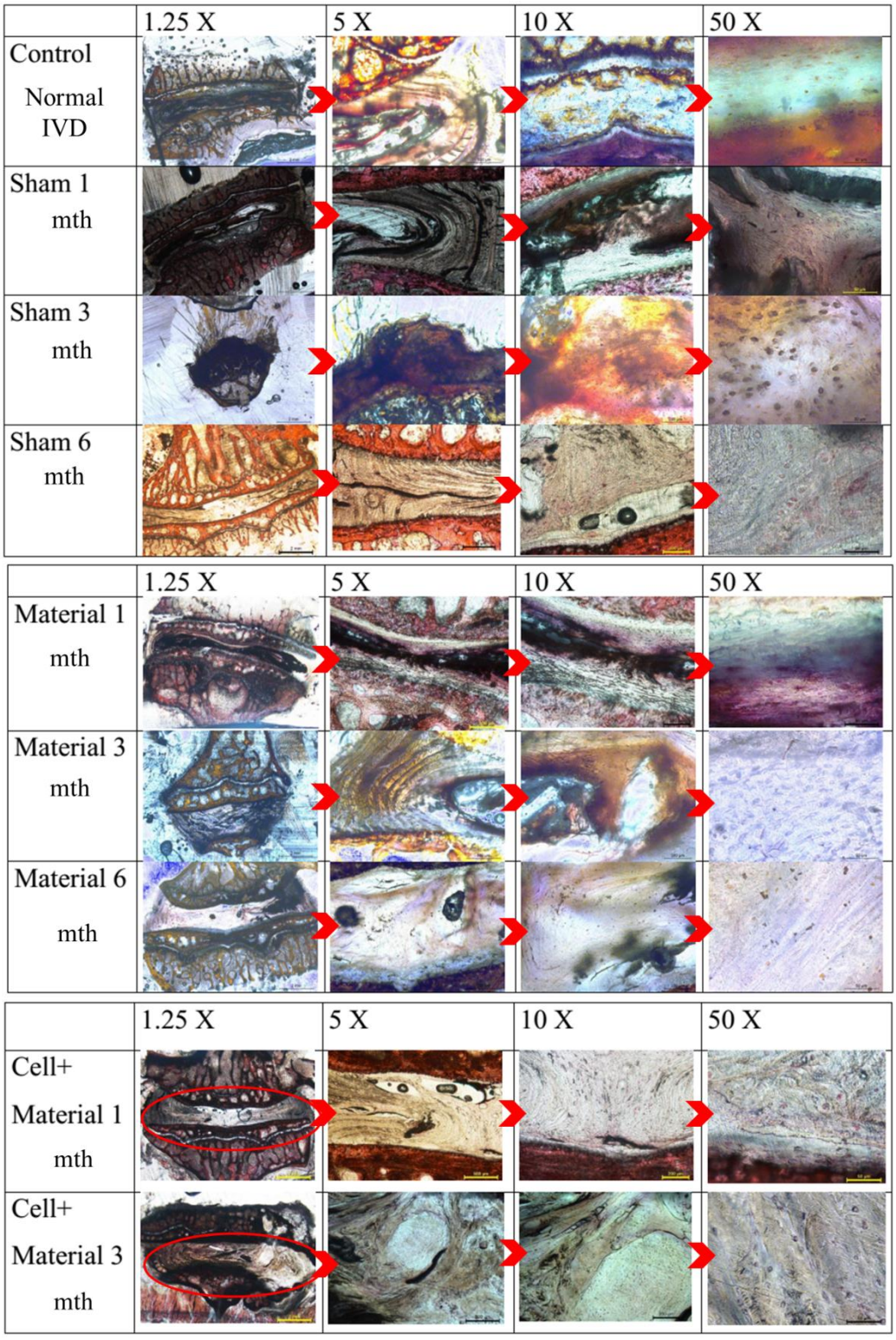


Fig 4-4-4: Light Micrographs of rabbit lumbar NP-IVDs of Control, sham, material and cells +material Groups at time intervals of one, three and six months.

4.4.1.1. Histomorphometric evaluation

Histomorphometry evaluation indicates maximum regeneration in the 3 months cells-material Group and 6 months materials alone Group.

