CURCUMIN ALBUMIN CONJUGATE: A NOVEL ANTI-INFLAMMATORY DRUG FORMULATION FOR THE POTENTIAL TREATMENT OF OSTEOARTHRITIS

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Ph.D THESIS

2021

SREE CHITRA TIRUNAL INSTITUTE FOR MEDICAL SCIENCES AND TECHNOLOGY

THIRUVANANTHAPURAM

INDIA

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A THESIS PRESENTED BY

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TO

SREE CHITRA TIRUNAL INSTITUTE FOR
MEDICAL SCIENCES AND TECHNOLOGY
THIRUVANANTHAPURAM
INDIA

IN PARTIAL FULFILMENT OF THE REQUIREMENTS
FOR THE AWARD OF
DOCTOR OF PHILOSOPHY

2021

CERTIFICATE

I, **Deepa S**, hereby certify that I have personally carried out the work depicted in the thesis entitled, ***"Curcumin Albumin conjugate: a novel anti-inflammatory drug formulation for the potential treatment of osteoarthritis",*** except where due acknowledgement has been made in the text. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

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This is to certify that **Ms. Deepa S**, in the Division of Thrombosis Research of this Institute has fulfilled the requirements prescribed for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram. The thesis entitled, ***"Curcumin Albumin conjugate: a novel anti-inflammatory drug formulation for the potential treatment of osteoarthritis",*** was carried out under my direct supervision. No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Note: Clearance was obtained from the concerned Institutional Ethics Committee (IEC), Institutional Committee for Stem Cell Research (IC-SCR) and Institutional Animal Ethics Committee (IAEC) for carrying out this study.

Thiruvananthapuram

25.01.2021

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for the degree of

Doctor of Philosophy

Of

SREE CHITRA TIRUNAL INSTITUTE FORMEDICAL SCIENCES AND TECHNOLOGY THIRUVANANTHAPURAM

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DEDICATED TO MY MENTOR

DR. LISSY K KRISHNAN & MY PARENTS

ACKNOWLEDGEMENT

With deep sense of gratitude, I take this opportunity with great pleasure to thank all those who have contributed in one or the other way for the success of this study.

I express my deepest sense of gratitude to my supervisor Dr. Lissy K. Krishnan for the constant encouragement, guidance and incessant support provided to me throughout the course of the study. I thank her for putting in great effort to train me with scientific principles and practices for tackling research problem and for preparation of this thesis. She has always been a pillar of strength and was available all the time for clearing my doubts.

The Director, SCTIMST and the Head, BMT Wing are greatly acknowledged for providing all the facilities for successful completion of this work. Also, I would like to express my sincere thanks to the former Dean, Dr. V. Kalliyana Krishnan, present Dean, Dr. Sankara Sharma, Associate Dean, Dr. Roy Joseph, former Deputy Registrar, Dr.Sundar Jaysingh, present Deputy Registrar, Mrs. Radha M, Registrar, Dr. Santhosh Kumar and all the members of academic division, Directors's office, Head BMT Wing's office, Research & Publication cell and Institute Library for their continuous support throughout the course of the study. I express my sincere gratitude towards institutional TDF project for providing me with the financial support throughout the study period and the SCTIMST travel grant for funding International conference participation.

I thank members of the doctoral advisory committee, Dr. Roy Joseph, Scientist 'G', Division of Polymeric Medical Devices, Dr. Maya Nandkumar, Scientist 'G', Division of Microbial Technology, Dr Nandakumaran Nair (Retd), Visiting faculty, Department of Physical Medicine and Rehabilitation, SCTIMST for their timely valuable suggestions and comments which helped in setting the study objectives, in planning experiments and improving presentation of data. I also take this opportunity to thank all the teaching faculty of my course work.

I express my sincere gratitude to Dr. Sachin J Shenoy, Scientist 'F', Division of In Vivo Models and Testing, for his constant effort and patience in developing the animal model for inflammation. I express my special thanks to Dr. Arya Anil from DLAS for her sincere support throughout the animal study till completion. I also thank Dr.Harikrishnan V.S and all members of DLAS, especially Mr. Sharath, Mr. Manoj, Mr. Sunil, Mr. Pradeep and Ms. Jolly for their sincere endeavour and assistance throughout the animal experiments.

I express my sincere gratitude to Dr.Manesh Senan, KIMS hospital, Trivandrum for providing lipoaspirate samples for hADMSCs isolation.

I take this opportunity to thank Dr. Sabareeswaran and all staff of Division of Histopathology for helping with the histological analysis of tissue sections, I am extremely grateful to Mr. Joseph and Ms. Sandhya for their support rendered for tissue sectioning and providing with all sorts of support for carrying out special staining procedures.

I express my sincere gratitude to Dr. Roy Joseph, Division of Polymeric Medical Devices and Dr. Renjith, Central Analytical Facility for FTIR and HPLC analysis.

