A DISSERTATION ON

To Study the Pathophysiology and Mechanism of Osteoarthritis

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DEGREE OF MASTER OF TECHNOLOGY

IN BIOTECHNOLOGY

BY

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UNDER THE SUPERVISION OF

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DECLARATION FORM

I, **Kahkashan Moheet,** a student of M. Tech Biotechnology (II Year / IV semester), Integral University have completed my six months dissertation work entitled **"To study the pathophysiology and mechanism of osteoarthritis"** successfully from CSIR-Central Drug Research Institute under the able guidance of **Dr. Ritu Trivedi**, Senior Principal Scientist, CSIR-CDRI, Lucknow.

I, hereby affirm that the work has been done by me in all aspects. I have sincerely prepared this project report and the results reputed in this study are genuine and authentic.

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Department of Bioengineering

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TO WHOM IT MAY CONCERN

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I wish her good luck and bright future.

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I wish her good luck and bright future.

Dr. Alvina Farooqui

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TABLE OF CONTENT

S.No.	PARTICULARS	PAGE No.
1.	**Introduction**	1-2
	Objectives	2
2.	**Review of Literature**	3-18
2.1	Cartilage	4
2.2	Types of cartilage	4
2.3	Ultra-structure of articular cartilage	4
2.3.1	Superficial zone	5
2.3.2	Transitional zone	5
2.3.3	Middle zone	5
2.3.4	Deep zone	5
2.3.5	Calcified cartilage zone	5
2.4	Composition of articular cartilage	6
2.4.1	Water	6
2.4.2	Collagen	7
2.4.3	Proteoglycan	8
2.5	Functions of the articular cartilage	9
2.6	Extracellular matrix (ECM) of cartilage	9
2.7	Functions of the matrix	10
2.8	Subchondral bone (SCB) and its role in osteoarthritis	11
2.9	Matrix metalloproteinases (MMP's)	13
2.9.1	Regulation of MMP's gene expression	14
2.9.2	The role of matrix metalloproteinase in healthy cartilage	14
2.9.3	MMPs and cartilage degradation	15
2.10	Changes in extracellular matrix during osteoarthritis	16
2.11	Chondrocyte death in osteoarthritis	16
2.12	Apoptosis as a cause of OA progression and during OA development	17
2.13	Cell culture	17
2.13.1	Primary cell cultures	17

2.13.2	Secondary cell cultures	17
2.14	Media preparations	18
3.	**Materials and Methods**	19-28
3.1	Materials	19
3.1.1	List of chemicals	19
3.1.2	List of solvent	20
3.1.3	List of instruments	21
3.1.4	List of plastic and glasswares	22
3.2	Methodology	23
3.2.1	Aseptic technique and good cell culture practice	23
3.2.2	DMEM media	24
3.2.3	Composition of DMEM media for 100 ml	24
3.2.4	Direction for preparation of DMEM media	24
3.3	Cell culture	24
3.4	Rat articular chondrocyte isolation	25
3.5	Cell viability assay (MTT)	25
3.6	Real time PCR	25
3.7	Development of a surgically induced OA model *in-vivo*	26
3.8	Alcian blue staining	27
3.9	Histological evaluation	27
3.10	Micro computed tomography (micro-CT)	27
3.11	Statistical analysis	28
4.	**Results and discussion**	29- 47
4.1	Objective 1	29
4.1.1	In-vitro study to analyse osteoarthritic pathophysiology in Dexamethasone treated chondrocytes	29
4.1.2	Cell viability assay of Dexamethasone	29
4.1.3	Long term usage of Dex aggrevates the proteoglycan content loss of articular cartilage in primary rat articular chondrocyte culture which is analysed through alcian blue staining	30
4.1.4	To check the gene expression of different genes in chondrocyte through RT-PCR in order to study the osteoarthritic pathophysiology with treatment of Dexamethasone	31

4.1.5	Anabolic gene expression in chondrogenesis	31
4.1.6	Catabolic gene expression in chondrogenesis	33
4.2	EGLN3 and ORM1 were examined using RT-PCR to gain a comprehensive understanding of the molecular-level pathogenesis of subchondral bone in osteoarthritis	35
4.3	**Objective 2**	36
4.3.1	To study an in-vitro ACLT model that mimics human osteoarthritic condition	36
4.3.2	ACLT model	36
5.	**Results**	39-47
5.1	Radiological appearances of the knee joint in ACLT animals	39
5.2	Histopathology	41
5.2.1	To study the tibial articular and subchondral bone in the control and ACLT groups	41
5.2.2	To study the tibial subchondral bone in the control and ACLT groups	42
5.2.3	Study of the chondrocytes in growth pate region of the contrl and ACLT groups	43
5.2.4	Effect of ACLT surgery on the cartilage thickness	44
5.2.5	Micro-architecture of the tibial subchondral bone in control and ACLT-induced osteoarthritic animals	45
6	**Discussion**	48-50
7	**Conclusion**	51-52
8	**References**	53-56

LIST OF FIGURES

FIGURE No.	FIGURE CAPTION	PAGE No.
1.	Phenotypes of Osteoarthritis (OA). Clinic evidence shows that the majority of OA patients have a diversity of OA phenotypes, including articular cartilage erosion, synovial hyperplasia, abnormal angiogenesis, synovial inflammation, subchondral bone disturbance, ligaments and tendons instability, and joint stiffness. Left-half side shows the structure of the normal synovial joint. Right-half side showed the possible alterations of synovial joint structure and symptoms in osteoarthritis.	3
2.	Cross sectional image of healthy articular cartilage and cellular arrangement in the healthy articular cartilage.	6
3.	The high content of water in proteoglycan helps the cartilage to act as a shock absorber.	7
4.	The organization of matrix component in articular cartilage and subchondral bone	8
5.	Proteoglycans, collagens, and other matrix components of cartilage.	9
6.	Hypothetical model of osteoarthritis (OA) pathogenesis in cartilage and subchondral bone.	14
7.	Cell viability assay on rat chondrocytes. The MTT assay was done to check the viability effect of dexamethasone for 48 hrs of treatment. Cell viability data were presented as the Mean±SEM.	30
8.	Alcian blue staining for proteoglycan content. A is undifferentiated, B is Dexamethasone treated and C is differentiated.	31
9.	Expression of chondrogenic and ECM producing genes: [A] Sox9, [B] Col2 and [C] Aggrecan. SOX9: The SRY-box transcription factor 9 (SOX9) is a master regulator of chondrogenesis. It plays a critical role in the early stages of chondrocyte differentiation and is essential for cartilage formation. Collagen Type II (COL2A1): Collagen type II is the main collagen component of cartilage. It is highly expressed	32

	during chondrogenesis and is considered a specific marker of mature chondrocytes. Aggrecan: Aggrecan is a large proteoglycan bearing numerous chondroitin sulfate and keratan sulfate chains that endow articular cartilage with its ability to withstand compressive loads. It is present in the extracellular matrix in the form of proteoglycan aggregates, in which many aggrecan molecules interact with hyaluronan and a link protein stabilizes each interaction.	
10.	Expression of genes: [A] MMP-3, [B] MMP-13, and [C] ADAMTS-5.	34
11.	EGLN3 and ORM1 both are found to be downregulated gene but in the effect of Dexamethasone, they are found to be upregulated. EGLN3 and ORM1 may be the new candidate targets for diagnosis and therapies on patients with OA in the future.	36
12.	Development of ACLT model in SD rats to study osteoarthritis.	37
13.	In fig: [A] Animal (SD Rat) has been made unconscious using a cocktail of ketamine and xylaxine. In fig: [B] patella has been dislocated for ACLT surgery. In fig [A] anterior cruciate ligament is identified.	37
14.	In fig [B] Cut has been made on the knee using surgical blade. In fig [C] cut on anterior cruciate ligament is made. In fig [D] post-operative care of animal has been taken using betadine and neosporin powder.	38
15.	2D images of subchondral bone of the control and ACLT-induced osteoarthritis model. Cross-sectional 2D images of the knee joints upper (coronal view) and lower (sagittal view), showing the significant subchondral bone loss in the tibia of the ACLT group.	40
16.	Histological evaluation of the tibial articular and subchondral bone in the control and ACLT groups. Haematoxylin and eosin staining showing the chondrocyte cells in the cartilage of the control and ACLT model. It shows the cartilage thickness, cell number, their alignment and tidemark duplication in ACLT group.	41
17.	Quantitative analysis of cartilage of Control vs. ACLT group. Stained sections were quantified by ImageJ software for the assessment of	42

	cartilage thickness. [A] Hyaline cartilage (HC), [B] Calcified cartilage (CC), and [C] total cartilage thickness (TC).	
18.	Histological evaluation of the tibial subchondral bone in the control and ACLT groups. Haematoxylin and eosin stain representing subchondral bone deterioration in the ACLT group.	43
19.	Histological representation of the chondrocytes in growth plate region of the control and ACLT groups. Haematoxylin and eosin staining showing the chondrocyte cells in the cartilage of the control and ACLT group.	43
20.	Histological evaluation of the growth plate region in the control and ACLT groups representing articular and hyaline cartilage layers. Toluidine blue staining of tibia showing that the altered subchondral architecture at the histology level is disrupted in the surgery group and relative peptidoglycan degradation was observed.	44
21.	Micro-architecture of the tibial subchondral bone in control and ACLT-induced osteoarthritic animals. 3D image of tibial plateau showing subchondral bone loss in the ACLT group.	45
22.	Quantitative analysis of subchondral bone in control and ACLT groups. BV/TV % (bone volume and tissue volume %) is decreased in ACLT group as compared to the control group.	45
23.	Quantitative analysis of subchondral bone in control and ACLT groups. Trabecular number (Tb.No.), Trabecular thickness (Tb. Th), and No. of closed pores is decreased in ACLT group as compared to the control group.	46
24.	Quantitative analysis of subchondral bone in control and ACLT groups. Structure model index (SMI) increased in the ACLT group.	47

LIST OF TABLES

TABLE No.	TITLE OF THE TABLE	PAGE No.
1.	List of chemicals used.	19
2.	List of solvents used.	20
3.	List of instruments used.	21
4.	List of plastic and glassware used.	22
5.	List of genes used	26

LIST OF ABBREVIATIONS

OA	Osteoarthritis
MMP	Matrix metalloproteinases
NSAIDS	Nonsteroidal anti1 inflammatory drug
ACLT	Anterior cruciate ligament transection
GAGs	Glycosaminoglycans
ECM	Extracellular matrix
FGFs	Fibroblast growth factors
SCB	Subchondral bone
AP-1	Activator protein
DMEM	Dulbecco's modified eagle medium
FBS	Fetal bovine serum
PBS	Phosphate buffered saline
MTT	3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
cDNA	Complementary DNA
GAPDH	Glyceraldehyde-3- phosphate dehydrogenase
Sox9	SRY-Box Transcription factor 9
Col2	Collagen type 2
ACAN	Aggrecan
ADAMTS-5	ADAM metallopeptidase with thrombospondin type 1 motif 5
ITS	insulin-transferrin-selenium
RAC	Rat articular cartilage
AB	Alcian blue
H&E	Hematoxylin and Eosin

BV/TV	Bone volume/tissue volume
Tb. No.	Trabecular number
Tb. Sp.	Trabecular separation
CT	Computed tomography
SMI	Structural model index
Dex	Dexamethasone
RT-PCR	Real time- polymerase chain reaction
DEGs	Differently expressed genes
KEGG	Kyoto Encyclopaedia of Genes and Genomes
EGLN3	Egl-9 Family Hypoxia Inducible Factor 3
HC	Hyaline cartilage
CC	Calcified cartilage
TC	Total cartilage thickness

1.Introduction

Osteoarthritis (OA) is a prevalent degenerative joint disorder that affects millions of individuals worldwide. It is the most common form of arthritis and a leading cause of pain and disability, posing a significant burden on affected individuals, their families, and healthcare systems. This chronic condition primarily affects the articular cartilage, synovium, and subchondral bone, leading to joint pain, stiffness, and functional impairment(Lespasio et al., 2017).

Osteoarthritis is not an inflammatory disease, but inflammation can be a result of cartilage degradation, affecting the growing part of the elderly population compromised by the age group 50-70 years. The physiology of osteoarthritis is very complex than simple wear and tear or aging(Hussain et al., 2016). It is a multifactorial joint disorder associated with chronic degeneration of cartilage. OA was primarily considered an articular cartilage disease, but recent studies have shown that the disease involves the whole joint(Armiento et al., 2018).

The pathophysiological alterations that are seen in osteoarthritic joints are articular cartilage degradation, subchondral bone plate thickening, the formation of peripheral osteophytes, synovitis, and narrowing of the joint space. (Giorgino et al., 2023).

The pathogenesis of osteoarthritis involves a progressive degradation of the articular cartilage, which acts as a cushioning and load-bearing structure in joints. The loss of cartilage integrity triggers an inflammatory response within the joint, leading to the release of pro-inflammatory cytokines, matrix metalloproteinases (MMP's), and other mediators that perpetuate cartilage breakdown and induce structural changes in the surrounding tissues. As a result, individuals experience symptoms of joint pain, stiffness, and functional limitations, which significantly impact their overall quality of life. Given the rising prevalence of osteoarthritis and its substantial impact on global healthcare systems, effective management strategies are of paramount importance (Kan et al., 2019). The management of osteoarthritis typically encompasses a multidisciplinary approach, involving lifestyle modifications, physical therapy, pain management, and, in severe cases, surgical interventions such as joint replacement. Non-pharmacological interventions, including exercise, weight management, and assistive devices, play a crucial role in improving joint function and reducing pain. Pharmacological therapies for osteoarthritis primarily focus on pain relief and inflammation control. Analgesics, nonsteroidal anti-

inflammatory drugs (NSAIDs), and intra-articular corticosteroid injections are commonly used to alleviate symptoms. However, it is important to balance the benefits of these medications with potential side effects, especially in individuals with comorbidities. However, further research is required to establish their long-term efficacy and safety. This thesis aims to provide a comprehensive analysis of osteoarthritis, exploring its pathogenesis, identifying key risk factors, and evaluating various management strategies (Martin & Buckwalter, 2012).

Elucidating the intricate interplay between these factors holds the potential to identify novel therapeutic targets and develop tailored interventions that address the underlying molecular abnormalities in OA. Furthermore, the advent of high-throughput technologies, such as genomics, proteomics, and metabolomics, has paved the way for the identification of biomarkers associated with OA. These biomarkers not only aid in early diagnosis and disease stratification but also provide insights into the molecular processes occurring within affected joints. The integration of these molecular profiling techniques with clinical data holds immense promise for precision medicine approaches, enabling personalized treatment strategies for individuals with OA.

The prevalence of osteoarthritis (OA) is considerable, exerting a significant impact on a substantial segment of the worldwide populace, with a particular emphasis on the elderly. The epidemiology of OA is influenced by various factors such as age, gender, genetic predisposition, and lifestyle choices. Understanding the epidemiological characteristics of OA is crucial for effective prevention, early diagnosis, and appropriate management strategies. According to the Global Burden of Disease Study, as of 2020, OA was the 11th leading cause of years lived with disability globally(Michael et al., 2010).

1.2.4 Objectives of the study:

1. *In-vitro* study to analyse osteoarthritic pathophysiology in Dexamethasone treated *chondrocytes*

2. To study an *in-vivo* ACLT model that mimics human osteoarthritic condition.

2. Review of Literature:

Osteoarthritis (OA) is a degenerative joint disease that is characterized by the breakdown of cartilage in the joints. Cartilage is a smooth, rubbery tissue that cushions the ends of bones in a joint. When cartilage breaks down, the bones rub against each other, causing pain, stiffness, and inflammation. OA is the most common form of arthritis, affecting an estimated 30% of adults over the age of 65(Whirl-Carrillo et al., 2012). It is more common in women than men, and the risk increases with age. OA can also be caused by injury, overuse, or certain medical conditions. There is no definite cure for OA, but there are treatments that can help relieve pain and improve function. These treatments include: Medications, such as nonsteroidal anti-inflammatory drugs (NSAIDs) or pain relievers, physical therapy, injections, such as cortisone or hyaluronic acid or surgery, in some cases The goal of treatment is to relieve pain and improve function so that people with OA can continue to live active lives.