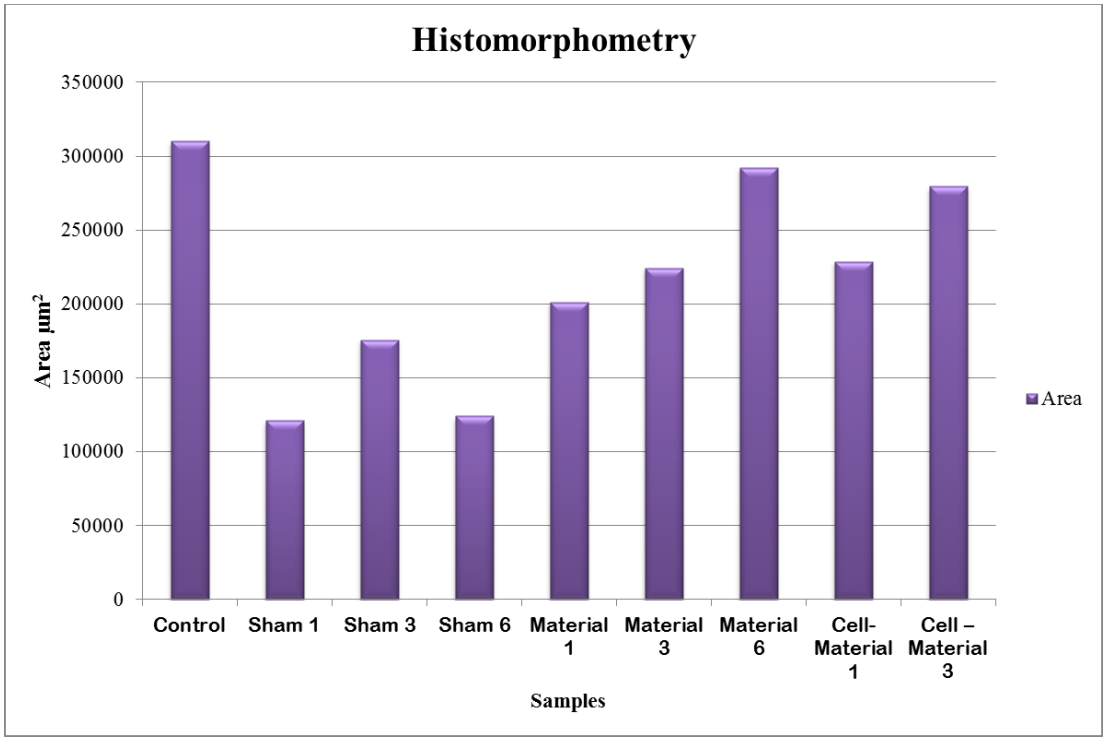
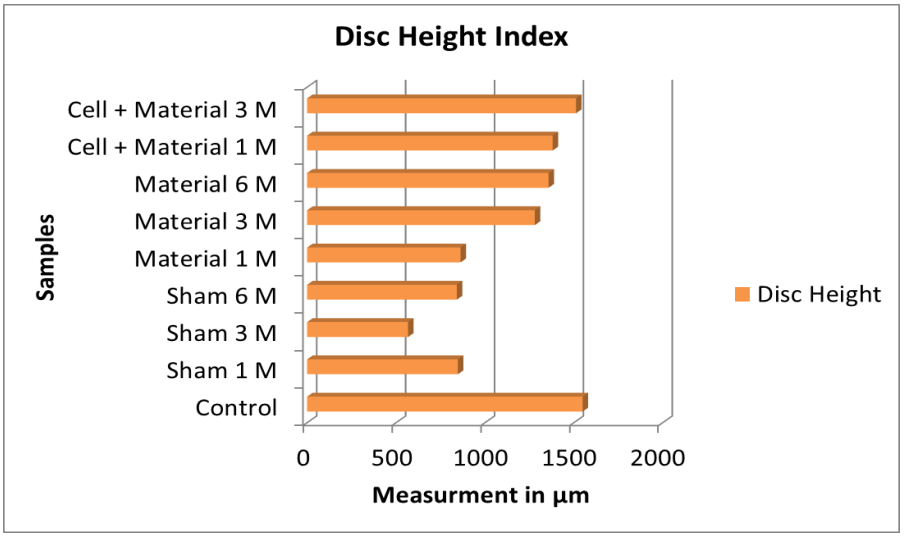


Fig 4-4-4-1: Regeneration of intervertebral disc by measuring total area of space between two vertebrae.

4.4.5. Disc Height Index and Disc Grading Evaluation of the regenerated disc in Rabbit NP-IVD defect model at different period of study.

Disc grading was done according to the disc height index which is a good indicator of IVD regeneration and repair. Disc height index observed at 3 months in the cells-material Group is higher than that observed at 6 months in the material alone Group and in turn equivalent to the Control Group too. Disc height index observed in the Cells+Materials Group at one month is equivalent to Materials alone group at 6 months followed by Materials alone Group at 3 months. So Cells+Materials at 3 months scored the highest grade of promoting regeneration of the injured NP.



1.	Control		1	2	3	4	5
2.	Sham 1 M		1	2	3	4	✓5
3.	Sham 3 M		1	2	3	✓4	5
4.	Sham 6 M		1	2	3	✓4	5
5.	Material 1 M		1	2	✓3	4	5
6.	Material 3 M		1	2 ✓	3	4	5
7.	Material 6 M		1	2 ✓	3	4	5
8.	Cell + Material 1 M		1	2✓	3	4	5
9.	Cell + Material 3 M		1 ✓	2	3	4	5

Fig 4-4-5: Disc Height Index (A) and Disc Grading remarks (B) of the regenerated disc in Rabbit NP-IVD defect model in – Control, Sham, Material, Cells+Material Groups at different period of one , three and six months.

CHAPTER 5

DISCUSSION

CHAPTER 5

DISCUSSION

5.1. Anatomy of IVD and its properties

Anatomy of nucleus pulposus (NP) of intervertebral disc and its mechanical properties indicates its small size and highly gel like appearance in rodents as well as in rabbits. Intervertebral disc degeneration and regeneration is multifactorial and complicated process. Any type of treatment for back pain should be tested in natural condition till all the elements and factors which are interfering in recovering of degenerated disc can be estimated and measured. There are structural differences and similarities between human skeleton and animal skeleton. Humans are biped but animals are quadruped (Alini et al. 2008). Therefore different types of animals can be used for various tests. In this study New Zealand White rabbits have been used due to easy handling and less food consumption of the animal (Alini et al. 2008; Daly et al. 2016). Rabbit lumbar region was selected as part of interest for this IVD research (fig 4.1). Intervertebral discs were separated from vertebral column and cleaned from extra muscles. White area among vertebrae bone appeared as location of intervertebral disc (fig 4.1.A). Location of intervertebral disc was also determined by using of X-Ray imaging. After sectioning of the vertebral column; IVDs with jelly center was visible in the Petridish (fig 4.1.B). Nucleus

pulposus dried after 60 minutes at room temperature due to their jelly structure(Greenaway et al. 2001). Access to the nucleus pulposus was determined by inserting needle under X-ray. For better understanding of IVD structure; intervertebral discs of five types of animals (rat, rabbit, goat, cattle and pig) were studied. Their compression and tensile strength has been tested(Zhang et al. 2014). Gross images of different types of intervertebral discs revealed jelly - like appearance of nucleus pulposus at the center of the IVD in the transverse plane of IVD (fig 4.1.1). Keyence microscopy result revealed that from the small rodent to bigger animals, the area of intervertebral disc increased. Cattle showed the largest area of IVD as well as NP size. Light micrographs of rodents and bigger animals showed big vacuoles in the notochordal cells in rodent and this is absent in the bigger animals (fig 4.1.2). In the smaller animals there are plenty number of collagen fibers compared to the bigger animals (Shankar, Scarlett, and Abram 2009). Ultrastructural micrographs supported the histological images (fig4.1.3).

Tensile strength measurements of these animals revealed the highest strength of pig lumbar region compare to other animals while the IVD area of cattle was more than pig (fig 4.1.4.1). These results showed that for the second trial of NP repair, pig is a suitable animal before entering clinical trial (Tschugg et al. 2016). All over checking and testing of different animals, New Zealand White rabbits showed better properties for phase I trial of new material for IVD regeneration.