I express my gratitude to Dr. R.S Jayasree for extending the facilities for In vivo imaging of animal tissue and fluorescence microscope. I also express my sincere thanks to Ms. Hema, Ms. Reshmi and Mr. Jibin for their timely help in acquiring the tissue images.

I express my gratitude to Dr. P.V.Mohanan and all staff and students from Division of Toxicology, for providing the facilities for animal blood cell counting.

I sincerely thank Mr. Ranjith S, Ms. Priyanka, Mr. Anilkumar from the Division of Thrombosis Research for providing the fibrin sealant components.

I express my sincere gratitude to the Director of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, for providing the flow cytometry facility. I also thank Ms. Indu and Ms. Arya of Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram for the technical assistance in performing flow cytometry.

I am extremely grateful to Mr. Sajithlal, Mr. Binu and all other staff of Network Services for providing the uninterrupted online services for conducing Ph.D Seminars and presentations.

I express my sincere gratitude to Dr.Anoop Kumar T, Scientist F, Division of Molecular Medicine and all staff and students of Division of Molecular Medicine for providing with Nanodrop facility. I also thank Dr. P.R. Anilkumar and all staff and students of Division of Tissue Culture; Dr Prabha. D. Nair and all staff and students of DTERT for helping with their facilities during the course of the study.

I am deeply indebted to all staff and colleagues at Division of Thrombosis Research (TRU). I express my sincere thanks to Dr.Anugya Bhatt for the flow cytometry analysis, her advice and support during the course of my work. I am extremely thankful to my all time supportive seniors Dr. Tara S, Dr. Renu Ramesh, Dr. Subha. S, Dr.AmitaAjit, Dr. Krishnapriya Chandrababu, and Dr. Rashmi. R for guiding and training me on various techniques for completing the experiments. I sincerely thank Ms. Priyanka Manoj, Ms. Safeena, Mr. Ranjith S, and Mr. Anilkumar V, for

their technical support rendered throughout the study. I also thank Dr. Renjith . P. Nair, Ms. Sreekutty, Ms. Rakhi, Mr. Mejo, Ms. Karthika, Ms. Hima, Ms. Silpa, Ms. Athira, Ms. Lakshmi, Ms. Riya and Dr. Suvanish for their support, encouragement and friendship. I also thank Ms. Usha, Ms. Bindu, Mr. Hari, Mr. Shibu, Mr. Arun and Mr. Vijith for helping me in maintaining the aseptic conditions in cell culture facility.

I express my sincere gratitude to all my fellow students from other departments of our campus, the Stores and Purchase Division, the Administrative section and Accounts section for their assistance support and friendship throughout the course of the study.

I owe a huge debt of gratitude to all my teachers and friends who have always encouraged me to move forward by overcoming the hurdles.

Last but the not the east, I am indebted to the encouragement and endless support from my parents who have enabled me realize this Ph.D. research and thesis submission possible.

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ABBREVIATIONS

Ab-Am	: Antibiotic-Antimycotic
ACI	: Autologous Chondrocytes Implantation
AGE	: Advanced Glycation End Products
AGE	: Angiotensin Converting Enzyme
Alb	: Albumin
BM	: Basal Medium
BSA	: Bovine Serum Albumin
CABG	: Coronary Artery Bypass
ChCs	: Chondrocytes
CM	: Curcumin
COX-2	: Cyclooxygenase-2
CVDs	: Cardiovascular diseases
DAB	: Diaminobenzidine
DAPI	: 4,6, diamidino-2-phenylindole
DC	: Dendritic Cells
DMARDs	: Disease Modifying Anti-Rheumatic Drugs
DMEM-LG	: Dulbecco's Modified Eagle's Medium Low-Glucose
DMSO	: Dimethyl Sulphoxide
ECM	: Extracellular marix
ECs	: Endothelial cells
EDTA	: Ethylene Diamine Tetra Acetic acid
ELISA	: Enzyme Linked Immune Sorbent Assay
ET-1	: Endothelin-1
FBS	: Fetal Bovine Serum
FITC	: Fluorescein Isothiocyanate
FTIR	: Fourier-Transform Infrared Spectroscopy
GAGs	: Glycosaminoglycans
H & E	: Haematoxylin and Eosin
hADMSCs	: Human Adipose Derived Mesenchymal Stem Cells
Hb	: Haemoglobin
HBSS	: Hank's Balanced Salt Solution
HCT	: Haematocrit
HPLC-GPC	:High Performance Liquid Chromatography-Gel Permeation Chromatography
HSA	: Human Serum Albumin
IAEC	: Institutional Animal Ethics Committe
ICAM-1	: Intercellular Adhesion Molecule-1
IEC	: Institutional Ethics Committee
IKK	: Inhibitor of Kappa b Kinase