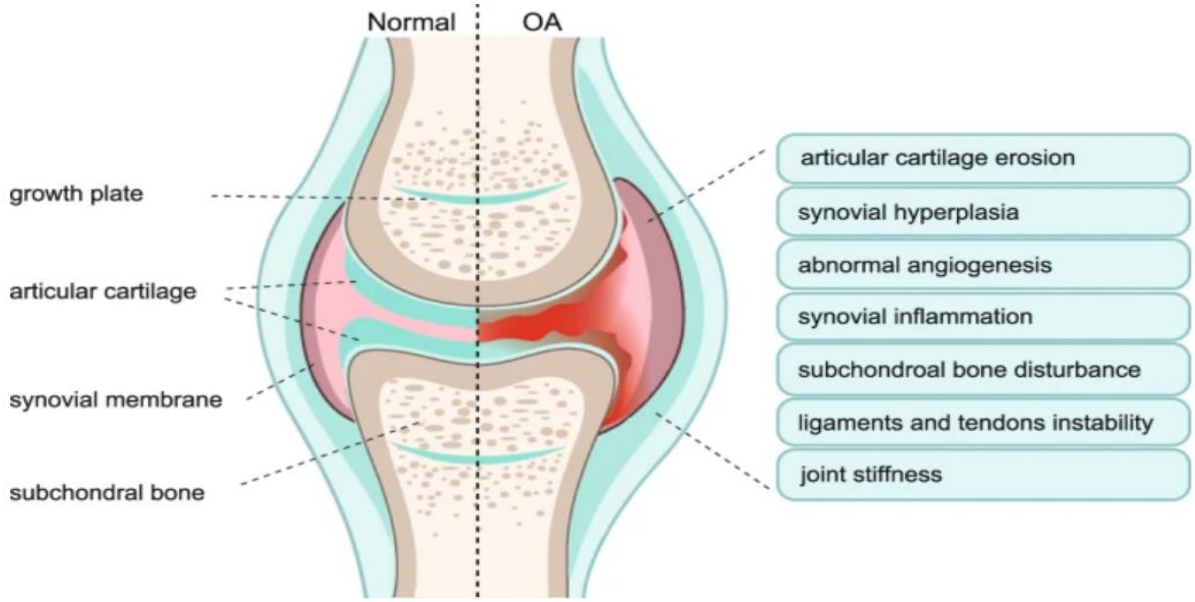

Fig 1: *Phenotypes of Osteoarthritis (OA). Clinic evidence shows that the majority of OA patients have a diversity of OA phenotypes, including articular cartilage erosion, synovial hyperplasia, abnormal angiogenesis, synovial inflammation, subchondral bone disturbance, ligaments and tendons instability, and joint stiffness. Left-half side shows the structure of the normal synovial joint. Right-half side showed the possible alterations of synovial joint structure and symptoms in osteoarthritis.*

Image source: Qing yao, Xiahao Wu et.al. Osteoarthritis: pathogenic signaling pathways and therapeutic targets. **(2023)**

2.1 Cartilage:

Cartilage is a type of dense connective tissue with specialised characteristics. Semi-rigid construction is designed to offer stability, support weight, and endure torsion, bending, and tension. It lacks blood vessels and nerve innervation. Cartilage can undergo both appositional and interstitial growth. The tissue contains single-cell chondrocytes that are embedded in an amorphous matrix composed of collagen and fibres. Cells are primarily composed of intracellular components.

Hyaline, elastic, and fibrocartilage are three distinct types of cartilage distinguished by their collagen content and presence of elastic fibres. (Fujii et al., 2022).

2.2 Types of cartilage

Hyaline, elastic, and fibro cartilage are the three types of cartilage that are classified according to the cellular matrix makeup. The most prevalent and distinctive kind of cartilage is hyaline cartilage.

1) Hyaline cartilage: Glass is the Greek word for hyaline cartilage. The chondrocytes build the hyaline cartilage matrix. The term "perichondrium" refers to the thin layer that covers hyaline cartilage.

2) Elastic cartilage: This type of cartilage resembles hyaline cartilage in certain ways, but the matrix also contains type-2 collagen fibres and more elastic fibres. The cartilage is covered by a perichondrium. There is an increased number of cells.

3. Fibrocartilage: This type of cartilage is made up of bundles of collagen fibres that are hardly covered by matrix. Normally, cells are positioned singly between collagen fibre bundles. There is no perichondrium covering it.

2.3 Ultra-structure of articular cartilage

According to Martin and Buckwalter, chondrocytes in cartilage combine secretory elements, peptidoglycan, collagen, and non-collagenous proteins in a highly specialized manner. The position of chondrocytes in the depth of the cartilage affects their structure, composition, and functions. There are five morphological zones based on the distance from the cartilage's surface(Martin & Buckwalter, 2012).

2.3.1 Superficial zone:

It is the thinnest layer compared to all the other zones. It has ellipsoid and flattened cells. They are shielded by a thin synovial fluid barrier known as lubricin and are situated parallel to the joint surface. The thin superficial zone of cartilage, which makes up 10 to 20% of the thickness of articular cartilage, protects deeper layers from shear forces. The superficial zone of cartilage has the largest concentration of water among the other zones because chondrocytes produce a lot of collagens there but little proteoglycan.

The parallel-arranged fibrils that deliver the highest tensile strength. Alterations in the superficial zone of articular cartilage contribute to changes in its mechanical properties, thereby facilitating the progression of osteoarthritis. The layer also acts as a protective barrier against immune system activity and synovial tissue, preventing macromolecules from reaching the cartilage.

2.3.2 Transitional zone:

The cells exhibit a spherical shape and their reduced density is primarily attributed to their incorporation within an extracellular matrix. The collagen fibres in this region exhibit disorganised arrangement with large diameters. Proteoglycan aggrecan concentrations are higher in this particular zone. The superficial zone of this area is alternatively referred to as its superficial tangential zone.

2.3.3 Middle zone:

Cells are spherical and arranged perpendicular to the surface. With the biggest diameter of collagen fibres, this region contains the highest concentration of proteoglycans. The zone's cell density is still low, though.

2.3.4 Deep zone:

The columnar orientation of chondrocytes in the deep zone is parallel to the collagen fibres and perpendicular to the line of the knee joint. Roughly 30% of the volume of articular cartilage comes from the deep zone. The chondrocyte population in this region exhibits hypertrophy.

2.3.5 Calcified cartilage zone:

The calcified zone exhibits low metabolic activity, as evidenced by a limited number of cells surrounded by a calcified matrix. The cells in this region exhibit a unique ability to

synthesise type X collagen, which collaborates with the subchondral bone beneath it to maintain essential structural integrity and function as a shock absorber. The chondrocytes in this region exhibit hypertrophy and reduced activity. (Buckwalter, 2002). Tidemark is the distinct line that can be seen separating the fourth and fifth layers. It prefers simple colours like toluidine blue. This region offers a crucial transition to the subchondral bone beneath.

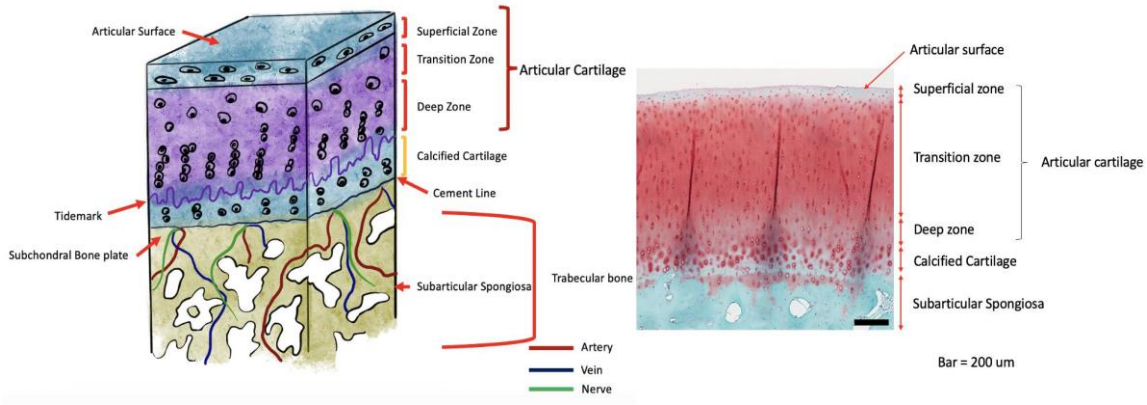

Fig 2: Cross sectional image of healthy articular cartilage and cellular arrangement in the healthy articular cartilage.

Image source: George Jacob et. al. Osteochondral Injury, Management and Tissue Engineering Approaches (2020) doi: 10.3389/fcell.2020.580868.

2.4 Composition of Articular Cartilage:

Cartilage is hydrated in nature so that it can absorb mechanical load. It has the following composition:

2.4.1 Water: Water alone makes up 65–80% of the cartilage's total wet weight. The water content of the cartilage zone varies with its depth. According to Newman, the superficial zone includes 80% water while the deep zone only has 65% (Newman, 1998). It serves as a lubricating medium and creates a gliding surface with low friction, which primarily serves as a shock absorber and feeds the cartilage. Due to increased permeability and disturbance in the matrix, the water content increases in disease conditions, rising to over 90%. According to Carballo, changes in permeability and matrix composition result in a drop in modulus of elasticity and a corresponding alteration in the articular cartilage's ability to absorb shock (Carballo et al., 2017).

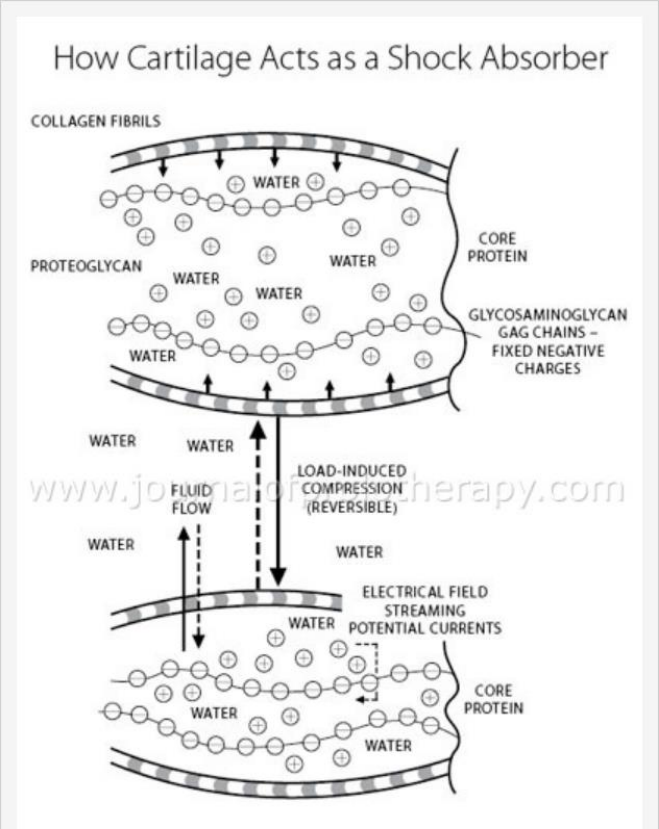

Fig 3: The high content of water in proteoglycan helps the cartilage to act as a shock absorber.

Image source: Ross A. Hauser et. al. Journal of Prolotherapy **(2010)**

2.4.2 Collagen: It contains between 10 and 20 percent of the articular cartilage's moist weight. The majority (90–95%) of the component, type II collagen, gives the articular cartilage its tensile strength. Cartilage contains various types of collagens, including collagen II. Additionally, collagen type VI is present in the pericellular matrix, where it assists chondrocytes in adhering to the matrix. (Armiento et al., 2018). The surface of the macro fibril, which possesses tensile characteristics and connects to the inter-fibrillar, is crosslinked with collagen type IX. The hypertrophied cells of the calcified cartilage layer, which sustain structural integrity and aid in cartilage mineralization, are associated to collagen type-X. The presence of collagen type-XI within or on macro fibrils serves to initiate the development of fibrils (Hayes et al., 2001).

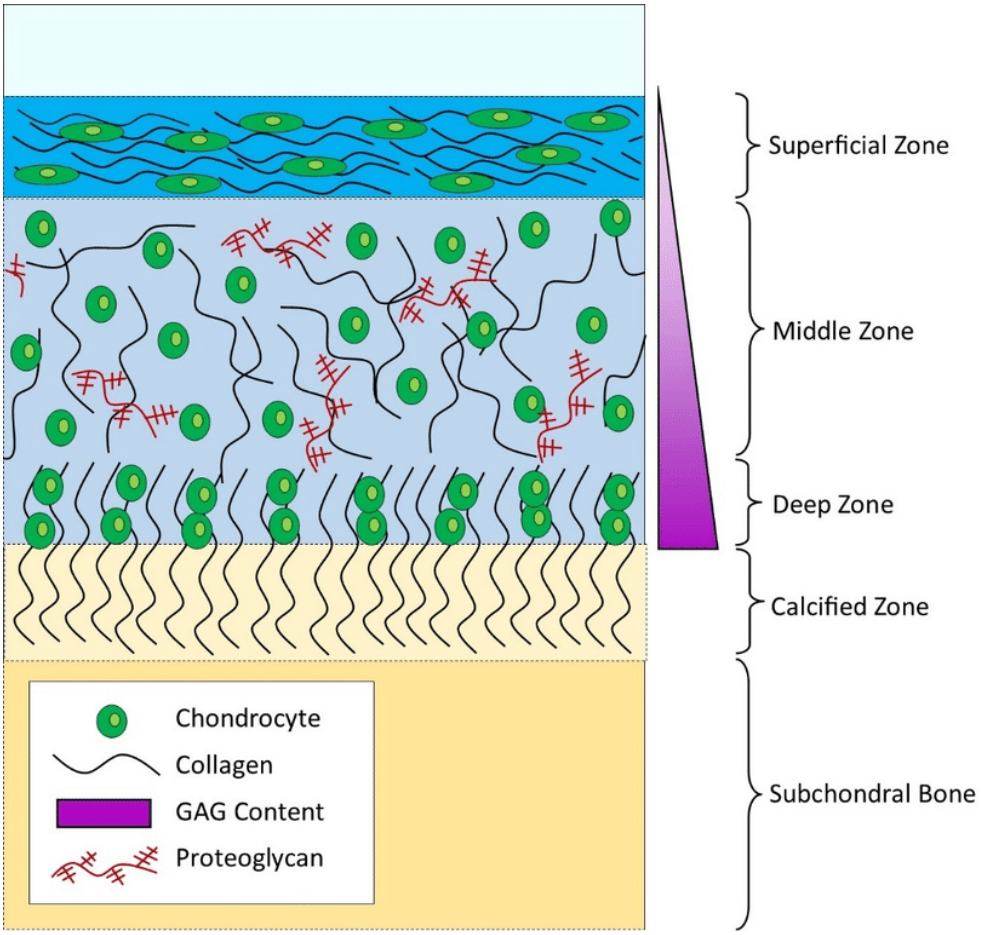

Fig 4: The organization of matrix component in articular cartilage and subchondral bone.

Image source: Rebecca L. Davies et. al, Bioengineering (2019).

2.4.3 Proteoglycans: Protein-containing polysaccharide molecules are known as proteoglycans. This gives the cartilage a 10–20% moist weight and gives the cartilage compressive strength. Articular cartilage contains two primary categories of proteoglycans: large aggregating proteoglycan monomers known as biglycans, and smaller proteoglycans including decorin, biglycan, and fibromodulin. Chondrocytes synthesise and subsequently release proteoglycans into the extracellular matrix of cartilage. Glycosaminoglycans (GAGs) are the subunits of proteoglycans. Mandelbaum and Elattrache identified two primary types of disaccharide molecules, namely keratin sulphate and chondroitin sulphate. The aggrecan molecule is created when GAGs are joined to the protein core by sugar bonds. A core chain of hyaluronic acid and link protein combine to form a complicated structure for the GAG molecule. Types 4 and 6 of chondroitin sulfate are the two varieties. While type 4 of chondroitin sulfate decreases with age, type 6 remains constant throughout life. According to Buckwalter (1983), the early sign of experimental arthritis is aggrecan depletion. Sulfate and carboxylate groups on these macromolecules, which are negatively

charged, draw only positively charged molecules and repel negatively charged ones. Consequently, the concentration of inorganic ions, specifically sodium, within the matrix rises, leading to an elevation in the osmolarity of the articular cartilage. This phenomenon is known as the "Donnan effect." In the articular cartilage, proteoglycans maintain the balance between the fluid and electrolyte (Ng et al., 2023).