Compression and tensile strength test of rabbit revealed from L1/L2 to L6/L7 intervertebral disc area, tensile and compression forces increased. In all animal models, iliac bone was selected as land mark for counting the vertebrae during the surgery to find out the exact location for puncturing the IVD. The L3/L4 and L4/L5 are suitable intervertebral discs in the rabbit according to their size and strength to withstand the surgery.

5.2. Material Characterization

Different hydrogel combinations of Alginate and strontium will give different properties to hydrogel for load bearing and cell encapsulation – example hydrogel like chitosan–poly (hydroxybutyrate-co-valerate) with chondroitin sulfate(Nair et al. 2015), PLLA/alginate(Li, Lu, and Zhou 2011), Type II collagen-hyaluronan(Calderon et al. 2010) and methacrylated gellan gum (GG-MA)(Silva-Correia et al. 2011) for total regeneration. For this, material characterization has been considered.

Mechanical properties, porosity and cell encapsulation was the main aim for designing of this type of hydrogel which can be injectable with applicator to form the hydrogel directly in the *in-situ* condition. Alginate mimicking the nucleus pulposus condition provides niche for cell to encapsulate inside the gel. Strontium played a big role as oxidizing agent in this hydrogel and provides covalent and ionic bond with alginate monomers(Sakai et al. 2008; Suguna and Sekar 2011).

Alginate and strontium solution were prepared in several concentrations. Finally the optimum concentration for alginate characterized on 4% passed through 22 G needle for injection in the central region of intervertebral disc. Strontium solution was adjusted in 3 different concentrations M_1=1.2%, M_2 = 1.8% and M_3= 2% (Graph 4.2.1.A). Alg/Sr hydrogel (M_2) showed high around 0.18 mPa stiffness like NP with easy pass

through 22 G needle. This property of material M_2 was the best option among the other two hydrogels. M_3 was not passing through 22G needle since stiffness was very high and M_1 was passing through 22G needle but stiffness was very low. In the comparision test displacement M_2 and M_3 was almost similar to each other (fig 4.2.1.A)

Assessment of porosity was determined with Micro-CT. 3 dimensional hydrogel block stained with osmium tetroxide to enhance contrast infra-structure of hydrogel block. Estimation of μCT revealed 74.28% porosity of hydrogel block (fig 4.2.2.1). This porosity is optimum for NP repair(Dadsetan et al. 2008), and exactly mimicking the structure of NP. Nucleus Pulposus contains 75% of water content and 25% of other ingredients like proteoglycans and collagens (Shankar, Scarlett, and Abram 2009; Colombini et al. 2008; Iatridis et al. 2007). Images of μCT indicate inter connectivity of pores in the hydrogel block with their wall thickness. The cells can migrate and attach to the walls of the hydrogel block and distribution of pores is visible in green color which indicate good porosity all over the Alg/Sr hydrogel block.

For comparing the results of hydrogel with 16 G needle and 22 G needle, SEM analysis was conducted. Hydrogel with 22 G needle had more porosity compared to 16 G needle (fig 4.2.2.2). Therefore thickness of the applicator needle has direct effect on the porosity of hydrogel. Thinner the needle, greater will be the hydrogel that pass out. But these techniques have

their own barrier. Designed hydrogel must be able to pass through the desired needle.

Alg/Sr hydrogel has been cryosectioned to ensure the porosity in normal condition (fig 4.2.2.3). Toluidine blue stain was used for each section. There were visible porosity overall on each section. Alg/Sr hydrogel was traced under the X-ray beam due to its radiopacity (fig 4.2.3). Radiograph properties of strontium reveal of alginate bead even in low percentage compared to hydroxyapatite material(Lam et al. 2015).

Researchers are trying to investigate any material under different conditions like normal body is facing. Three dimensional hydrogel networks include ionic and covalent bonding. By increasing the heat hydrogel release the fluid and later on absorb water from the surrounding tissue. Thermally reversible hydrogel are not only interesting but also they are important for commercial purposes (Roorda et al. 1988; Megeed, Cappello, and Ghandehari 2004). An enthalpic change does not occur by heat on this hydrogel. The graph showed structural changes at 66.2 ^{0}C which revert to main structure after 70 ^{0}C (fig 4.2.4.1).

Further investigation of thermogravimetric analysis of Alg/Sr hydrogel indicates material mass loss is 87% at 37^{0}C. It means that by reaching temperature to 37^{0} C which is like human body. Material water loss is just 13% and still 87% of material is remaining at that temperature. By

increasing the temperature in the open area, the rest of the hydrogel water will vaporize at 79.01^{0}C (fig 4.2.4.2).

Components of this hydrogel was tested by fourier transform infrared spectroscopy (FTIR). Alginate peaks were similar to Alg/Sr hydrogel due to the ionic and covalent bond in hydrogel.

5.3. *In Vitro* analysis of Alg/Sr Hydrogel

Before initiation of *in vivo* experiments, any material should be biocompatible to be used safe in the human body (Annunziata et al. 2006). Alg/Sr hydrogel passed the cytotoxicity test with fibroblast cells (L-929) without cell population reduction (Ozdemir, Yilmaz, and Yilmaz 2009). RADMSCs isolated from Rabbit Adipose tissue (Brayfield, Marra, and Rubin 2010; Gimble, Katz, and Bunnell 2007) were cultured and expanded *in vitro* and i*n vivo* (fig 4.3.1)(fig 4.3.2) and characterized by_ specific markers like CD45, CD 90 and CD 105 by flow cytometry (fig 4.3.3) (Ong et al. 2014; Camilleri et al. 2016). Cell-surface immunophenotype for RADMSCs were positive for CD 90 and CD 105 and negative for CD 45. Cells were counted with hemocytometer and confirmed by scepter cell counter -1.2 * 10^5 Cells (fig 4.3.4) (Halloran et al. 2008; Peroglio et al. 2012) for encapsulation within the 3D hydrogel where cells proliferated and remained viable. High cell numbers like $2*10^6$, $3.3*10^6$ may easily lead to cell clusters and ultimately to apoptosis. Researchers could reduce the speed of degeneration but production of extra cellular matrix was less (Chiba et al. 1997; Yu 2002). In this study the cell number used was less ($1.2*10^5$) than elsewhere and the 3D design of hydrogel encapsulated the cell very nicely and is functional. Many researchers preferred to isolate NP cells rather than adult stem cells. The disadvantage of using NP cells is that they are already under pressure and these cells are exhausted and under the influence of different types of internal cytokines to form cell clusters (Kong et al. 2008; Melrose et al. 2008;

Sun et al. 2016). The advantage of RDAMSCs is that they are fresh cells and encourage co-culture systems under hypoxy condition when they are injected into the nucleus pulposus. The newly arrived cells in the NP are induced to differentiate into NP-like cells influenced by the host NP cells for which growth factors are not required.