IL-1β	: Interleukin-1β
IL-6	: Interleukin-6
ISCT	: International Society for Cellular Therapy
IVIS	: *In vivo* imaging system
LDL	: Low Density Lipoprotein
MCP-1	: Monocyte Chemo-Attractant protein
MMPs	: Matrix Metalloproteinases
MSCs	: Mesenchymal stem cells
MTT	: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NADP	: Nicotinamide Adenine Dinucleotide Phosphate (NADP)
NaOH	: Sodium Hydroxide
NBF	: Neutral Buffered Formalin
NF-kB	: Nuclear Factor-kB
NO	: Nitric oxide
NSAIDs	: Non-Steroidal Anti-Inflammatory Drugs
OARSI	: Osteoarthritis Research Society International
PBS	: Phosphate Buffered Saline
PCNA	: Proliferating Cell Nuclear Antigen
PGE	: Prostaglandins
PVDF	: Polyvinylidene Fluoride
PVDs	: Peripheral vascular diseases
qRT-PCR	: Quantitative Real Time Polymerase Chain reaction
RIPA	: Radioimmunoprecipitation Buffer
ROS	: Reactive Oxygen Species
RT	: Room temperature
SD	: Standard Deviation
SDS-PAGE	: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
TACE	: TNF-α Converting Enzyme
TCPS	: Tissue Culture Polystyrene
TIMP-1	: Tissue Inhibitor of Metalloprotease-1
TNF-α	: Tumor Necrosis Factor-α
VCAM-1	: Vascular Cell Adhesion Molecule-1
VLA-4	: Very Late Anitgen-4
vWF	: Von Willibrand Factor
WHO	: World Health Organization

ANNOTATIONS

%	: Percentage
<	: Less than
>	: Greater than
µg	: Microgram
µl	: Microlitre
µm	: Micrometer
h	: Hours
IU	: International Unit
Da	: Dalton
min	: Minutes
mM	: Millimolar
nm	: Nanometer
µM	: Micromolar

SYNOPSIS

Inflammation is a vital physiological process in healing. However, chronic inflammation is a major reason for developing pathological process that lead to various diseases causing morbidity and mortality. Therefore, control of inflammatory conditions is a major step to prevent and cure such diseases. Currently available therapeutics falls under the category of Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) but are associated with harmful side-effects upon long term use. In order to cope with this, research focuses on development of new technologies to enable effective treatment with minimum side-effects. Novel drug development and efforts for reducing side effects of identified molecules form a major area of research. In this background, the major goal of this study is to establish the anti-inflammatory effect of a plant derived natural molecule, curcumin in a soluble and stable formulation.

The thesis consists of 6 chapters.

Chapter 1 introduces the research problem and search for the gaps in the current knowledge on the research topic. Based on the identified gaps a research hypothesis is proposed. Two inflammatory diseases causing major problems in public health across the world are atherosclerosis and osteoarthritis. In the case of atherosclerosis, inflammatory changes in endothelial cells (ECs) are considered to initiate the disease which progress into endothelial dysfunction resulting into major morbidity. Osteoarthritis is another inflammatory disease causing cellular changes in the chondrocytes (ChC). In both EC and ChC an interplay between cytokines can be exploited to evaluate the action of newly developed drug systems. Curcumin (CM) is a molecule purified from *Curcuma Longa* and is known to possess immense anti-inflammatory property. But, owing to poor aqueous solubility, CM has low bioavailability limiting its effective use in clinical scenario. Studies have shown that conjugating CM with albumin (Alb) increased solubility by many folds while retaining its anti-cancer effects both *in vitro* and *in vivo*. In this background, this study hypothesized that anti-inflammatory effect of water soluble CM may be studied *in vitro* using TNF- α induced human endothelial cells/chondrocytes in culture, by quantifying relative gene expression of specific markers; and *in vivo*

using inflammation induced knee joints in rabbits upon local administration of soluble and stable conjugate. Based on this hypothesis, following objectives were formulated for the study:

1. To establish solubility and stability of soluble CM-Alb conjugate at physiological pH.
2. To establish endocytosis and minimally toxic and safe concentration of CM-Alb for testing anti-inflammatory role in characterised human ECs and ChCs.
3. To establish inflammatory *in vitro* models in ECs and ChCs upon stimulation with cytokines and quantifying relative gene expression of specific markers.
4. To study the anti-inflammatory effect and dose range of CM-Alb conjugate using *in vitro* stimulated cultures of EC and ChCs.
5. To develop an *in vivo* inflammatory model in rabbit by intra-articular injection of TNF-α in knee joint.
6. To establish the reversal of inflammatory changes upon intra-articular administration of different formulations comprising CM-Alb.