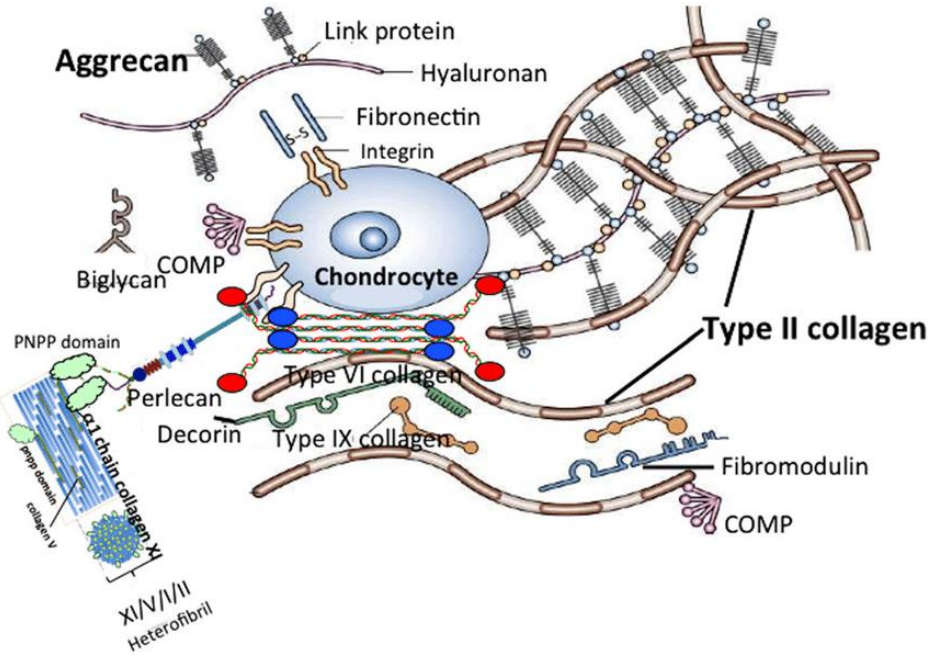

Fig 5: Proteoglycans, collagens, and other matrix components of cartilage.

Image source: Anthony J. Hayes et. al, Advanced Therapeutics (2019)

2.5 Functions of the articular cartilage

1. Provides gliding surface with low friction.
2. Acts as a shock absorber.
3. Minimizes pressure on the subchondral bone.

2.6 Extracellular matrix (ECM) of cartilage:

The extracellular matrix (ECM) is a component of cellular structure that typically gives cells and the environment around them structural support and opportunities for biochemical interactions. The extracellular matrix is a non-cellular component that serves as a physical framework and skeleton for the cellular elements. It also serves as a network for biochemical and biomechanical communication and is essential for cell proliferation, adhesion, differentiation, remodelling, and homeostasis. Depending on the necessity and intended use of the tissue, an ECM with a unique composition is created during tissue

formation. In fact, the ECM's physical and biochemical components are not only tissue-specific, but also noticeably heterogeneous in composition. Proteoglycans and fibrous proteins are the two types of bio-macromolecules that make up cartilage's ECM. The surrounding tissue affects the ECM's composition. The surrounding tissue's function and composition affect the ECM's makeup. According to Hasler et al. (2000), the ECM has multiple functions, such as providing support, facilitating tissue separation, and regulating intracellular communication. It regulates the dynamic behaviour of cells and serves as a local depot for a variety of growth factors. The ECM is a dynamic structure that undergoes several post-translational alterations on a regular basis, either enzymatically or by non-enzymatic means. Through all stages of osteoarthritis, the remodeling mechanisms are extremely active. Because of the rapid turnover rate and unique composition architecture of the newly generated ECMs, the quality of the extracellular matrix (ECM) is compromised during the dynamic remodelling (Song et al., 2022). The cartilage ECM is unique in its surroundings. According to Afzali et al. (2002), the ECM of articular cartilage is essential for chondrocyte activities, chondrocyte cell matrix interaction, cell cytoskeleton, and integrin-mediated signaling. There is an interaction between cartilage's chondrocytes and ECM. The ECM is created and regulates cell behavior. Chondrocyte-derived substances may also have an impact on the ECM. Interleukin-1 (IL-1), fibroblast growth factors (FGFs), bone morphogenic proteins (BMPs), or insulin-like growth factors (IGFs) are possible candidates for the factor (Simon & Jackson, 2018). The homeostasis and repair of articular cartilage depend on the regulation of biological processes, such as cell proliferation, attachment, differentiation, and survival, via interactions between the extracellular matrix (ECM) and chondrocytes. The ECM of cartilage tissue can be modified in response to any changes in the functional demand. ECM compounds have various half-lives and turnover rates. Major collagen type-II has a half-life between 100 and 400 years, whereas cartilage ECM proteoglycan (aggrecan) can take approximately 25 years. For tissue homeostasis and cartilage integrity, healthy cartilage with functional ECM requires a balance between anabolic and catabolic activity (Goldring, 2006).

2.7 Functions of the matrix:

1. Shields the chondrocytes from mechanical loading, hence helping to maintain their phenotype.
2. Storage of required growth factors and cytokines.
3. Matrix regulates the concentration and diffusion rate of the nutrients to chondrocytes.

4. It acts as a signal transducer for the chondrocytes. Matrix deformation produces signals (mechanical, electrical, and chemical) influencing the functions of chondrocytes. Thus, the matrix also plays a role in recording a loading history of the articular cartilage.

2.8 Subchondral bone (SCB) and its role in osteoarthritis:

The term "subchondral bone" refers to the bone that is distal to the calcified cartilage, but this concept confuses physiological and mechanical variations that may be crucial to understanding the function of subchondral bone in osteoarthritis. Directly beneath the calcified cartilage lies a 1-3 mm thick plate of cortical bone, which is physiologically and functionally comparable to the cortical bone in other cortical sites but somewhat less stiff than diaphyseal cortical bone. Distal to the structure is the subchondral cancellous bone, which is significantly more porous, metabolically active, and stiffer than the cortical plate (Y. Li et al., 2023). It is also important to remember that calcified cartilage may be a factor in the sclerosis that distinguishes non-mineralized articular cartilage from subchondral bone in advanced OA. This rejuvenated developing process leads to thickened calcified cartilage, which is more highly mineralized than bone. The upper layer of articular cartilage, which is not highly active and is unable to produce enough new cartilage to preserve its volume, becomes thinner due to the calcified cartilage thickening.

It is important to keep in mind that different conditions determine the beginning of cartilage degradation and the gradual loss of articular cartilage that leads to OA. These conditions also represent different pathophysiological approaches to understanding the pathophysiology of OA (Grässel et al., 2009). Each person loses some cartilage by the time they turn 60 years old, which is an inevitable result of aging. However, not everyone develops OA. Laboratory investigations have shown that the destruction of articular cartilage does not always lead to osteoarthritis (OA), suggesting that certain circumstances may contribute to the progression of cartilage deterioration. According to Aigner et al. (2007), the subchondral bone's function in the joint is to distribute forces and modify in ways that maintain joint alignment and prevent stress buildup (Hashimoto et al., 2008). Experimental research utilising arthritis rabbit and mouse models has provided valuable insights into the pathogenesis of the disease. These models have demonstrated that the degradation of articular cartilage primarily occurs in the enlarged subchondral plate. Furthermore, a rabbit model has uncovered a significant correlation between increased cartilage injury and thinning of the subchondral bone. Interestingly, when comparing

sclerosis and bone remodelling, it is observed that greater bone remodelling is associated with lower density, heightened porosity in the subchondral bone zone, and overall bone loss. It is important to note that sclerosis and fast remodelling in osteoarthritis (OA) should not be viewed as contradictory findings, but rather as distinct temporal stages of the disease or potentially different spatial positions, or even both. (Shi et al., 2019).

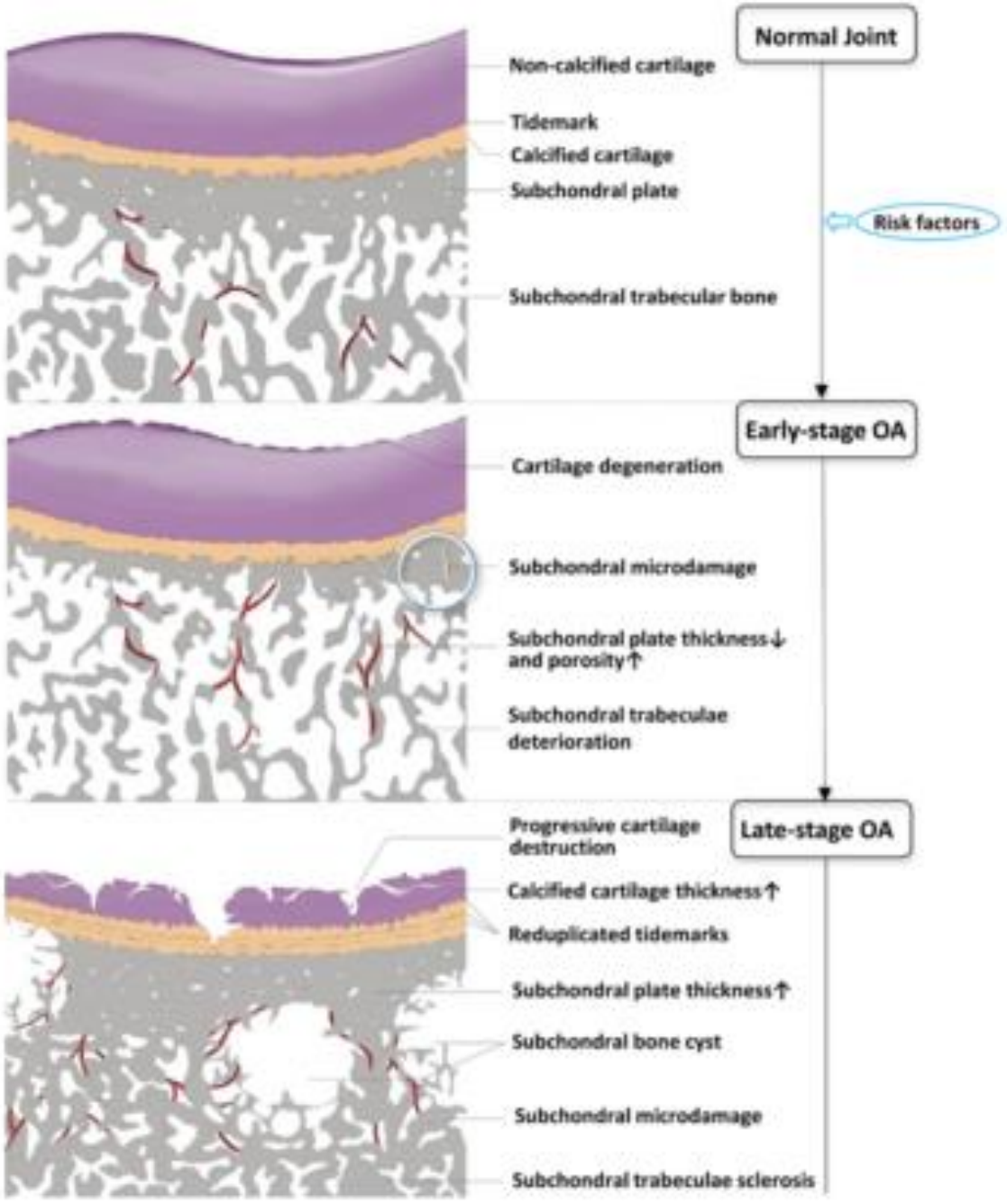

Fig 6: Hypothetical model of osteoarthritis (OA) pathogenesis in cartilage and subchondral bone.

Image source: Li et al. Arthritis Research & Therapy (2013).

2.9 Matrix metalloproteinases (MMP's)

The enzymes belonging to the matrix metalloproteinases (MMP) family are endopeptidases that rely on zinc as a cofactor for their catalytic activity. These enzymes exhibit a remarkable structural homology, indicating a common evolutionary origin. The assemblage of 23 discrete matrix metalloproteinase (MMP) proteins, characterised by a remarkably conserved architecture, is observed in humans. Additionally, the human genome encodes 24 distinct genes responsible for the production of these MMP proteins. (Hu & Ecker, 2021). The interaction and composition of the cell-matrix is regulated by the MMP family of endopeptidases(Grässel et al., 2009). Any development or regulation must inevitably involve cell-matrix interaction. Homeostasis and regulation can be altered by any modification or minute shift in the relationship. MMPs are the likely culprits for changing the cell matrix interaction in cartilage, which leads to the deterioration of cartilage(Li et al., 2017). The onset of articular cartilage degeneration in the early stages of osteoarthritis (OA) has been associated with several biomarkers, including matrix-degrading enzymes like the matrix metalloproteinase (MMP) family, the disintegrin and metalloproteinase with thrombospondin (ADAMTS) family, aggrecanases, etc. Matrix-forming cells, chondrocytes, and synovial fibroblasts all produce these matrix-degrading enzymes. These very powerful MMP enzymes have a catabolic action. MMPs can break down both non-matrix and extracellular matrix proteins in cartilage. MMPs have a uniform structure and are made up of a broad family of proteases that have similar structural and functional properties. Depending on which domains are included in the structure, MMPs have different structures(Mehana et al., 2019).

Each member of the MMP family consists of a pro-peptide and a catalytic domain that houses conserved methionine and a zinc-binding site, among other catalytic machinery(Fan et al., 2004). The catalytic region of MMP contains additional zinc and calcium ions to preserve its three-dimensional structure, stability, and enzymatic activity(Mehana et al., 2019). The expression of genes responsible for encoding Matrix Metalloproteinases (MMPs) is subject to stringent regulatory mechanisms. These mechanisms tightly control the activation and localization of MMPs through the binding of proteins to proteoglycan core proteins and other constituents of the extracellular matrix. The matrix metalloproteinase (MMP) enzyme family plays a pivotal role in orchestrating tissue remodelling processes. Notably, MMPs are actively engaged in facilitating the intricate transition from cartilage to bone, a fundamental process known as ossification.

Additionally, these remarkable enzymes contribute to the intricate mechanisms underlying wound healing. By participating in these dynamic processes, MMPs demonstrate their significance in maintaining tissue homeostasis and facilitating regenerative events.(Song et al., 2022). On the other hand, abnormal MMP activity has been linked to a number of pathophysiological conditions, such as rheumatoid arthritis, periodontics, and tumor cell metastasis(Hu & Ecker, 2021).

2.9.1 Regulation of MMPs gene expression:

Under normal conditions, extracellular matrix homeostasis is a strictly regulated process. Normal cell migration may be hampered by less degradation, whereas pathologic destruction of connective tissue and lack of interaction with the extracellular matrix would arise from excessive degradation. MMP production is tightly controlled, both tissue-specifically and at the transcriptional level of the gene. MMPs have low physiologic expressions in healthy connective tissues, but they dramatically increase in the pathologic conditions of both types of arthritis. The activator protein 1 (AP-1), whose binding site is -73 bp, is the primary regulator of MMP gene transcription. The promoters of the endopeptidase MMPs genes are well known. The complex protein known as activator protein (AP-1) is made up of either homodimers or heterodimers. This proximal AP-1 site is present in the promoters of the MMP-1, MMP-3, MMP-9, and MMP-13 genes and is necessary for their expression(Kim et al., 2020).

2.9.2 The Role of Matrix Metalloproteinase in Healthy Cartilage:

It has been discovered that seven different kinds of zinc-dependent matrix metalloproteinases express themselves in articular cartilage under different conditions. MMP-1, MMP-2, MMP-3, MMP-8, MMP-9, MMP-13, and MMP-14 are the matrix metalloproteinases. Four of those seven MMPs—MMP-1, MMP-2, MMP-13, and MMP-14—have been demonstrated to express constitutively in adult cartilage, suggesting that they probably play a role in tissue turnover. Their expressions are also elevated in pathological conditions. The remaining three MMPs (MMP-3, MMP-13, and MMP-9) in cartilage seem to be exclusively useful for pathological state differentiation. Based on their domain organization, sequence similarity, and substance affinity, MMPs can be further categorized.

2.9.3 MMPs and cartilage degradation:

MMPs have the ability to degrade all components of the extracellular matrix of the basement membrane. There are five distinct types. The first category of collagenases includes MMP-1, MMP-2, MMP-8, and MMP-13, which target collagen types I, II, and III. MMP-2 and MMP-9, also known as Gelatinous A and B, respectively, are enzymes that degrade type IV collagen within the basement membrane. Stromelysins (MMP-3, -10, -11) belong to the category of enzymes that cleave non-collagen matrix proteins. The fourth category consists of membrane-type MMPs, which include MMP-14, -15, -16, -17, -24, and -25. Finally, the diverse subgroup comprises MMP-7, -11, -12, -20, and MMP-23, encompassing a broad spectrum. In healthy joints, the expression of all these MMPs is basal. Osteoarthritic joints exhibit a substantial rise in the expression of these MMPs. Although the helix of collagen can be broken by any collagenase, substrate affinities and preferences vary. The chondrocytes that make up cartilage express MMP-13, which cleaves type II collagen more readily. Compared to MMP-1, MMP-13 cleaves type-II collagen 5–10 times more frequently. MMP-1, on the other hand, is more active against type-III collagen, while MMP-8 is most active against type-I collagen. Remembering that they can efficiently split on type-II collagen in the collagen helix is crucial.