Proliferation assay of RADMSCs revealed better growth of cells in 3 D Alg/Sr hydrogel increasing in number compared to cells alone as control (fig 4.3.5). Cell growth in 3D is better than 2D culture conditions (Chen et al. 2015). Alg/Sr hydrogel encapsulated the cells and provided a habitat for them to multiply and expand profusely retaining their spindle shape visible in the environment friendly 3D hydrogel block (fig 4.3.6).

Illustration of actin by confocal microscopy revealed nucleus of clustered cells at different depths of the hydrogel. Cells were alive and 3D z-stack mode thickness of cell population could be captured (fig 4.3.7).

Live-Dead assay of cells within Alg/Sr hydrogel revealed healthy encapsulated cells in the hydrogel block. Green cluster of cells were visualized by confocal imaging and in 3D z-stack mode imaging, cells were visualized in the center of the hydrogel block (fig 4.3.8). This indicated eco-friendly environment of Alg/Sr hydrogel for cell growth, expansion, attachment and differentiation.

5.4. *In Vivo* analysis of Alg/Sr Hydrogel

After a study of the IVD from different animals, rabbit appeared as the best choice for *in vivo* experiments. Rabbit lumbar vertebrae was examined for its anatomy, histology, tensile and compression measurements. After dissecting the lumbar region of rabbit vertebral column, IVD were separated in the location L1/L2 to L7/S1. Transverse section of intervertebral disc revealed intervertebral disc anatomy. Keyence microscope confirmed the distance from the central part of rabbit IVD which is the jelly NP to the outer layer of AF to aid the puncturing and injection of Alg/Sr hydrogel(Greenaway et al. 2001).

According to the measurements from the keyence micrograph, the distance between outer layers of IVD to center of NP is equal to 3.89 mm (fig 4.4). It means that for puncturing and insertion of hydrogel needle the distance to be covered is 3.89 mm and further insertion of the needle more than 5 mm will prick the spinal cord and ultimately the rabbit will paralyze. During surgery, tip of the needle is marked for a maximum of 4 mm prior to insertion into the intervertebral disc(Kong et al. 2008).

By setting up all requirements for the first surgery, rabbit undergoes anesthesia for developing the NP-IVD degenerative model by opening up from the left mid dorsolateral side. Pelvic bone can be used as a land mark for counting vertebrae from L7 to L1. The physical injury with the needle is

done on the NP of the IVD mainly on L4/L5 or L5/L6 to create the NP-IVD model. Thereafter post one month of physical injury, rabbit was taken for X-ray radiography to confirm NP degeneration of L5/L6 region compared to the control animal (fig.4.4.2).

After every surgery rabbit was checked for any pain and behavioral changes(Morton and Griffiths 1985). Here, the rabbits were able to curl and walk which indicated that surgery was fine and there was no harm to spinal cord and only the intervertebral disc was targeted. In this study, no animals were paralyzed due to the expertise surgical skill (fig.4.4.1).

Subsequently, Alg/Sr hydrogel alone and with cells were injected into the injured NP, post one month of degeneration for a period of one , three and six months respectively. Thereafter radiography evaluations indicated degeneration still persisted in the L5/L6 sham group while considerable regeneration of NP after 3 months in the Alg/Sr alone group and Alg/Sr in cooperated with cells were observed (fig 4.4.3.1). MRI imaging also confirmed increase of disc height index (DHI) in Alg/Sr and Alg/Sr combined cell group (fig 4.4.3.2) by T2 MRI system (Menezes et al. 2004; Krueger et al. 2007).

Many reports explained the production of extra cellular matrix and COL II which indicates NP is under the healing process rather than evaluate the disc height (Li et al. 2014; Fontana et al. 2014; H et al. 2010). After

expression of the extra cellular matrix by cells, the environment of NP must be stable and stiff to sustain the disc height for a period of time. Normally the disc height increases temporarily but continuation of healing process is the most challenging part of IVD regeneration. In this study, NP regeneration was evident by histomorphometric analysis and in turn by the disc height index over a period of time (fig.4.4.4.1) (fig.4.4.5).

Histology evaluation revealed NP regeneration on Alg and cell -Alg groups. In the 6 months Alg-Sr group there was significant regeneration compared to sham 6 months. In 3 months cell-Alg group, regeneration rate was better than 3 months Alg-Sr and sham (fig 4.4.4). Histology was evaluated with Disc Height Grading Index - one month sham as grade 5 (total degeneration) while in sham 3 and 6 months there was minimum regeneration of the degenerated tissue initiating healing (Roberts, Gratin, and Whitehouse 1997; Wilke et al. 2006). Alg-Sr group after 3 months showed good progress of healing. However, in the cell-Alg/Sr group, good regeneration of intervertebral disc was observed in the first month itself and thereafter 3 months it was more than 90%.

Alg-Sr & cell combination product healing at 3 months is comparable to bare Alg-Sr hydrogel at 6 months. Hence, “Alg-Sr & cell combination product” definitely helped in enhancing the healing of the damaged NP and increasing the disc height index.

Compared to other studies, this preclinical study mainly focused on the stiffness and load bearing of hydrogel to permit cells to proliferate, expand and provide extra cellular matrix to increase the disc height by regeneration of the defected disc and revert to normal function. Further study in a larger animal NP-IVD model is warranted prior to clinical trials, The Alg/Sr hydrogel scaffold is a potential candidate for *tissue* engineered *nucleus pulposus*. In the clinical perspective, "Alg-Sr & cell combination product" may be an interim relief to improve the quality of life of the younger generation affected by low back pain. Though results from all animal models are impressive, questions remain unanswered - as to whether a quadruped disc with its very different load can serve as an adequate model for biped disc repair. Again, can an acute disc model created in all animal models be a surrogate for chronic degenerated disc normally encountered in patients (Yoshikawa et al., 2010). There's a long way to go before an optimal scaffold for NP-IVD repair is identified and a combinatorial application of adult stem cells and injectable hydrogels will be advantageous in NP tissue engineering. Besides conservative and surgical treatments (removal of disc and vertebra fusion), cell therapy foresees the possibility of regenerating the damaged NP where cells can proliferate *in vitro* and re-implanted to alleviate the pain and further prolong the damage to sustain quality of life. Hence, the prospect of cell-based Nucleus Pulposus (NP) tissue-engineering strategy has become attractive and relevant in Regenerative Medicine.