MMP-1 expression is roughly ten times higher than MMP-13 expression. Elevated levels of MMP-1 may compensate for its limited ability to degrade type II collagen. Within the layers of cartilage, MMPs are distributed differently. The cartilage's deeper layers contain MMP-13. This well-organized pattern would suggest that MMP-1 and MMP-8 originate from joint synovial cells and neutrophils, which are found close to the cartilage, respectively. MMP-13 expression is primarily seen in chondrocytes. The additional collagenases, gelatinases A and B (MMP-2 and -9), can further break down the collagen triple helix into denatured collagen after it has been cleaved.

2.10 Changes in Extracellular Matrix during Osteoarthritis:

The primary mediators of the alterations in the ECM's architectural quality are matrix proteases and inflammatory cytokines. A change in the amount of extracellular matrix (ECM), particularly aggrecan, causes the tissue to undergo mechanical stress, which significantly modifies the chondrocyte's mechanical environment in the cartilage matrix.

Proteoglycan synthesis is reduced, collagen production is elevated during the early stages of OA, and the ratio of collagen synthesis is improved. It has also been demonstrated that collagen type II can convert to type I collagen. Collagen type II is present in the matrix of healthy cartilage, whereas collagen type I is mostly present in subchondral tissue. The microenvironment of extracellular matrix (ECM) is altered during inflammation, which modifies cartilage ECM formation and further disrupts cell function. Consequently, OA progresses due to a continuous cycle that occurs between the chondrocytes of the cell and the newly generated extracellular matrix. According to reports, there are a series of events that happen during OA that change the integrity of ECM homeostasis. Aggrecan content decreases, whereas collagen content increases.

2.11 Chondrocyte death in Osteoarthritis:

It has been observed that apoptosis, necrosis, chondroptosis, autophagy, or a mix of these processes can cause chondrocyte death. The morphological perspective displays membrane blebbing, chromatin condensation of the nucleus, cell shrinkage while preserving membrane integrity, and finally, the creation of apoptotic bodies. Apoptosis is a series of successive molecular processes that include activation, effector, degradation, and clearance phase.

2.12 Apoptosis as a cause of OA progression and during OA development:

The induction of programmed cell death, known as apoptosis, in chondrocytes is an essential requirement for the initiation and progression of osteoarthritis (OA). The process of chondrocyte apoptosis encompasses the intricate phenomenon of cartilage degradation, culminating in the ultimate transformation of hypertrophic chondrocytes within the growth plate. The pathogenesis of autoimmune disease, specifically osteoarthritis (OA), has been elucidated to commence through the process of apoptosis in the cartilage of human beings. In the context of osteoarthritis (OA), the cartilage exhibits macroscopic characteristics that are within the realm of normalcy. However, an intriguing observation arises: there is an occurrence of either an upsurge in apoptotic chondrocytes or a decline in the population of fully functional chondrocytes. Furthermore, an intriguing possibility emerges, suggesting a potential disruption in the expression of genes associated with the process of apoptosis.

2.13 Cell Culture:

2.13.1 Primary Cell Cultures:

Primary cell cultures are derived directly from typical animal tissue and culture either as an explant or by enzyme digestion dissociated into a single cell suspension. The primary cell retains most of the distinguished features of the cell in vivo within its relatively limited life span. Important Note: According to the definition of primary cultures, they are not passaged; after passaging, they are converted into a cell line, and at that point they are no longer primary anymore. 'Primary' cells obtained from most providers are cell lines which are low passaged (Chen, Mallon et al. 2014).

2.13.2 Secondary Cell Cultures:

Secondary or continuous cell culture comprises a single cell type propagated either by cell division or indefinite time. Cell lines which have a determinate lifespan are generally diploid and uphold a point of differentiation. The observation that these particular cell lines undergo senescence after approximately thirty rounds of division underscores the need to establish a robust and sustainable banks system to ensure the prolonged maintenance of such cell lines. Continuous cell lines that exhibit indefinite propagation typically acquire this capacity through their transformation into tumorigenic cells. The induction of tumorigenesis can be achieved through the utilisation of viral oncogenes or the application of chemical treatments, thereby facilitating the transformation of neoplastic cells. Altered

cell lines offer the advantage of nearly limitless availability, yet come with the disadvantage of retaining only a fraction of the original in vivo characteristics. (Pham and Ichikawa 2013).

2.15 Media preparations

Whilst all media might be prepared from essential ingredients Numerous frequently used media are accessible as readymade powders or available as 10x and 1x liquid media. All widely used media mentioned in the Sigma-Aldrich named online catalogue. If the powder or 10x media are bought, the water which is used to re- 19 form the powder or used to dilute the concentrated liquid is free from microbial, organic, and mineral contaminants. It should also be free from pyrogen and should be of tissue culture grade. In most of the cases, the prepared water which is suitable should be prepared by resin cartridge purification and reverse osmosis with an ultimate resistance of 16-18MΩ. When prepared, the medium pH is changed and then fixed the media filter before use (Sung, Ferlay et al. 2021). For the preparation of DMEM (Dulbecco's modified eagle medium), we took 3.5 g/l $NaHCO_3$, 10 ML (1%) of non-essential amino acid, 10 ml (1%) of antibiotics. We took DMEM in a powdered form and added in 1 litre autoclaved water. We romove 20 ml, from the remaining 980 ml of media, we took 50 ml for mixing $NaHCO_3$. We prepare a total volume of 1000 ml DMEM media in that manner.

3.0 Material and Method

Cell culture media and supplements such as Dulbecco's Modified Eagle Medium (DMEM), Fetal bovine serum (FBS), Phosphate buffered saline (PBS), antibiotic cocktail, Decalcifying solution, Haematoxylin & Eosin, Toluidine Blue, and Fast Green, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Complementary DNA (cDNA) synthesis kit was purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA) and SYBR Green kit was obtained from Genetix (New Delhi, India).

3.1 Materials

3.1.1 List of Chemicals

Table 2: List of chemicals used.

Sl. No.	Chemical name	Company
1	DMEM	Sigma Aldrich
2	Fetal bovine serum	Gibco, Brazil
3	Phosphate buffer saline	Gibco, Brazil
4	Antibiotics	Gibco, Brazil
5	Trypsin	Gibco, Brazil
6	Sodium Pyruvate	Gibco, Brazil
7	TRIzol	MP
8	Chloroform	Thermo Fisher ScientificSigma Aldrich
9	MTT	Sigma Aldrich
10	Dexamethasone	MP
11	ITS	MP
12	Elisa Kit	Elabscience
13	cDNA Reverse Transcription Kit	Thermos Scientific applied biosystems
14	RNA kit	Thermos Scientific
15	Betadine	Life pharma

16	Neosporin Powder	GSK Pharmaceuticals
17	Alcian blue	Sigma Life Science
18	Hematoxylin	Sigma Life Science
19	Eosin	Thomas Baker
20	Toluidine blue	Sigma Aldrich
21	Fast green	Sigma Aldrich
22	SYBR Green	SMOBIO Genetix Biotech
23	Decalcifying Solution	Sigma Aldrich
24	DPX mount	Sigma Aldrich
25	RNAase ZAP	Sigma Aldrich

3.1.2 List of Solvents

Table3: List of solvents used

Sl. No.	**Solvents**	**Company**
1	Isopropanol	Thermo Fisher Scientific
2	Ethanol	Thermo Fisher Scientific
3	Methanol	Thermo Fisher Scientific
4	Formaldehyde	Amresco
5	Xylene	Merck
6	Nuclease free water	Thermo Fisher Scientific
7	Butanol	CDH

3.1.3 List of Instrument

Table 4: List of Instruments used

Sl. No.	**Instruments**	**Company**
1	Autoclave	New Brunswick Global Scientific Technology
2	Centrifuge	Eppendroff Thermo scientific
3	Nanodrop	Thermo scientific
4	Spinwin (micro centrifuge)	GeNei
5	Freezer (-80^{o}c) (-20^{o}c) (4^{o}c)	New Brunswick, Eppendorf Samsung, LG Iceberg, Vestfrost
6	CO_2 IncubatorR	Binder
7	Laminar air flow chamber	Telstar Bio II Advance
8	Inverted microscope	Evos XL
9	Spinwin (micro centrifuge)	Genetix
10	Weigh balance	Aczet Sartorius
11	Ice machine/Ice flaker	Labman
12	Orbital shaker	Thermo Scientific
13	Shaking water bath	Alcon
14	Plate reader molecular device	SpectraMax Paradigm

15	QuantStudio 3, PCR Machine	Thermo Fisher Cloud
16	Micro Computed Tomography	Bruker SkyScan 1276 Bruker SkyScan 1275
17	Dry bath	GeNei
18	Pipettes	Thermo Scientific

3.1.4 List of Plastic and glass wares

Table 5: List of Plastic and glass wares used

Sl. No.	**Wares**	**Size**	**Company**
1	Beakers	100ml, 200ml, 500ml	Borosil
2	Centrifuge tubes	15ml, 50ml	Thermo Scientific Genaxy
3	Flask	25cm, 75cm	Thermo Scientific
4	Measuring cylinder	1000, 250, 150, 100, 25ml	Borosil
5	Tips	100µl, 200µl, 1000µl	Genaxy
6	Micro centrifuge tube	1,5ml, 2ml	Axygen
7	PCR Plates	0.1ml, 0.2ml, 96well plate	Genaxy
8	Glass funnel	Big small	Borosil
9	Cryovial	1.5ml	Cryovial Nunc,Apogent
10	Cell Culture Plate	12, 24, 48 well plate	Thermo Fisher, Costar, CytoOne, Nest
11	Prafilm	Medium	Bemis

3.2 Methodology

3.2.1 Aseptic Technique and Good Cell Culture Practice

Procedure:

1. Before the commencement of work, cabinet is sanitized by using 70% isopropanol.

2. Prior to starting of the work, gloves were sanitized by spraying 70% isopropanol on them and permitting them to air dry for about 30 seconds.

3.All the equipment were kept in the cabinet or the required materials were brought inside the cabinet such as pippete tip boxes, bottles and pipette aids. They must be wiped off with a tissue drenched with 70% isopropanol before usage.

4. Gloves should not be contaminated by touching the things kept outside the cabinet specifically face and hair and if gloves got contaminated during the work, 70% isopropanol is used to re-spray on them as above before starting the next proceeding.

5. Gloves were discarded after working with the cultures which got contaminated during the work and after completing the work.

6. Movement must not be rapid inside and instantly outside the cabinet. The slow movement will permit the air to circulate correctly within the cabinet.

7. Coughing, speech and sneezing must not be in the direction of the cabinet so the airflows should not be disrupted.

8. All the equipment and materials were disinfected before removing it from the cabinet after completing the work. Work surfaces inside the cabinet must be sprayed with 70% isopropanol and then wiped dry with a tissue, and then tissue must be disposed of by incineration.

9. Waste of liquid cell culture were discarded in sodium hypochlorite (10,000 ppm). It should be kept preferably for overnight in the cabinet or for a minimum of two hours before discarding drain with abundant amounts of water.

10. Occasionally cabinet surfaces were cleaned with the use of a disinfectant or the cabinets were fumigated as per the instructions of the manufacturer. However, it should be confirmed that fumigating the laboratory environment is safe due to the formation of formaldehyde in gaseous form and on-site Health and Safety Advisor should be consulted.

3.2.2 DMEM media

Dulbecco's Modified Eagle Media is the most commonly used media for culturing of adherent cell lines. For the culturing of A549 cells DMEM media is used**.**

3.2.3 Composition of DMEM media for 100ml

• DMEM media powder - 1.337 gm • HEPES - 0.59 gm • NaHCO3 -0.16 • FBS - 10% foe flask and 20% for reviving • Antibiotics- 1ml

3.2.4 Direction for preparation of DMEM media

Before preparation of DMEM media FBS bottle was heat inactivated at 56º C in water-bath for 30 minutes.

1- Weighed all the components of the media as mentioned above.

2- Taken all the components inside the LAF

3- Took a beaker and added around 50 ml of autoclaved water into it.

4- Added all the components one by one.

5- Adjusted the pH of the media in between 7.2-7.4 using HCL (1N) / NaOH (1N). Optimised the volume to 100 ml by adding autoclaved water.

6- Using an autoclaved syringe and micro-filter, filtered the media into an autoclaved falcon / bottle.

7- Store the liquid media at 2-8ºC and in dark till use.

3.3 Cell culture

For the in vitro experiments, primary rat chondrocytes were harvested from the knee joints of neonatal pups. In-vitro studies were done with primary cultured chondrocytes(Shi et al., 2019). These cells were grown in DMEM high glucose media and seeded into T-25 flasks. Cultures were trypsinized at 80% confluency and seeded in well plates or flasks according to experimental requirements(Shi et al., 2019).

3.4 Rat articular chondrocyte isolation

Enzymatic digestion of knee joints isolated from 2-3 days old pups yield pure population of chondrocytes. Chopped pieces of cartilage were subjected to enzymatic cocktail of collagenase II (0.6mg/ml) and dispase (0.1mg/ml) in DMEM, high glucose (Gibco, MD, USA) for a period of 6 hours at the temperature of 37 degree Celsius(Malemud, 1993). To remove any unwanted and undigested clump, the isolated chondrocytes were passed through 70µm filter (Genetix, biotech Asia, India). The collected cells were centrifuged and resuspended in DMEM, high glucose with 10% fetal bovine serum (FBS, Gibco, MD, USA) with antibiotics (penicillin, streptomycin 100 U/ml, Sigma, MO, USA) and incubated in 5% CO2 at 37-degree Celsius in humified atmosphere (Binder), and at every alternate day, media was changed.

3.5 Cell viability assay (MTT)

Cells were seeded into 96 well plates in 10% DMEM-high glucose media for cell viability experiments. After 50% confluency, the cells were treated with different concentrations of dexamethasone (1µM). Rat articular chondrocytes were used to assess dexamethasone for cell viability. At the end of the treatment duration, MTT (5 mg/ml) was added to each well and incubated for 2–4 hrs. Following this, 100 µl of DMSO was added to each well to dissolve the formazan crystal, and absorbance was measured at 570 nm.

3.6 Real time PCR

Primary articular chondrocytes were seeded in six-well plates and treated with varying concentrations of dexamethasone ranging from 1 nM to 1 µM for 48 hrs. At the end of the time point, RNA was collected in TriZol and cDNA was synthesised by the high-capacity cDNA reverse transcription kit (Thermo Fisher). Following this, real-time PCR was done (Quant Studio 3, Applied Biosystems) using a SYBR green master mix for the assessment of the chondrogenic genes Sox-9, Col2A1, and Aggrecan.

Gene Symbol	Gene Name	Primer Sequence
GAPDH	**Glyceraldehyde-3-phosphate dehydrogenase**	**Forward: GGAGTCAACGGATTTGGTCGTA Reverse: GGCAACAATATCCACTTTACCAGAGT**
Sox9	**SRY-Box Transcription factor 9**	**Forward: CTTCGCAGGGAGTTCTCA Reverse: AGCTGTGTGTAGACGGGT**
Col2	**Collagen type 2**	**Forward: AGAGCGGAGACTACTGGA Reverse: TCTGGACGTTAGCGGTGT**
ACAN	**Aggrecan**	**Forward: TGGTGTTTGTGACTCTGAGG Reverse: GATGAAGTAGCAGGGGATGG**
MMP13	**Matrix metalloproteinase-13**	**Forward: TTCTTGTTGCTGCGCATGA Reverse: TGCTCCAGGGTCCTTGGA**
ADAMTS-5	**ADAM metallopeptidase with thrombospondin type 1 motif 5**	**Forward: ATGCAGCCATCCTGTTCAC Reverse: CATTCCCAGGGTGTCACAT**

3.7 Development of a surgically induced OA model in vivo

Animals procured from CDRI were acclimatised for a week before starting the experiments. Three animals were kept in each group for experiments; three for control group and three for ACLT group. For anaesthesia, a cocktail of ketamine and xylazine in 3:1 ratio was injected into rats for surgery to transect the anterior cruciate ligament transection (Kothari et al.) in the right leg. Animals with surgery but not transection were considered as control group. Four weeks after surgery, both ACLT and control animals were sacrificed(Adhikary et al., 2019). Healthy animals in which ACLT was not done were used as a positive control.

3.8 Alcian blue staining

To determine the proteoglycan content, chondrocyte cells were differentiated for 21 days. The cells were given treatment of Dexamethasone and different plate was supplemented with 1% ITS (insulin-transferrin-selenium) for differentiation. The medium was changed and treated with Dexamethasone after 48 h intervals. After 21 days of treatment in differentiation medium, Rat articular cartilage (RAC) cells were fixed in plate for 30 minutes with methanol. At room temperature cells were stained with lcian Blue (AB). In a readymade solution of alcian blue, fixed cells were incubated for AB staining (Sigma-Aldrich) for 30 min before extra dye is extracted by means of sterile water.

3.9 Histological evaluation

Histological staining was performed for the analysis of the cellular features and matrix content of the cartilage and the micro-architecture of subchondral bone. During euthanization, the tibia as a sample from the right leg was fixed in 4% paraformaldehyde and decalcified after cleaning of the adjacent muscle tissue. The decalcified bones were then processed to get 5 µm thin paraffin sections(Kothari et al., 2020). The sections were stained with hematoxylin and eosin (H&E), fast green and toluidine blue. Morphological analysis of the bone and subchondral bones was performed in a double-blinded manner, and images were taken using a microscope (Evos XL, Life Technologies). To evaluate the thickness of cartilage, the toluidine-blue-stained sections were quantified with the help of ImageJ software. Further, for histological analysis of the subchondral architecture, the bone volume, bone volume/tissue volume (BV/TV), trabecular number (Tb.No), and trabecular separation (Tb.Sp) were quantified using the Bioquant Osteo software 2014(Kothari et al., 2022).

3.10 Micro computed tomography (micro-CT)

Computed tomography (CT) is an additional important tool that provides an understanding of high-resolution bone anatomical images and enables three-dimensional post-processing of imaging data of particular importance for orthopaedic surgery. However, its main disadvantage is the limitations in the assessment of soft tissue structures compared to MRI. CT arthrography may be useful in assessing focal cartilage defects or meniscal tear. Arthrography technique combined with either X-ray/computed tomography (CT) is used to measure cartilage surface contour. It does not provide information for soft tissues, and cartilage is one of them. However, its applicability may be limited due to its invasive nature.

The intact knee joint of the femur and tibia from each group was scanned using micro-computed tomography (Sky Scan 1276 scanner; Sky Scan, Aartselaar, Belgium). Scanning was done at a voxel size of 18 µm, at a voltage of 70 kV, a current of 142 mA, and a field of view of 35 mm, by using a filter of 1.0 mm aluminium plate, a 0.8-degree rotation step, and full width. The scanned file was reconstructed using the NRecon software. 3D images were drawn using the CT volume software, and 2D images were obtained by CTAn from the ROI in the data viewer software. 3D microarchitecture parameters such as bone volume (BV), bone volume/tissue volume% (BV/TV%), trabecular number (Tb. No), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and structural model index (SMI) were measured using software provided with the instrument(Kothari et al., 2023).

3.11 Statistical analysis

Results are expressed as mean ± SEM. One-way ANOVA was applied for experiments with multiple comparisons followed by Tukey's test of significance. For the statistical analysis, GraphPad Prism v.8 was used. The probability value of $P < 0.05$ for the experiments were considered to be significant. For the animals for gross appearance, 2D images, histological assessment, and micro-CT anal

4.0 Results and Discussion

4.1 Objective 1

4.1.1 *In-vitro* study to analyse osteoarthritic pathophysiology in Dexamethasone treated *chondrocytes*

The aim of this objective is to study the detrimental effect of Dexamethasone on chondrocytes at its higher concentrations as it is the most widely used glucocorticoid, worldwide.

4.1.2 Cell viability assay of Dexamethasone

Cell viability after treatment with different concentrations of Dexamethasone was determined by MTT assay on rat articular chondrocytes cells. Chondrocytes isolated from rat articular chondrocyte culture and treated with different concentrations of Dexamethasone (100 µM to 1pM). Treatment of Dexamethasone was given to chondrocytes for 48 hours. Dexamethasone is a corticosteroid generally used in re-differentiation of chondrocytes and stimulates the synthesis of cartilaginous matrix. Since at higher concentrations and when used for longer duration glucocorticoids impact bone, it is crucially important to understand the conditions Dexamethasone imposes on the chondrocyte cells that make up the cartilage. This would help determine the potential for safe use of glucocorticoid clinically as a disease-modifying drug. So, the study of control vs. dexamethasone is being done through *in-vitro* model mimicking osteoarthritic condition. Data in fig. shows that the dexamethasone concentration from 100µM to 1pM was examined. This graph shows that dexamethasone at 1µM concentration shows detrimental effects on the chondrocytes. So, we can use this concentration for the study of pathophysiology of osteoarthritis. At 100µM concentration, dexamethasone is toxic to the chondrocytes so we cannot use this concentration for our study. At other concentrations like 10nM, 100pM, and 1pM shows similar effect like the normal growing cells in the control so we did not use these concentrations for our further study.

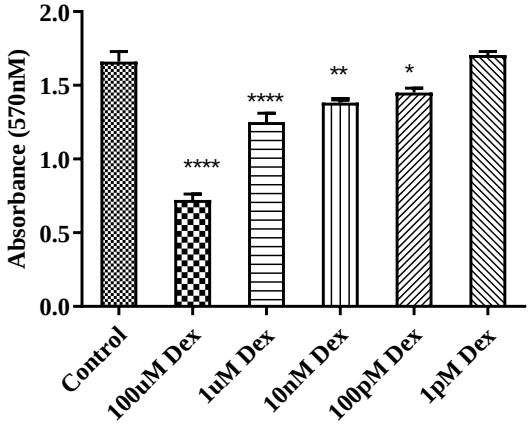

Fig.7. : Cell viability assay on rat chondrocytes. The MTT assay was done to check the viability effect of dexamethasone for 48 hrs of treatment. Cell viability data were presented as the Mean±SEM.

4.1.3 Long term usage of Dex aggravates the proteoglycan content loss of articular cartilage in primary rat articular chondrocyte culture which is analysed through alcian blue staining.

To determine the proteoglycan content, chondrocytes were differentiated for 21 days. After trypsinization, the cells were given treatment of dexamethasone (1µM) along with the supplementation of 1% ITS (Insulin-Transferin-Selenium) (5µl/ml in 10% DMEM media) for differentiation. Plating of wells for undifferentiated, differentiated and dexamethasone was done. The medium was changed and treated with Dexamethasone in one well and ITS on another well at 48 hours intervals for 21 days. After 21 days of differentiation, the cells were fixed with methanol for 30 min at room temperature then stained with alcian blue. Individual wells were photographed

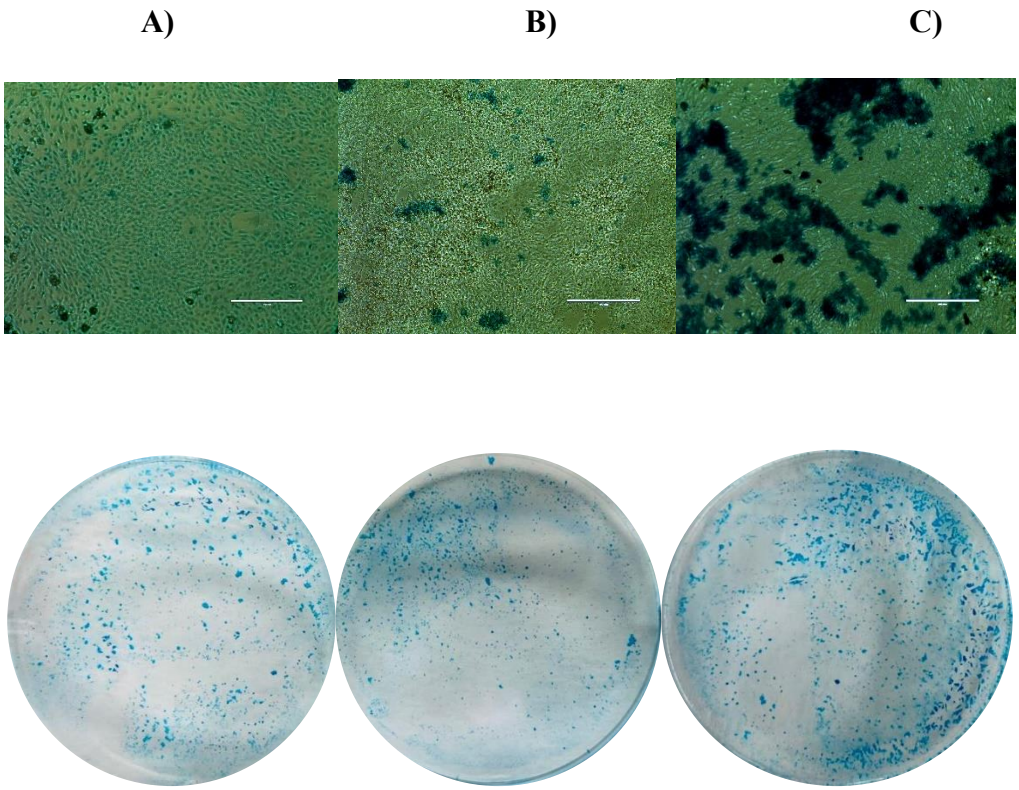

Fig 8: Alcian blue staining for proteoglycan content. A is undifferentiated, B is Dexamethasone treated and C is differentiated.

4.1.4 To check the gene expression of different genes in chondrocyte through RT-PCR in order to study the osteoarthritic pathophysiology with treatment of Dexamethasone

Cells are counted manually by haemocytometer using a vital stain trypan blue and then plated for further molecular experiment like RNA isolation by TRIzol method is used out for studying normal vs osteoarthritic pathophysiology. After isolation of RNA, cDNA was prepared and then Real- Time (RT-PCR) was setup for studying the impact on cartilage catabolic genes like MMP-3 (Matrix metalloprotein-3), MMP-13 (Matrix metalloprotein-13), and anabolic genes sox-9 (Sex determining region Y-box 9), aggrecan, type-2 collagen.

4.1.5Anabolic gene expression in chondrogenesis

Sox9

Sox9 is a major transcription factor. It remains expressed throughout lifespan of healthy articular chondrocytes. It helps in increasing the expression of other anabolic genes. Sox9 is required for chondrogenesis: it secures chondrocyte lineage commitment, promotes cell

survival, and transcriptionally activates the genes for many cartilage-specific structural components and regulatory factors.

Col-2

Cells treated with Dexamethasone for 48h were analysed at transcriptional level. In Dexamethasone treated cells, chondrogenic genes Sox9 and matrix producing gene col2 expression were decreased. In our study we found that Dexamethasone treated chondrocytes showed decreased expression of sox9, and col2. These altered levels of genes affect chondrocytes and its matrix production ability.

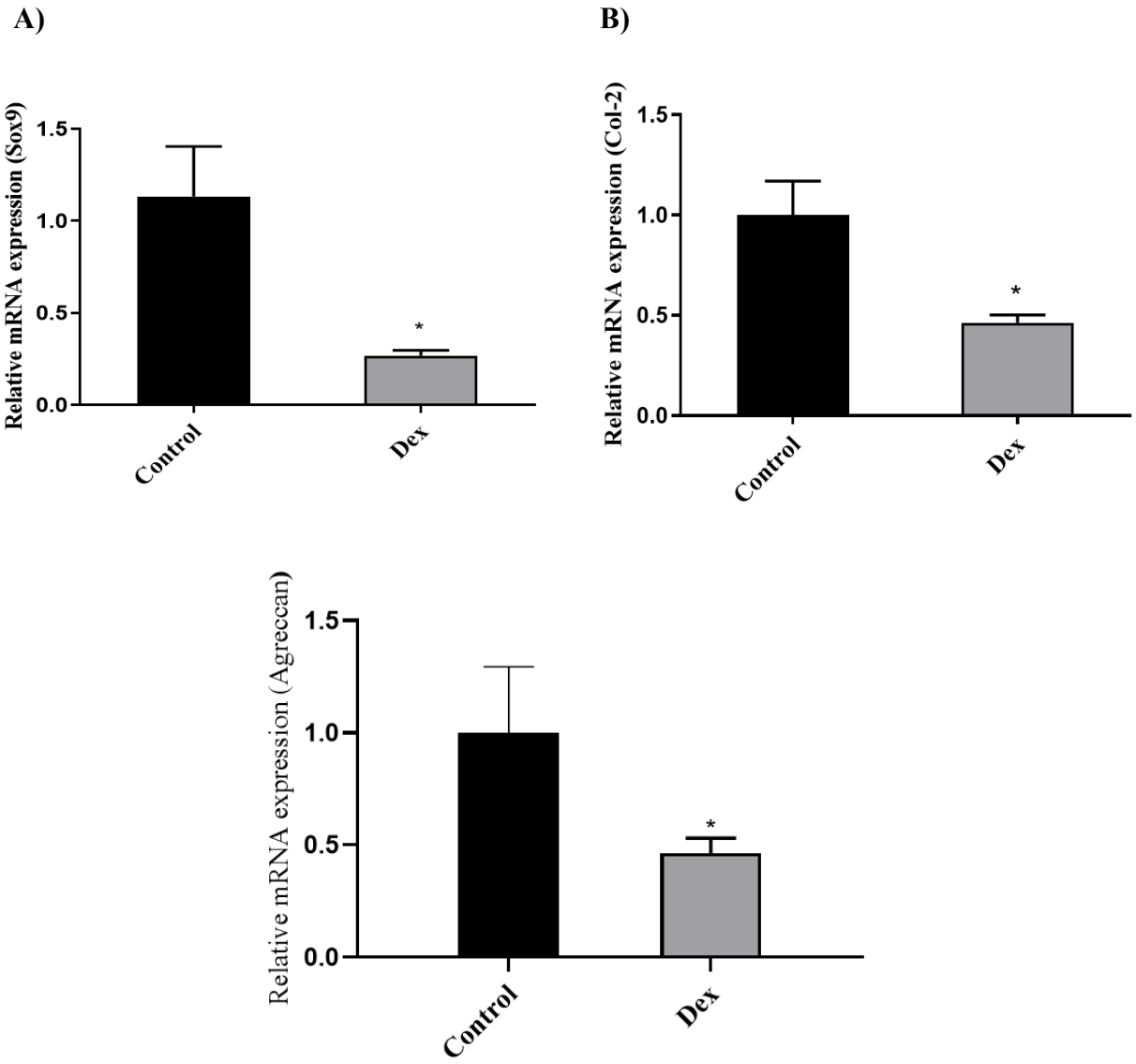

Fig 9: Expression of chondrogenic and ECM producing genes: [A] Sox9, [B] Col2 and [C] Aggrecan. SOX9: The SRY-box transcription factor 9 (SOX9) is a master regulator of

chondrogenesis. It plays a critical role in the early stages of chondrocyte differentiation and is essential for cartilage formation. Collagen Type II (COL2A1): Collagen type II is the main collagen component of cartilage. It is highly expressed during chondrogenesis and is considered a specific marker of mature chondrocytes. Aggrecan: Aggrecan is a large proteoglycan bearing numerous chondroitin sulfate and keratan sulfate chains that endow articular cartilage with its ability to withstand compressive loads. It is present in the extracellular matrix in the form of proteoglycan aggregates, in which many aggrecan molecules interact with hyaluronan and a link protein stabilizes each interaction.

4.1.5 Catabolic gene expression in chondrogenesis

MMP-3

MMP-3 is a proteinase synthesized and secreted by synovial fibroblasts and chondrocytes in the joints. The MMP3 enzyme degrades collagen types II, III, IV, IX and X, proteoglycans, fibronectin, laminin, and elastin.

MMP-13

Matrix metalloproteinases (MMP's) family of enzymes which are structurally associated and characterized as zinc-dependent endopeptidases. Matrix metalloproteinases (MMPs) have been identified as the primary mediators involved in modulating the interaction between cells and the extracellular matrix (ECM) within cartilage tissue. This intricate interplay ultimately leads to the degradation of cartilage. In dexamethasone induced cells, the expression of the matrix regulating protease i.e., MMP-13 was found to be upregulated. In the context of osteoarthritis, the presence of inflammation triggers the activation of matrix-degrading proteinases, leading to the degradation of matrix proteins. This process can be considered as a potential consequence of the inflammatory response or the activation of genes associated with inflammation. (Li et al., 2017) .