CHAPTER 6

SUMMARY & CONCLUSION

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Intervertebral (IVD) is a major pathological process (age, slip or rupture) implicated in low back pain. A damaged disc may press spinal cord or spinal nerves towards the intervertebral foramen of the vertebral column and cause pain or contribute towards loss of the disc height. This has emerged as the most expensive global healthcare problem ranging from $20 to $100 billion per year. Besides conservative and surgical treatments (removal of disc and vertebra fusion), cell therapy foresees the possibility of regenerating the damaged NP where cells can proliferate *in vitro* and re-implanted to alleviate the pain and further prolong the damage to sustain quality of life. Hence, the prospect of cell-based Nucleus Pulposus (NP) tissue-engineering strategy has become attractive and relevant in Regenerative Medicine.

The fibrocartilaginous structure of IVD comprising of Nucleus Pulposus (NP), Annulus Fibrosus (AF) and End Plate (EP) that lie in between the vertebrae are responsible for the movement and bending of the vertebral column. IVD prevents the vertebrae from grinding against one another and absorbs shock caused by such movements as running, jumping and even walking. NP is the inner core of the IVD that

usually decreases its height and thereby the Annulus Fibrosis bulges out impinging on the nerves, ultimately causing pain. When NP cells are not able to provide sufficient Extra Cellular Matrix (ECM), NP looses the water content resulting in reduction of the disc height and subsequently low back pain

The Thesis is divided into five chapters - The first chapter describes the structure of the spine, vertebral column and the IVD and its composition. Degenerative Disc Disease and its pathogenesis contributing to low back pain.. Current therapeutic options for degenerative disc disease such as non-operative management, surgical management, spinal fusion, total disc arthroplasty, nucleus pulposus replacement, cellular and molecular Therapy, Nucleus Pulposus Tissue Engineering are described in detail.

In the second chapter, a review of literature is carried out on different types of natural polymers like Polycaprolactone (PCL), Gelatin/chondroitin-6-sulfate/hyaluronan, Agarose/collagen, Collagen/Glycosaminoglycan, Collagen, Polyglycolic acid/alginate have been used for repairing the intervertebral disc in different animal models like – Rat, Rabbit, Pig but all of them are far from clinical usage. The major clinical problem of tissue-engineered disc is the insufficient biomechanical behavior and stiffness for long term usage. The attraction of fabricating tissue-engineered disc is by using either the cultured disc cells or by converting stem

cells into cartilage cells in combination with the scaffold. This appears to be a better option for NP tissue engineering.

The third chapter deals with Materials and Methods pertaining to the study of the IVD of different animals like – rat, rabbit, goat, sheep, cattle and pig; preparation and characterization (FTIR, DTG, TGA, Micro-CT (porosity) & mechanical testing) of the alginate strontium hydrogel; *in vitro* of Rabbit Adipose-derived Stem cells (RADMSCs) and fabrication of the tissue- engineered construct substitute; *in vivo* development of the NP-IVD Rabbit defect model by physical injury and finally transplantation of the substitute into the NP defect site. Radiographs of the Rabbit vertebral column showed the prolapse of the Disc at L5/L6 in degenerative IVD model after needle injury – post injury 7 days. Thirty animals survived injury. Further, post-surgery one month, a re-surgery was done (from right dorsolateral side) to inject bare Alg-Sr and Alg-Sr/cell combination product *via* a dual in-house applicator after radiographic confirmation of the planned damaged IVD.

The fourth chapter describes the anatomy, histology and mechanical properties of the IVD of different animals prior to embarking on the study of the Rabbit IVD; Combination of alginate (4%) with Strontium chloride ($SrCl_2$ _ 1.8%) (via a dual in-house applicator with 22G needle) gave a porous Alginate-strontium (Alg-Sr) hydrogel similar to the NP which proved noncytotoxic; *in vitro* RADMSCs proliferated and differentiated and were viable within the encapsulated Alg/Sr hydrogel to form the cell-

construct substitute; and transplanted Alg/Sr-cell construct in the NP defect site *in vivo* showed regeneration of the NP evidenced by the disc height evaluated by radiography. Post injection one, three and six months, X-ray and MRI were done and retrieved IVD samples were subjected to histology and histomorphometric analysis.

The Fifth chapter deals with Discussion with the outcomes of the study. The IVD of different animals showed different tensile and compressive strength. TGA and DTA showed the mass loss and enthaplic changes of the Alg-Sr Hydrogel. FTIR spectra showed no significant difference between Alginate and Alg-Sr.Alg-Sr passed the Cytotoxicity test. RADMSCs were cytocompatible with Alg-Sr Hydrogel and favored proliferation. Histomorphometric analysis showed that: Bare Alg-Sr hydrogel improved healing compared to sham at one month. Alg-Sr & cell combination product enhanced healing compared to bare Alg-Sr at one and 3 months. Alg-Sr & cell combination product healing at 3 months is comparable to bare Alg-Sr hydrogel at 6 months. Hence, "Alg-Sr & cell combination product" definitely helped in enhancing the healing of the damaged NP and increasing the disc height index.

Conclusion

Alg/Sr hydrogel scaffold is a potential candidate for *tissue* engineered *nucleus pulposus*. In the clinical perspective, "Alg-Sr & cell combination product" may be an interim relief to improve the quality of life of the younger generation affected by low back pain. Though results from all animal models are impressive, questions remain unanswered - as to whether a quadruped disc with its very different load can serve as an adequate model for biped disc repair. Again, can an acute disc model created in all animal models be a surrogate for chronic degenerated disc normally encountered in patients (Yoshikawa et al., 2010). A long way to go before an optimal scaffold for NP-IVD repair is identified and a combinatorial application of adult stem cells and injectable hydrogels will be advantageous in NP tissue engineering.

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APPENDIX

APPENDIX

1. **SBF Buffer**

Sodium Chloride	:8.05g
Sodium carbonate	:0.355g
Potassium chloride	:0.225g
Dipotassium hydrogen phosphate	:0.261g
Magnesium chloride hexahydrate	:0.311g
1 M Hydrophilic acid	:35ml
Calcium chloride Dihydrate	:0.55g
Sodium Sulphate	:0.072g
Tris	:6.60g

2. **H and E stain**

- **Harri's Hematoxylin stain**

A = 1 gm hematoxylin in 10 ml ethanol

B = 20 gm ammonium alum in hot distilled water

Mix A & B, boil and add 0.5 gm of mercuric oxide and filter.

- **Eosin solution**

Yellow eosin = 1 gm

Distilled water = 80 ml

Ethanol = 320 ml

Glacial Acetic Acid = 2 drops

- **0.5% HCl**
- **Dilute ammonia water**

LIST OF PUBLICATION

1. **Mir Mahmoud Mortazavi R** , Annie John; Intervertebral Disc Damage & Repair - its Pros and Cons. (World Journal of Research and Review, ISSN: 2455 - 3956 , Volume-2, Issue-2, February 2015 Pages 01-12).

2. **Mahmoud Mortazavi R** , Annie John, Harikrishnan VS, Suresh Babu, A functional injectable hydrogel for Nucleus Pulposus Regeneration, (Under preparation)**.**

3. **Mir Mahmoud Mortazavi R** , Annie John; Intervertebral disc repair and obstacles (Under preparation)**.**

4. **Mir Mahmoud Mortazavi R** , Annie John; A proper animal model for IVD regeneration (Under preparation)**.**

World Journal of Research and Review (WJRR)
ISSN:2455-3956, Volume-2, Issue-2, February 2015 Pages 01-12

Intervertebral Disc Damage & Repair – its Pros And Cons

Mir Mahmoud Mortazavi R ,Annie John

***Abstract*— Incidence of Low Back Pain (LBP) is attributed to the degeneration of the intervertebral disc (IVD) occurring during the second or third decade of life. This has emerged as the most expensive global healthcare problem with costs in billion. The IVD comprises of an inner nucleus pulposus (NP) and an outer Annulus Fibrosis (AF). They act as cushions between the vertebrae of the vertebral column. Current treatment modalities involve conservative management (medication and physical therapy) or surgical intervention (spine fusion, total disc replacement (TDR), or NP replacement. Since the last decade, there has been a surge of interest in applying tissue-engineering principles (scaffold and cells) to treat spinal problems associated with IVD.**

***Index Terms*— Low back pain, Intervertebral disc, Nucleus pulposus, Annulus Fibrosis,**

I. Structure of the Vertebral Column

The vertebral column consists of five regions (cervical vertebrae, thoracic vertebrae, lumbar vertebrae, sacrum region and coccyx region)[1].

Vertebrae in vertebral column are separated by cartilaginous intervertebral discs[2]. "S" shape of back bone gives special features and function to the body to support skull and upper extremities, trunk of muscles, bipedalism, movement and flexibility[3] (Fig1).

Vertebrae consist of special shape on the anterior side "Drum Shape", which is connected to the intervertebral disc above and below. The vertebral arch is connected to the posterior side of the body with two pedicles and two arched laminae. Vertebrae contain special foramen for the passing spinal cord. Spinal cord nerves are passing through intervertebral foramina to the anterior organs[3].

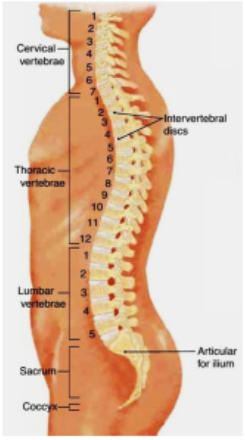

Figure1: Vertebral Column of human body with S-shape. Adapted from clinical anatomy of spine, Gregory D et al)

There are distinctive features for the different vertebrae in cervical vertebrae the transverse foramen is visible. In thoracic vertebrae facets join for connecting to the ribs and the lumbar region vertebrae contain flat spineous process for muscle attachment[3] (Fig2).

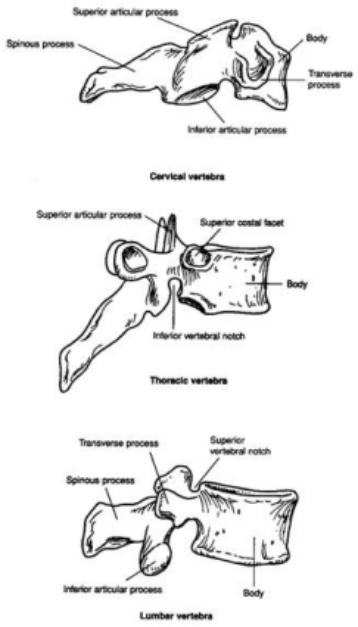

Figure2: Examples of vertebrae from different vertebral regions (adapted from human anatomy and physiology, Kent et al)

II. Intervertebral Disc Development and Composition

➢ Intervertebral Disc Development:

Notochord with mesodermal origin makes axial cord at the center of embryo. Mesenchymal cells which are available surrounding the notochord provide a prichordal shape[4]. This structure takes the notochordal cells inside a clear notochordal sheath. By pressure of the sheath some notochordal cells push to the vertebra bodies while the segmentation of

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A FUNCTIONAL INJECTABLE HYDROGEL FOR NUCLEUS PULPOSUS REGENERATION IN LAPINE INTERVERTEBRAL DISC DEFECT MODEL

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ABSTRACT

Nucleolus Pulposus (NP) of the Intervertebral disc (IVD) is the first tissue to initiate degeneration especailly from a younger age which eventually leads to low back pain. IVD degeneration happens due to unusual sitting, trauma, genetic inheritance and impaired nutrient exchange between nucleus pulposus and blood vessels. If the extracellular matrix of NP remains the same throughout life, the disc height will not collapse. In this study, alginate was selected as the material of choice to mimic the NP. Alginate hydrogel was made in different concentrations incorporating strontium and tested for the required compression strength to mimic the real NP which is always under constant pressure. The designed and fabricated Alg/Sr hydrogel was tested for cytotoxicity to depict the safety of the material and further tested *in-vitro* by mimicking an *in vivo* 3 dimensional structure incorporating cells which was ultimately injected *in situ* at the physically damaged NP site of the lumbar vertebrae (L3-L4/L5-L6) of New Zealand White rabbits *in situ.* This study showed that Alg/Sr Hydrogel is permissive and a promising hydrogel which can encapsulate cells for secreting extra cellular matrix (ECM) to restore the collapsed disc height to a certain extent to enable temporary relief of low back pain.