ADAMTS-5

ADAMTS-5 is involved in the pathogenesis of OA. As the major aggrecanase degrading articular cartilage matrix, ADAMTS-5, has been regarded as a potential target for OA treatment.

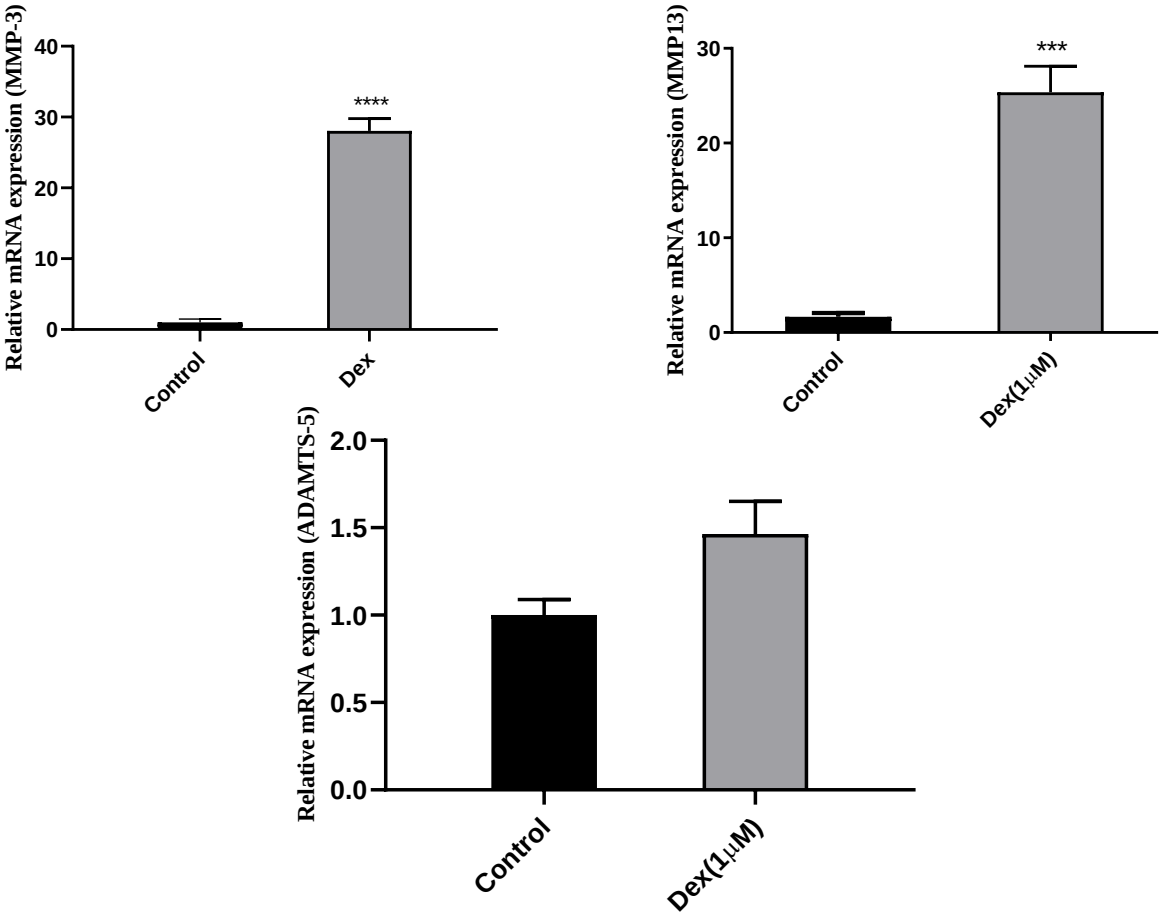

Fig10 : Expression of genes: [A] MMP-3, [B] MMP-13, and [C] ADAMTS-5.

Chondrogenic genes and matrix degrading proteases were investigated from knee cartilage samples. Osteoarthritic symptoms in the Dexamethasone treated group were induced due to down-regulated expression of chondrogenic genes, including Sox-9 and, Col2 and upregulation of matrix-degrading genes such as MMP-13 and, ADAMTS-5 which act as proteases and cleave matrix protein and degrade cartilage as evidenced in the RT-PCR. Decreased Sox-9 expression affects cartilage homeostasis and their matrix producing capacity by down-regulating Col2, which are major proteins in the extracellular matrix, and provides the skeleton of cartilage and resistance to compressive load. In addition to these deleterious effects, MMP-13 levels were up-regulated in the Dexamethasone treated chondrocytes. Here Dexamethasone has shown to play a dual role in cartilage degradation

by decreasing chondrogenic expression and by up-regulating MMP-13 and ADAMTS-5 activity.

4.2 EGLN3 and ORM1 were examined using RT-PCR to gain a comprehensive understanding of the molecular-level pathogenesis of subchondral bone in osteoarthritis.

Along with the known anabolic genes like Sox9, Col2, and Aggrecan and catabolic genes like MMP-3, MMP-13, and ADAMTS-5, we have also examined some differently expressed genes (DEGs). To obtain differentially expressed genes Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment analysis were performed. A few genes were identified, from which I have selected EGLN3 and ORM1 for further studies through RT-PCR. The enrichment analyses of the DEGs and significant modules indicated that DEGs were mainly involved in the inflammatory response, extracellular space, RAGE receptor binding, and amoebiasis pathway. The present study provides a novel and in-depth understanding of the pathogenesis of OA EGLN3 and ORM1 have potential as candidate targets for future diagnosis and therapeutic interventions in patients with OA. The association between osteoarthritis and these specific genes has not been documented. ORM1 encodes a key acute phase plasma protein, Alpha-1-acid glycoprotein 1, which acts to modulate the activity of the immune system in the acute phase responses. During OA, the hypoxic microenvironment could induce endoplasmic reticulum stress in chondrocytes and further disturb extracellular matrix (ECM) secretion. We did detect several regulators or target genes of the HIF-1 signalling pathway, such as Egl-9 Family Hypoxia Inducible Factor 3 (EGLN3). The present study provides a novel and in-depth understanding of the pathogenesis of the OA subchondral bone at the molecular level, however, further studies are necessary to clarify the biological function of these genes in the pathogenesis of OA(Yang et al., 2020).

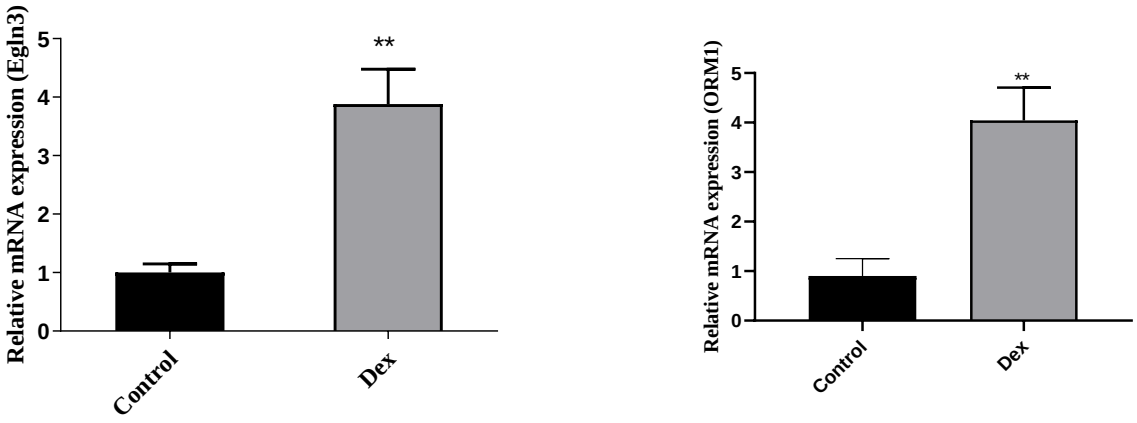

Fig11: EGLN3 and ORM1 both are found to be downregulated gene but in the effect of Dexamethasone, they are found to be upregulated. EGLN3 and ORM1 may be the new candidate targets for diagnosis and therapies on patients with OA in the future.

4.2 Objective 2:

4.2.1 To study an *in-vivo* ACLT model that mimics human osteoarthritic condition.

The aim of this objective is to study the changes on cartilage and subchondral bone in the surgically ACLT model which is a human mimic and more appropriate model to mimic pathophysiology of human osteoarthritis. Total six animals were taken, 3 for control group and 3 for ACLT.

4.2.2 ACLT model

Therapeutic model for osteoarthritis was set up. ACLT surgery was performed to create osteoarthritis. After performing anterior cruciate ligament transection, animals were kept for 1 month and then terminated. After completion of 1 month, animals were terminated and collection of knee sections i.e., tibia and femur bone were done for the analysis. Tibia was processed for micro-CT and analysis, and histology for subchondral bone micro-architecture and cartilage respectively. The cartilage and subchondral bone deterioration is the result of the abnormal mechanical loading that occurs concomitantly as a result of the ACLT surgery. Cartilage with bone sections were histologically examined for haematoxylin and eosin (H&E), and toluidine blue staining. Stained sections were captured at 10x and 40x magnification. In the same sections, along with cartilage, subchondral bone was also

analysed at 10x magnified images. Altered thickness of total cartilage, hyaline cartilage and calcified cartilage were measured by ImageJ software of both control and ACLT group animals and the data was analysed. Stained sections were captured by microscope for calculation of subchondral bone architecture via bioquant software.

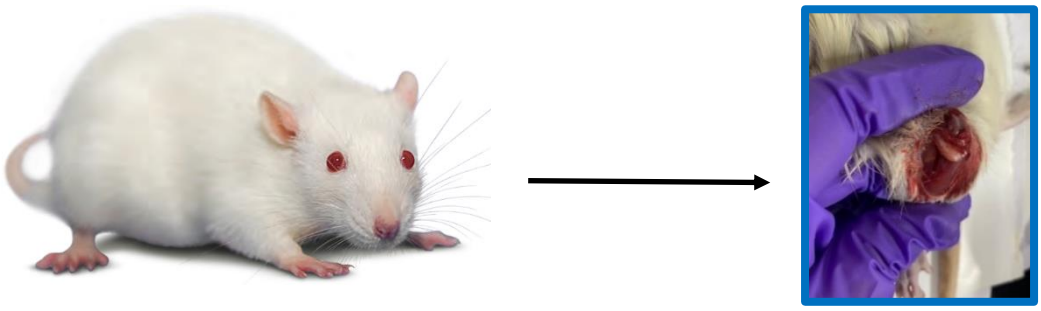

Fig 12: Development of ACLT model in SD rats to study osteoarthritis.

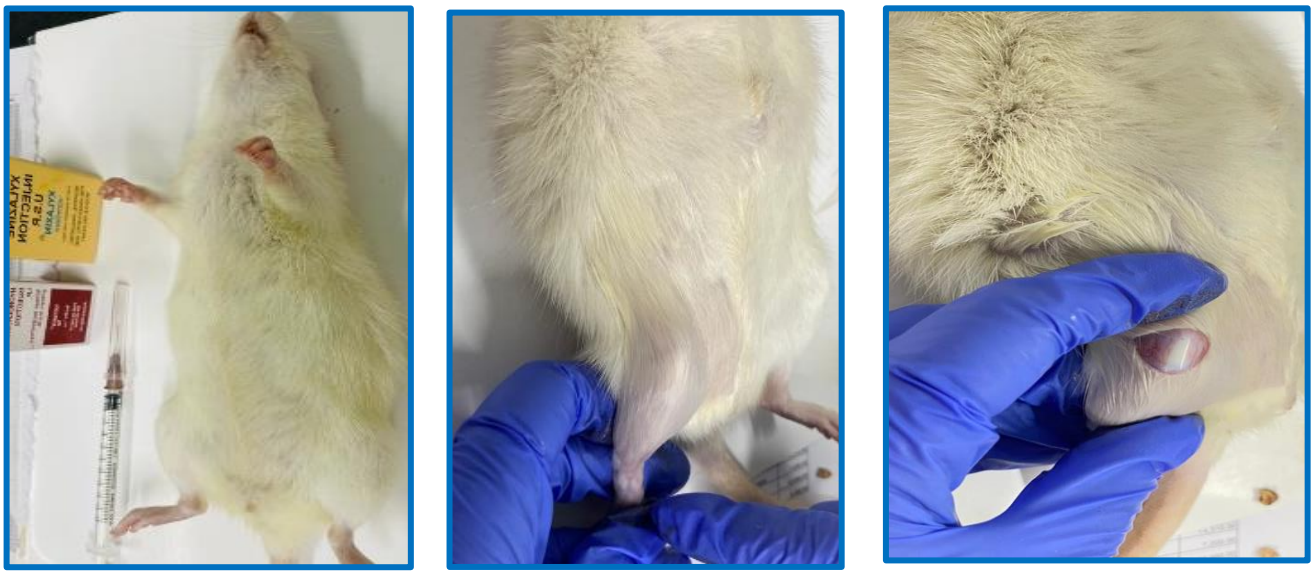

Fig 13: In fig: [A] Animal (SD Rat) has been made unconscious using a cocktail of ketamine and xylaxine. In fig: [B] patella has been dislocated for ACLT surgery. In fig [A] anterior cruciate ligament is identified.

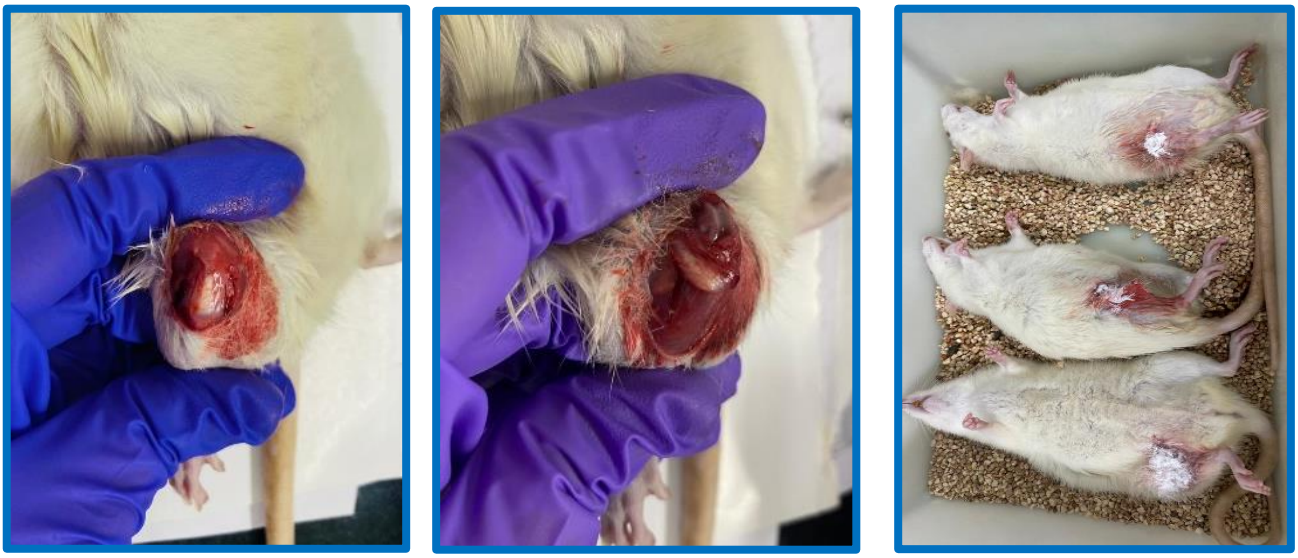

Fig 14 : In fig [B] Cut has been made on the knee using surgical blade. In fig [C] cut on anterior cruciate ligament is made. In fig [D] post-operative care of animal has been taken using betadine and neosporin powder

5.Results:

To study the difference on cartilage and subchondral bone in the surgically ACLT model which is a human mimic and more appropriate model to mimic pathophysiology of human osteoarthritis at the cellular level H&E staining was done. After termination of experiment, tibia was processed to obtain 5µm section and stained with haematoxylin & to visualize cells, their number, and alignment at the 40x magnification. In the control, cells were uniformly distributed and good number of chondrocytes were observed. Duplication and triplication of tidemark is also observed in the ACLT group. By analysing the stained sections, we found that a lesser number of chondrocytes were observed in the ACLT group. This result may correspond with the result of the ACLT surgery compared with the control group. Randomly and unevenly scattered chondrocytes in the cartilage of ACLT group was also observed. Chondrocytes numbers in both the layers were decreased irrespective of the thickness of both individual layers. Along with chondrocyte number, the overall cartilage thickness decreased. The tibia from the knee joint section were stained with H&E. Articular cartilage covers the end of the bone and specially knee joint. It has two types of layers. 1. Hyaline cartilage is the upper most layers, while below to it there is a calcified cartilage. Hyaline has more thickness as compared to the calcified cartilage. Both of these layers collectively called as articular cartilage. Articular cartilage in the control group shows intact cartilage and integrated structure. The matrix and cartilage homogeneity are disturbed in case of the ACLT group. The width of the articular cartilage decreased significantly in case of the ACLT group as compared to the control group. Altered microarchitecture of subchondral bone is also observed in the ACLT group. The clear discernible cartilage layer and the chondrocytes number are the representative of healthy joint which is deleteriously affected in ACLT group.

5.1 5Radiological appearances of the knee joint in ACLT animals

Knee radiological images state about osteoarthritis. Radiographic images of the knee joint of ACLT represent altered joint space and architecture. In the case of osteoarthritis, along with cartilage, the architecture of subchondral bone also gets affected. The altered subchondral bone architecture of ACLT animals was depicted by sagittal and coronal views respectively. In the ACLT animals, joint space is increased and architecture of subchondral bone gets altered.

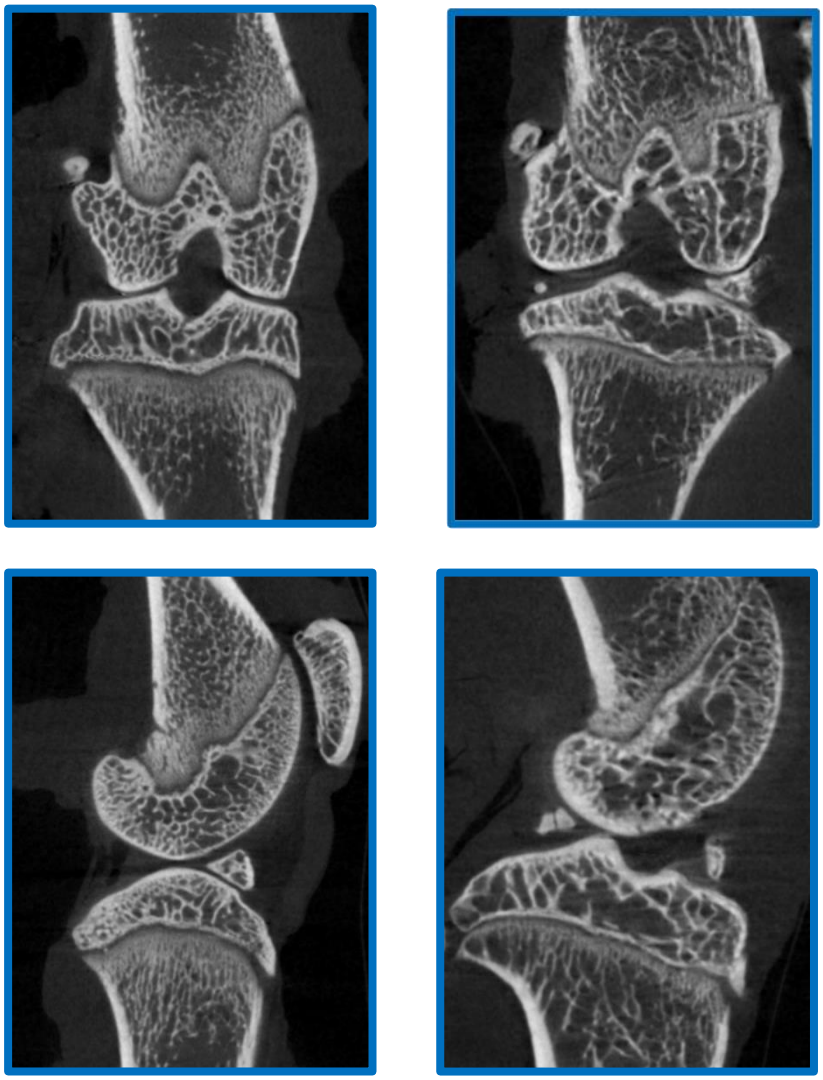

Fig 15: 2D images of subchondral bone of the control and ACLT-induced osteoarthritis model. Cross-sectional 2D images of the knee joints upper (coronal view) and lower (sagittal view), showing the significant subchondral bone loss in the tibia of the ACLT group.

5.2 Histopathology

5.2.1 To study the tibial articular and subchondral bone in the control and ACLT groups.

Intact subchondral bone microarchitecture is the representation of a healthy osteochondral junction. This intactness and integrity is deteriorated in the case of any mechanical injury to the joint tissue. The injury of the ligament causes the malalignment of the entire joint, affecting both cartilage and the underlying bone. In the ACLT animals; integrity of cartilage, chondrocytes number, matrix content i.e., proteoglycan as well as cartilage thickness is reduced when compared to the control animals. Along with all these parameters, the microarchitecture of subchondral bone has been observed to be deteriorated in the ACLT group.

CONTROL **ACLT**

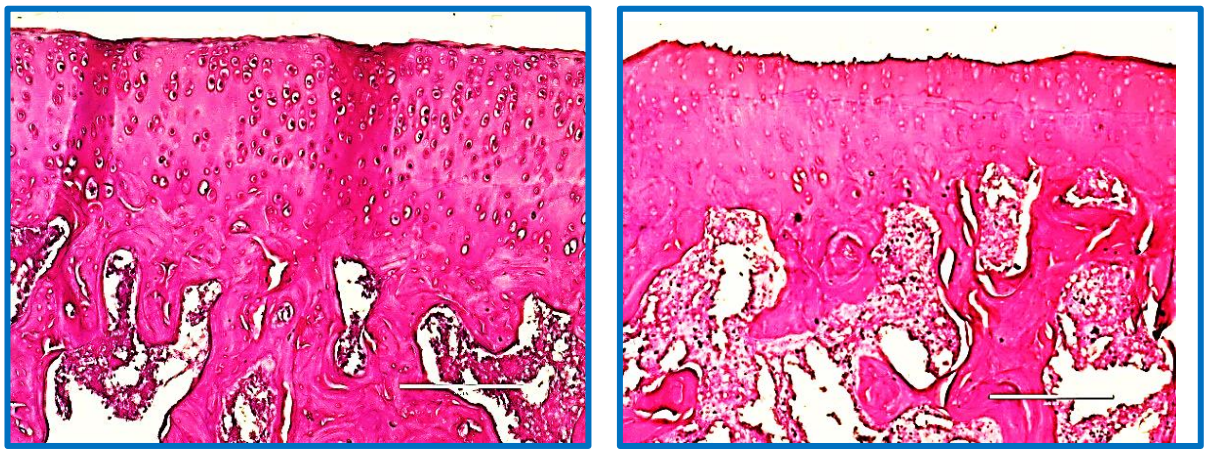

Fig 16: Histological evaluation of the tibial articular and subchondral bone in the control and ACLT groups. Haematoxylin and eosin staining showing the chondrocyte cells in the cartilage of the control and ACLT model. It shows the cartilage thickness, cell number, their alignment and tidemark duplication in ACLT group.

A) B) C)

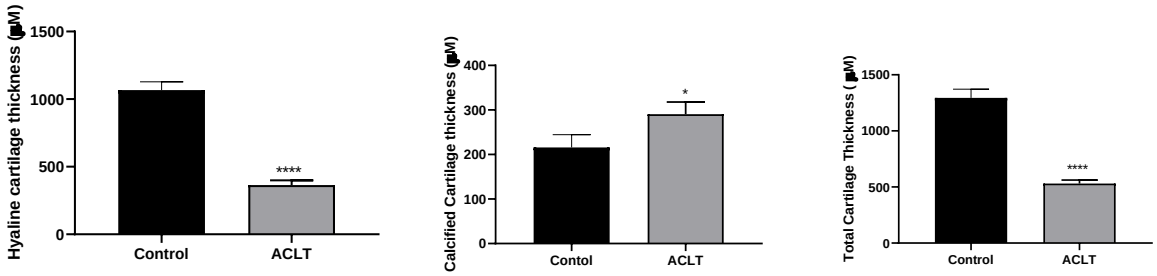

Fig 17: Quantitative analysis of cartilage of Control vs. ACLT group. Stained sections were quantified by ImageJ software for the assessment of cartilage thickness. [A] Hyaline cartilage (HC), [B] Calcified cartilage (CC), and [C] total cartilage thickness (TC).

5.2.2 To study the tibial subchondral bone in the control and ACLT groups.

Cartilage bears and transfers mechanical forces to the subchondral bone. The architecture of subchondral bone in individuals with osteoarthritis is disrupted. At the knee joint, cartilage and subchondral bone work as single osteochondral unit. Cartilage reduces friction during motion and transmits the load on the underlying subchondral bone. In osteoarthritis, the osteochondral unit gets affected in the subsequent progression of cartilage degradation that transmits more mechanical load on the tibial plateau, which ultimately alters the microarchitecture of subchondral bone. In four weeks of ACLT surgery, cartilage along with subchondral bone, represented significant changes in their architecture. Alteration in the knee joint was seen in both tibial plateau and femur epiphysis. Bone volume/tissue volume% (BV/TV%), trabecular number (Tb. No), trabecular thickness (Tb.Th.), No. of closed pores were reduced, with an increase in structure model index (SMI) reflecting altered trabecular interconnections. Decreased bone volume is the outcome of decreased trabecular number and thickness which increases the trabecular separation in the ACLT group. 3D images of subchondral bone are representing changes in the microarchitecture in ACLT group.

CONTROL **ACLT**

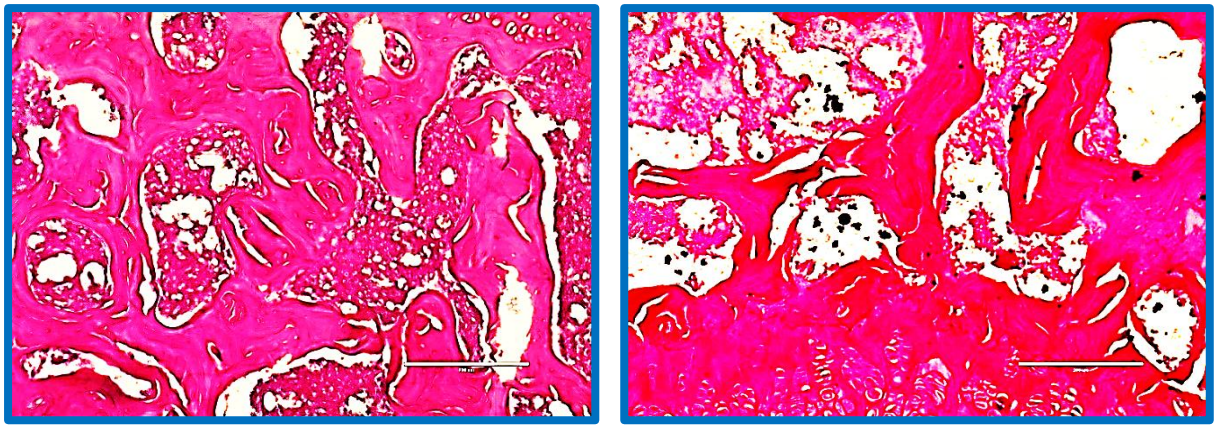

Fig 18: Histological evaluation of the tibial subchondral bone in the control and ACLT groups. Haematoxylin and eosin stain representing subchondral bone deterioration in the ACLT group.

5.2.3 Study of the chondrocytes in growth plate region of the control and ACLT groups.

The growth plate is the area of tissue near the ends of long bones that determines the future length and shape of the mature bone. Each long bone has at least two growth plates, one at each end, and they are longer than they are wide. The growth plate is more organised in the control as compared to the ACLT group. Cells gets diminished and scattered in the ACLT group.

CONTROL **ACLT**

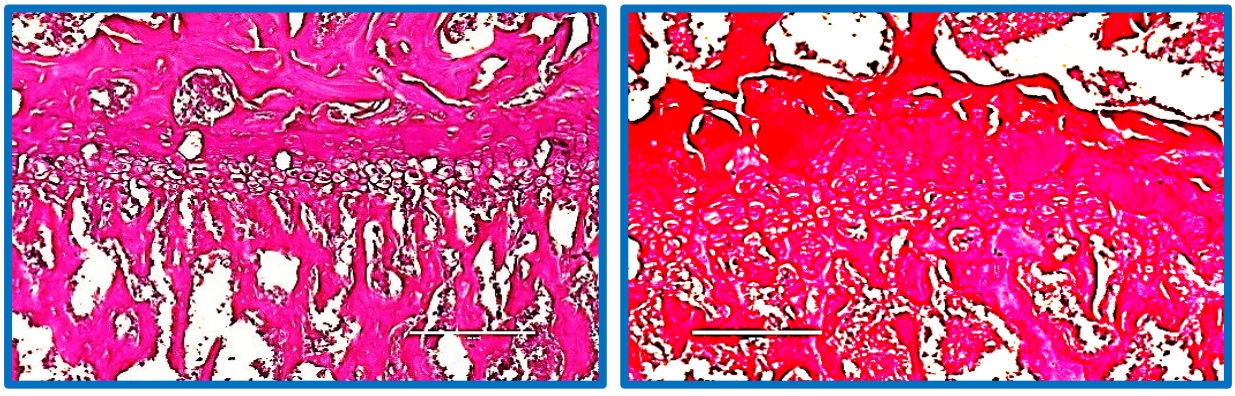

Fig 19 : Histological representation of the chondrocytes in growth plate region of the control and ACLT groups. Haematoxylin and eosin staining showing the chondrocyte cells in the cartilage of the control and ACLT group.

5.2.4 Effect of ACLT surgery on the cartilage thickness

Chondrocyte numbers and decreased proteoglycan/ glycosaminoglycans (GAG's) content in the ACLT mode markedly reduced cartilage thickness. Decreased proteoglycan/ glycosaminoglycans (GAG's) content and reduced thickness of cartilage in ACLT were evaluated by Toluidine Blue staining. Hyaline and calcified cartilage is well demarcated by the presence of the tidemark.

CONTROL **ACLT**

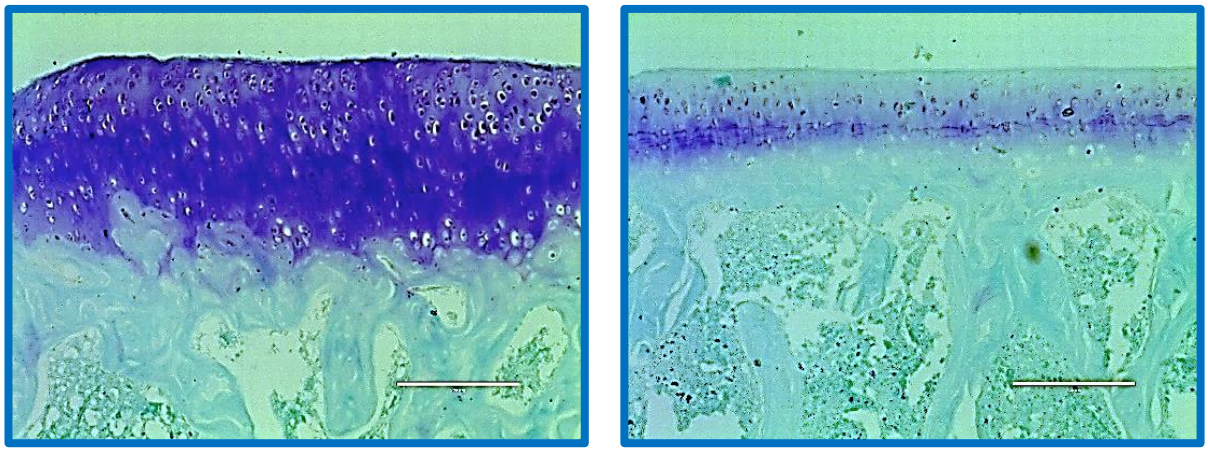

Fig 20 : Histological evaluation of the growth plate region in the control and ACLT groups representing articular and hyaline cartilage layers. Toluidine blue staining of tibia showing that the altered subchondral architecture at the histology level is disrupted in the surgery group and relative peptidoglycan degradation was observed.