KEYWORDS: Nucleus pulposus, Alginate/Strontium Hydrogel, Intervertebral Disc Lapine IVD model.

INTRODUCTION

In the first year of life, NP cells as well as notochordal cells are abundant to enhance secretion of extra cellular matrix like proteoglycans and aggrecans. They make a rich fiber network with different ions including Na^+ and K^+ and are responsible for fluid absorption towards the central region of NP. This rich environment act like a semipermeable membrane to absorb water from the surrounding tissue.[1] More the water absorption, greater is the increase in the disc height. Degeneration of intervertebral disc may occur in the nucleus pulposus (NP) especially from childhood. After the disappearance of notochordal cells from NP in the first year of life, from NP, the chondrocyte cells are the only cells at the center of NP which can continue enriching the extra cellular matrix by secreting collagen fibers.

As age steps in, NP looses stamina; proteoglycans and aggrecans get fragmented by different types of matrix metalloproteinase (MMPs).[2] Long chains of proteoglycans and aggrecans get shorter and shorter. They lose the properties of acting like a semipermeable membrane. By decreasing the density of Na^+ and K^+ ions, disc looses water content and disc height will collapse[3], resulting in disc bulging and herniation. There are different grades of disc herniation which leads to severe surgery like total disc replacement or partial disc replacement.[4] Herniation of intervertebral disc (IVD) leads the vertebral column to different types of deformities like scoliosis, spondylolisthesis, kyphosis and lordosis.[5] In the US, back pain is the most expensive treatment where the overall cost for treatment is around $ 91 billion per year.[6],[7] Over 80% of the younger generation below 45 years old complains of back pain which is related to less activity of their body.[7]

Tissue engineering technique can be applied for the regeneration of the damaged intervertebral disc before it reaches a severe condition,[8],[9] There is a tremendous surge to use natural biomaterials for regeneration and repair.[10],[11] One of the natural biomaterials is alginate[12],[13] suitable for such applications.

Alginate is extracted from large marine brown alga like Laminaria hyperborean - a hydrophilic polysaccharide that give stamina to brown algae. They are present in the cell wall and intercellular matrix providing the strength and flexibility to withstand the force of water.[10]

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- **Best Researcher among Iranian student in India on 2013 - selected by Iranian Embassy- NewDelhi**
- **نفر اول پروژه دانشجویی در مقطع فوق لیسانس در دانشگاه کرالا .**
- **Highest mark on my project work and industrial report among Indian student of Department of Biotechnology, University of Kerala.**
- **ارائه دهنده بهترین پوستر دانشجویی در بین محققین هندی در سال 2012**
- **"Best Poster Presentation Award"-Mir Mahmoud- SCTIMST Trivandrum,During XXIII National Conference of the Society, December 9-11,2012 at Indian Institute of Science, Bangalore, India.**
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RESEARCH EXPERIENCE

تجربیات کاری:

- **فعالیت استخوانسازی سلولهای بنیادی جدا شده از استخوان بز و تبدیل آن به سلولهای استخوانساز بر روی پولک ماهی.**
- **M.Sc Project: "Osteogenic activity of goat bone marrow-derived stem cells on fish scale – an in vitro study"**
- **باز تولید دیسک کمر با استفاده از کشت بافت**
- **PhD Project: "Alginate – Strontium Hydrogel with stem cells *in situ* for the repair of nucleus pulposus of the intervertebral disc in Lapine Model"**

TRANING INDIAN, AFRICAN AND IRANIAN STUDENT AND HOLDING INTERNATIONAL WORKSHOP FOR AFRICAN, INDIAN AND IRANIAN STUDENT.

- **برگزاری کلاسهای آموزشی بزبان انگلیسی برای دانشجویان هندی در هندوستان**
- **برگزاری ورکشاب در زمینه Reference Management and Research Methodology برای دانشجویان ایرانی ، هندی و آفریقایی.**

مهارتها:

Skills and Techniques

1. Confoucal Microscopy
2. Scanning Electron Microscopy
3. Transmission Electron Microscopy
4. SDSPage
5. Gel electrophoresis
6. Spectrophotometer
7. TLC -Thin Layer Chromatography
8. Gene Expression

Working knowledge on cell culture and tissue engineering including:

1. Preparation of a- MEM (Minimum Essential Medium)
2. Preparation of DMEM (Dulbecco's Modified Eagle's Medium)
3. Isolation of Mesenchymal Stem cells from Bone Marrow
4. Isolation of Mesenchymal Stem cells from Adipose Tissue
5. Trypsinization
6. Cryopreservation.

Embryology Techniques

A- Advanced Primer Design
B- Advanced PCR - DNA Extraction - PCR and Electrophoresis
C- Advanced Real Time PCR - RNA extraction - cDNA Synthesis - Real Time PCR and analysis
D- In Vitro Fertilization (IVF) in Mouse - mouse handling and mouse Sperm preparation- Oocyte Retrieval and Vasectomy- Mouse embryo transfer
E- Vitrification and Ovarian Tissue and Culture on the Thick Embryo
F- Mouse Oocyte and Embryo Vitrification- Vitrification of Mouse oocyte and Embryo- Warming of Mouse Oocyte and Embryo
G- Semen Analysis - Evaluation of Sperm Morphology With Diff Quick
H- Sperm Preparation for Intrauterine insemination (IUI) Semen Analysis- Swim up- Density Gradient
I- Sperm DNA and chromatin Structure Assay - TUNEL Assay- Aniline Blue and Chromomycin A3 Staining- Toluidine Blue Staining

- **Scaffolds Design**

- **Designing of In Vitro and In Vivo experiments**

POSTERS

پوسترها:

- Titled: **"Osteogenic Activity of Goat Bone Marrow – Derived Stem Cells on fish (Mugil cephalus) scales –An In vitro study"**. Mir Mahmoud Mortazavi R, Susan Mani and Annie John at International Conference on Futuristic Science and Technology in Frontier areas & 2nd Annual Conference of Indian JSPS Alumni Association Thiruvananthapuram, Kerala, India August 5-6, 2011, held ,at Thiruvananthapuram, Kerala, India.