5.2.5 Micro-architecture of the tibial subchondral bone in control and ACLT-induced osteoarthritic animals.

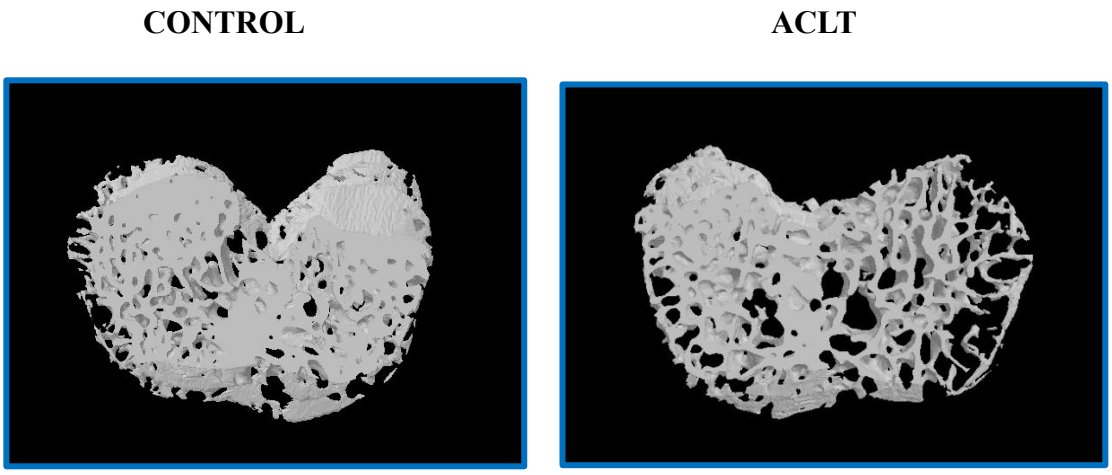

Fig 21: Micro-architecture of the tibial subchondral bone in control and ACLT-induced osteoarthritic animals. 3D image of tibial plateau showing subchondral bone loss in the ACLT group.

The analysis of subchondral bone shows that the bone volume is 50.22% in the control group animals and decreased by 37.02% in the ACLT group.

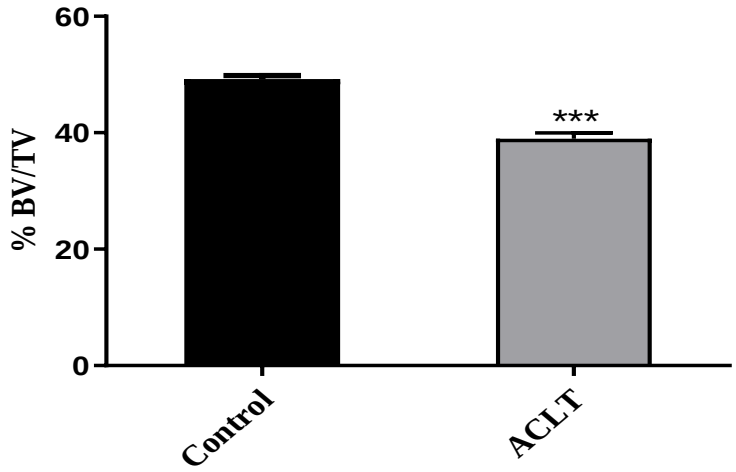

Fig 22: Quantitative analysis of subchondral bone in control and ACLT groups. BV/TV % (bone volume and tissue volume %) is decreased in ACLT group as compared to the control group.

Trabecular number (Tb.No) decreases in ACLT group as compared to the control group.

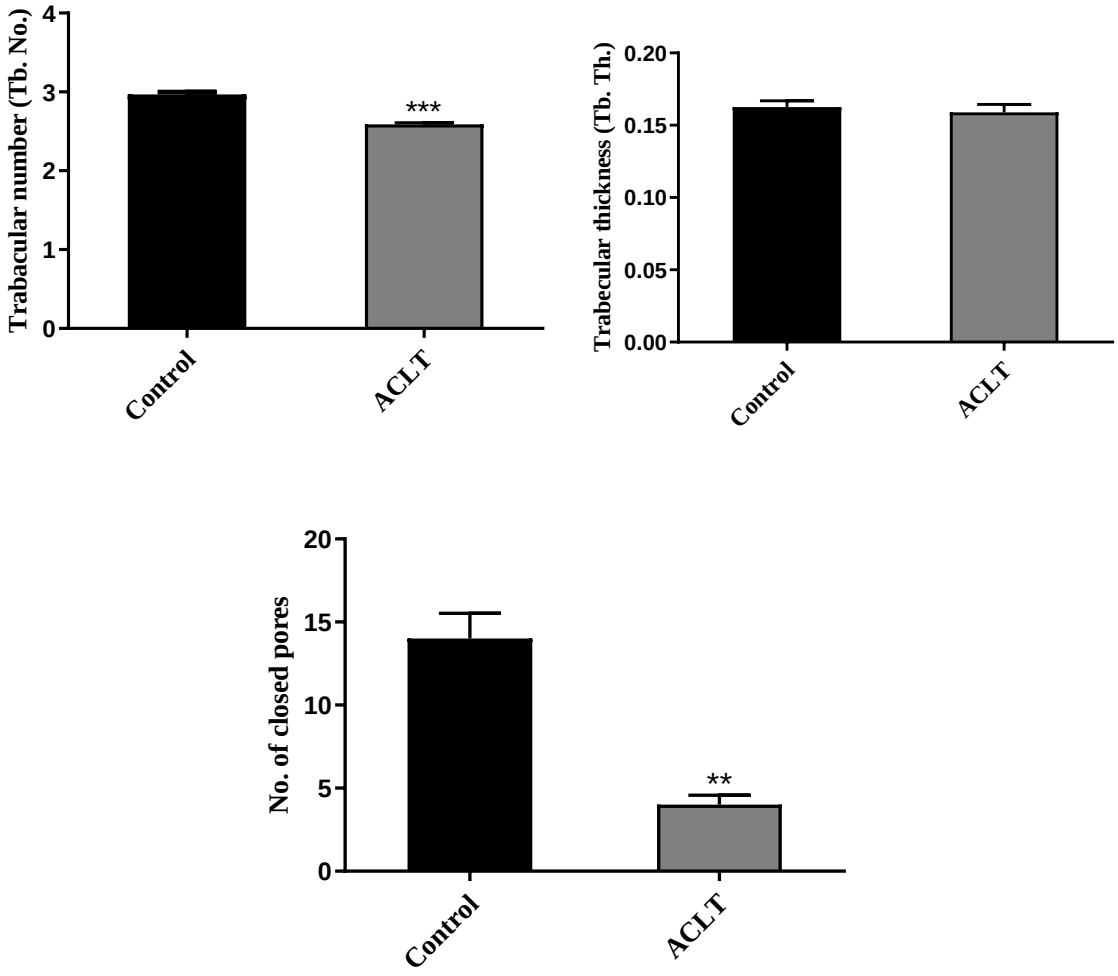

Fig 23: Quantitative analysis of subchondral bone in control and ACLT groups. Trabecular number (Tb.No.), Trabecular thickness (Tb. Th), and No. of closed pores is decreased in ACLT group as compared to the control group.

Altered microarchitecture is also depicted in the structure model index parameter, which indicates surface creature; higher SMI is related to higher alteration in microarchitecture. SMI of transacted animals was significantly increased as compared to the control.

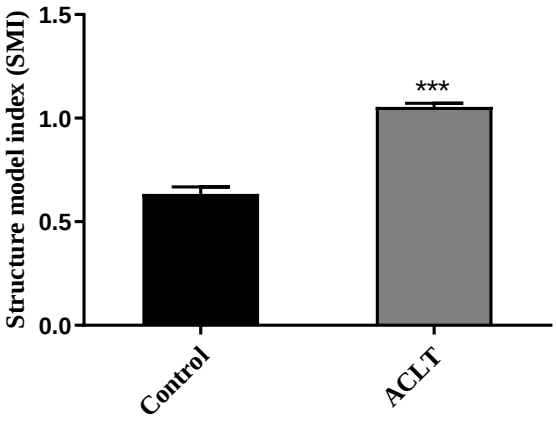

Fig 24 : Quantitative analysis of subchondral bone in control and ACLT groups. Structure model index (SMI) increased in the ACLT group.

6 Discussion:

The current investigation was conducted with the aim of comprehensively examining the pathophysiological underpinnings and intricate mechanisms underlying osteoarthritis. The present investigation was conducted to examine the pathophysiology of osteoarthritis and establish a comparative analysis with the physiological state through comprehensive in-vitro and in-vivo examinations. Osteoarthritis is the most common form of arthritis, characterised by degradation of the articular cartilage, formation of osteophytes, subchondral sclerosis, synovial inflammation, and ultimate loss of joint function

For in-vitro study, we have studied the catabolic response of dexamethasone on chondrocytes. Dexamethasone (Dex) is a synthetic corticosteroid generally used in re-differentiation of chondrocytes and stimulates the synthesis of cartilaginous matrix. It is crucially important to understand under what conditions Dex may be beneficial or harmful to cartilage and other joint tissues. The comparative effects of control cells and dexamethasone treated cells at varying concentrations (ranging from 0.1nM to 100 μM) within an in-vitro model that accurately replicates the pathophysiological characteristics of osteoarthritis. We conducted mRNA profiling using qRT-PCR and assessed cartilage extracellular matrix deposition using Alcian blue staining to investigate the changes in osteoarthritic pathophysiology compared to healthy conditions. After isolation of RNA, cDNA was prepared and then qRT-PCR was setup for studying the impact on cartilage catabolic genes like MMP-3, MMP-13, and anabolic genes sox-9, aggrecan and type-2 collagenase. The creation and turnover of the extracellular matrix (ECM) in cartilage are the responsibilities of chondrocytes, the sole resident cells in the cartilage. Master transcription factor Sox9 is crucial for controlling chondrocytes at the cellular level. It does this by activating several genes that are expressed in chondrocytes, most of which are cartilage extracellular matrix (ECM) genes including Col2 and Aggrecan. As the primary structural element of cartilage, collagen type II is most widely distributed there and accounts for roughly 60% of the tissue's dry weight. Aggrecan is a key proteoglycan that provides supports to articular cartilage and helps to withstand compressive stress. It contains chains of chondroitin and keratin sulfate Proteoglycan aggregates are present in the matrix of cartilage. Dexamethasone treatment resulted in downregulation of Col2 and aggrecan gene expression in chondrocytes, leading to alterations in cartilage structure and load-bearing capacity. This was determined through quantitative polymerase chain reaction analysis. There exists a relationship between extracellular matrix (ECM) production and

the cellular function of chondrocytes. Inflammation, cytokines, and matrix proteases are key factors affecting the quantity and functionality of extracellular matrix (ECM) production. Increased expression of matrix-degrading proteins, such as matrix metalloproteinases (MMPs), is mostly dependent on inflammation. Matrix-degrading proteins, such as matrix metalloproteinases (MMPs), which work on proteoglycans to break down the matrix, are highly expressed when there is inflammation. The primary cause of proteoglycan degradation is MMPs. Additionally, we observed that the cells treated with dexamethasone exhibited increased expression of MMPs. MMP activity-induced degradation of matrix structural components affected chondrogenesis and cartilage thickness. The osteochondral unit experiences age-related and mechanical loading-induced changes. These alterations are closely linked to a decrease in hyaline cartilage and an increase in the thickness of calcified cartilage.

In order to study the osteoarthritic condition *in-vivo*, various models are reported. Here we studied surgically induced ACLT (Anterior cruciate ligament transection) model, where we compared normal vs. ACLT group that mimics post traumatic osteoarthritic condition. The study involved female SD rats that underwent surgery and were then observed for a period of one month to develop osteoarthritis. Bone samples were collected from these animals for the purpose of studying subchondral bone microarchitecture using micro-computed tomography. Histological staining techniques such as haematoxylin and eosin staining, toluidine blue staining, and safranin orange staining were employed to examine cartilage morphology and assess the pathophysiology of normal and osteoarthritic conditions. The purpose of this study was to compare the human mimic model of osteoarthritis with the healthy one. Anterior cruciate ligament transection is a highly effective model to study the changes in cartilage and subchondral bone degradation that is observed during OA. The histology and radiological results provided valuable insights into the structural alterations in cartilage and subchondral bone associated with osteoarthritis, respectively. It is possible to see cartilage degradation and roughness in the ACLT model of OA. A visual distinction can be observed between the Control group and the ACLT group in terms of cartilage. Hematoxylin and eosin (H&E) staining, as well as toluidine blue staining, were employed to assess the reduction in cartilage thickness. The total cartilage thickness and the ratio of calcified to hyaline cartilage thickness were both lower in ACLT, which ultimately led to a rise in joint width and the indication of cartilage deterioration. The main type of cell found in cartilage that is embedded in the matrix is called a chondrocyte. In articular cartilage,

these chondrocytes are extremely mechanosensitive cells. The matrix or external stimuli elicit reactions when damaged. Osteoarthritis hinders the metabolic and functional activities of chondrocytes, leading to a decrease in the synthesis of proteoglycans and the production of the extracellular matrix. Changes in chondrocyte numbers, shape, and alignment occur at the cellular level. and Haematoxylin and eosin (H&E) staining and Toluidine blue staining were used to evaluate the decreased cartilage thickness. The total cartilage thickness and the ratio of calcified to hyaline cartilage thickness were both lower in ACLT, which ultimately led to a rise in joint width and the indication of cartilage deterioration. Haematoxylin and eosin staining illustrates these changed structural modifications, whereas toluidine blue staining illustrates the inhibited proteoglycan secretion in ACLT animals.

In joints, the close physical association between the cartilage and underlying subchondral bone has hosted the model of biochemical and molecular interplay across the affected region. In knee OA, chondrocytes can react to direct biomechanical load by modulating synthetic action or by elevating the production of the inflammatory cytokines. In this interplay, subchondral bone displayed the microarchitecture alteration in osteoarthritis. In our experiments, we also found this sort of change in micro-CT parameters of subchondral bone. 10x and 40x images of hematoxylin & eosin and 10x images of toluidine blue staining visibly represent an alteration of subchondral bone architecture. Decreased bone volume/tissue volume ratio, trabecular number, and trabecular thickness while increased structural model index parameters were observed by the micro-CT.

7. Conclusion:

Osteoarthritis is a disease of cartilage degradation and is associated with subchondral bone loss. The aim of this study was to understand the mechanism and pathophysiology of osteoarthritis. This may provide a strategy to check the impact of osteoarthritis on the subchondral bone. The cartilage, which acts as a shock absorber, is less efficient to bear the load and transmits more load on the underlying subchondral bone. During cartilage degradation, inflammation takes place, which also induces joint degradation and creates a vicious cycle.

Cartilage has a restricted nutrition supply, with a hypoxic environment, which makes it inefficient to repair itself. Apart from avascularity, cartilage has a denser and more compact matrix, which hurdles the delivery of a drug in the tissue and imparts additional challenge to the therapeutics of osteoarthritis. The symptoms of osteoarthritis take more time, and as it is a slow process, pain is felt sometimes.

Keeping in mind the discovery of drugs in the future, we have studied the mechanism and pathophysiology of osteoarthritis. To achieve the objectives, we have done both *in-vivo* and *in-vitro* studies in SD rats. For the *in-vitro* study, we have studied the catabolic response of dexamethasone on chondrocytes. Dexamethasone (Dex) is a synthetic corticosteroid generally used in the re-differentiation of chondrocytes and stimulates the synthesis of cartilaginous matrix. It is crucially important to understand the dark side of excessive usage of steroids. We, through our set experiments, have established the detrimental dose of Dex and its catabolic action on chondrocytes and the genes affected.

Few genes were checked through RT-PCR which may provide a novel and in-depth understanding of the pathogenesis of the OA subchondral bone at the molecular level. EGLN3 and ORM1 may be the new candidate targets for diagnosis and therapies in patients with OA in the future. The interaction between osteoarthritis and these genes has not been reported yet and needs further validation.

In order to study the osteoarthritic condition *in-vivo*, various models are reported. Here in this we have studied the surgically induced ACLT (Anterior cruciate ligament transection) model, where we have compared the normal vs. ACLT group that mimics post-traumatic osteoarthritic condition. The study was conducted on female SD rats and after surgery, animals were left for 1 month to develop the osteoarthritic condition. From these animals, bone samples were collected to study subchondral bone microarchitecture through micro-

computed tomography, cartilage morphology through histological staining such as haematoxylin and eosin staining, toluidine blue and safranin orange staining was performed to evaluate normal vs osteoarthritis pathophysiology. In conclusion it was interesting to gain lots of first-hand information regarding the development of in-vitro and *in-vivo* models and work on it.

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