- Titled: **"Tissue engineering of Intervertebral disc –A review"**; Mir Mahmoud Mortazavi R, Annie John; Second International Conference on Nanotechnology at the Bio-Medical Interface; Amrita Centre for Nanosciences & Molecular Medicine, Amrita Institute of Medical Sciences, Kochi, Kerala, India. 21-23 February, 2012.

- **Best Poster Presentation:** Titled: **"Sr-Alginate Scaffold for Nucleus Pulposus Tissue Engineering"**. Mir Mahmoud Mortazavi Roudmiane, S.S Babu, H.K. Varma and Annie John; At BIND 12, International Conference on Design of Biomaterials XXIII Annual meeting of SBADI and & Annual General Meeting of STERMI, December 9-11, 2012; Held at Indian Institute of Science, Bangalore, India.

Workshops

ورک شاپ ها:

- Titled: **"Basic Course on Flow Cytometry"** ; Held at C-CAMP, NCBS-TIFR, Bangalore, India; 5th to 8th July 2011.
- Titled: **"Research Methodology, Writing practices and Language Skills"**; Held at Kerala State Council for Science, Technology And Environment Sasthra Bhavan, Pattom, Thiruvananthapuram, Kerala; India; 3rd and 4th August 2012.
- Titled: **"Vision Genomics" Hands on Training in Ocular Genetics**; Held at Department of Genetics, Aravind Medical Research Foundation from October 3-9, 2012.
- Titled: **"Continuing Medical Education (CME) Programme on Scopes in Immunology**; Held at Madurai kamaraj University, School of Biotechnology, Madurai,Tamil Nadu, India October 11-12, 2012.
- Titled: **"Practice in PERL" Conducted by DBT Government of India**; Held at Bioinformatics Department at University of Kerala, India 30 November 2007.
- Titled: **"Electron Microscopy"**; Held at Electron Microscopy Laboratory, Biomedical Technology Wing, Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram, Kerala, India 5 July 2008.
- Titled: **"Advanced Primer Design";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 8 Aug 2016.
- Titled: **"Advanced PCR";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 9-10 Aug 2016.
- Titled: **"Advanced Real Time PCR";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 11-12 Aug 2016.
- Titled: **"In Vitro Fertilization (IVF) in Mouse";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 31 Jul - 1 Aug 2016.

- Titled: **"Vitrification of Ovarian Tissue and Culture on the Thick Embryo Chorioallantonic Membrane";** Held at Yazd Reproductive Medicine Center, Yazd, Iran, 5Aug 2016.
- Titled: **"Mouse Oocyte and Embryo Virification";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 4 Aug 2016.
- Titled: **"Semen Analysis";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 3 Aug 2016.
- Titled: **"Sperm preparation for Intrauterine insemination (IUI)";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 7 Aug 2016.
- Titled: **"Sperm DNA and Chromatin Structure Assay";** Held at Yazd Reproductive Medicine Center, Yazd, Iran 6 Aug 2016.

SEMINARS

- Titled: **"Genomics and Transgenics – Challenges and Prospects"**; Organized by Department of Biotechnology, University of Kerala, Karriavattom campus, Thiruvananthapuram city, Kerala state, India 14 November 2008.

- Titled: **"Chikungunya Prevention and Control"**; Organized by Department of Environmental Science, University of Kerala, Karriavattom campus, Thiruvananthapuram city, Kerala state, India 21 November 2006.

- Titled: **"Current Trends in Stem Cell Biology"**; Held at Rajiv Gandhi Center for Biotechnology, Thiruvananthapuram city, Kerala state, India 15-16 December 2007.

- Titled: **"Biodiversity for posterity"**; Held at Department of Botany, University of Kerala, Thiruvananthapuram city, Kerala state, India. 2 November 2007.

ARTICLES

1- Mortazavi R, Mir Mahmoud, and Annie John. **"Intervertebral Disc Damage & Repair – Its Pros And Cons."** World Journal of Research and Review (WJRR) 2.2 (2015): 01–12. Print.

SOCIETY FOR BIOMATERIALS AND ARTIFICIAL ORGANS (INDIA)

(Member, International Union of Societies for Biomaterials Science and Engineering)
Registration No. 110/86, Thiruvananthapuram, India

CERTIFICATE

This is to certify that

MIR MAHMOUD
SCTIMST, TRIVANDRUM

has won the
Best Poster Presentation Award (Third)
during XXIII National Conference of the Society,
December 9-11, 2012 at Indian Institute of Science,
Bangalore

BANGALORE, INDIA
DECEMBER 9, 2012

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December 9-11, 2012 | Indian Institute of Science, Bangalore
PRESENTATION CERTIFICATE
This is to certify that MIR MAHMOUD MORTAZAVI
of SREE CHITRA THIRUNAL INSTITUTE OF MEDICAL SCIENCES & TECHNOLOGY, THIRUVANANTHAPURAM, INDIA
has presented a poster on Sr ALGINATE SCAFFOLD FOR NUCLEUS PULPOSUS TISSUE ENGINEERING
and this contribution is co-authored by S.S. BABU, H.K. VERMA & ANNIE JOHN
Prof. Bikramjit Basu
Conference Chair

هوالعلیم

رایزنی علمی جمهوری اسلامی ایران در شبه قاره هند
I.R. Iran, Scientific and Educational Advisory Council
India and Subcontinent.

تکریم چهره های برجسته، موجب حرکت کشور به سمت قله های علمی می شود.

(مقام معظم رهبری حضرت آیت ا... خامنه ای مد ظله العالی)

دانشجوی عزیز **جناب آقای میر محمود مرتضوی رودمیانه**

حضور فعال جنابعالی را در جشنواره ی انتخاب دانشجویان برتر علمی شبه قاره هند به عنوان یک دانشجوی مستعد و سختکوش، ارج می نهیم و از اینکه با توکل بر خدا و پشتکار و اعتماد به نفس توانسته اید در سنگرهای علم و دانش بدرخشید صمیمانه به شما تبریک می گوئیم. این لوح تقدیر به پاس شایستگی های علمی به جنابعالی تقدیم می گردد.

انشاء الله در آینده ی نزدیک نامتان به عنوان یک دانشمند ایرانی در سطح جهان بدرخشد.

دکتر علی اعظم خسروی

رایزن علمی وسرپرست دانشجویان ایرانی

در هند وکشورهای شبه قاره

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