Chapter 2 provides summarized review of the literature related to the study topic with relevant citations. Topics covered are: inflammatory changes in atherosclerosis; endothelial dysfunction; isolation and maintenance of ECs in culture; inflammatory changes in arthritis; ChCs dysfunction in progressive arthritis; development of ChCs from mesenchymal stem cells; biochemical pathways in inflammatory responses at cellular level; inflammatory response to cytokines in cell cultures; mechanism of action of various anti-inflammatory drugs; strategies for development of new drugs; methods and markers of inflammatory/anti-inflammatory response in cells; anti-inflammatory action of CM- biochemical pathways; attempts for improving bioavailability of CM; albumin as drug carrier; animal models for studying inflammatory response and anti-inflammatory action of drugs.

Chapter 3 describes the details of experimental methods adopted for achieving the objectives of the study. This sections explained are: the preparation of CM-Alb conjugate and tagging the conjugate with fluorescein isothiocyanate (FITC); characterisation of CM-Alb conjugate by Fourier transform infrared spectroscopy (FTIR), determining storage stability of conjugate using UV-Visible spectroscopy and High performance liquid chromatography (HPLC). Isolation of human umbilical

vein endothelial cells (HUVEC), isolation of human adipose derived mesenchymal stem cells (hADMSCs), characterisation/differentiation of hADMSCs and ChCs. The cytotoxicity dose determination by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, Endocytosis of the FITC tagged conjugate by cells by microscopic imaging and flow cytometry are explained. For establishing inflammatory cells and for determining effect of CM-Alb, relative gene expression (RGE) using Quantitative Real Time polymerase chain reaction (qRT-PCR) for Nuclear factor-kB (NF-kB), monocyte chemoattractant protein-1 (MCP-1), endothelin-1 (ET-1), cyclooxygenase-2 (COX-2) and vascular cell adhesion molecule (VCAM-1) in ECs; Nuclear factor-kB (NF-kB), cyclooxygenase-2 (COX-2), interleukin-8 (IL-8), matrix-metalloproteinase-13 (MMP-13) and tissue inhibitor of MMPs (TIMP-1) in ChCs were used. Establishing *in vivo* cartilage disintegration in animal model and assessing reversal upon CM-Alb administration by qRT-PCR, histochemical [hematoxylin and eosin (H&E), alcian blue and picro-Sirius staining)] and immunohistochemical staining (MMP-13 and TIMP-1) that were carried out are explained.

In Chapter 4, the compiled results are presented as graphs and microscopic images. Representative FTIR, UV-Vis spectral recordings and HPLC chromatograms demonstrates purity, solubility and stability of CM-Alb conjugate. The proof of endocytosis is shown in immunofluorescence images and safe dose suitable for anti-inflammatory treatment in ECs and ChCs is presented graphically. Up-regulation of inflammatory markers in response to TNF-α established suitability of EC and ChC as *in vitro* models of inflammation. Significant and dose dependent regulation of inflammatory marker expressions in TNF-α stimulated ECs and ChCs upon treatment with conjugate is demonstrated. In ChCs, the expression of TIMP-1 molecule was significantly enhanced upon treatment with conjugate indicating chondroprotective role. From these consistent observations, a low concentration of conjugate was selected for *in vivo* studies in rabbit knee model. H&E staining showed chondrocytes with columnar alignment in normal cartilage with intact outer membrane lining but scattered arrangement of chondrocytes/infiltration of lymphocytes/diapedesis/disrupted outer membrane and up-regulated MMP-13/ IL-8

expression were observed in TNF-α (500ng/ml) administered knee joints confirming suitability of the model for testing effect of the conjugate. Administration of 1 ml of 5μM conjugated curcumin 3 times, in 1 week interval, produced significant reversal of the inflammatory changes in terms of columnar alignment of cells, collagen deposition and GAG deposition.

Chapter 5 discusses the results in the light of published studies in the literature that developed drugs and tested using cell culture and animal model. This study is the first attempt to prove the possibility of conjugating CM with human Alb and demonstrating cellular uptake. The anti-inflammatory effects at safe concentrations both *in vitro* and *in vivo* are novel findings. The effectiveness of drug in *in vivo* evaluation hence provides opportunities to explore studies with improved animal models which can serve as a base for clinical translation of the drug conjugate. Other animal studies reported will be used to discuss the potential of the CM-Alb conjugate as an effective therapeutic agent for minimising cartilage degradation observed during osteoarthritis.

Chapter 6 summarises the results and conclusions of the study. The half-life of the conjugate in solution was found to depend on the drug concentration in solution and the temperature at which the solution is stored. CM-Alb exposed to ECs and ChCs was transported effectively into cell cytoplasm. High concentration of curcumin (5mM) was achieved in aqueous solution and proved to exhibit short term stability upon storage as concentrated solution and long term stability upon storage in freeze dried form.The ECs and ChCs were found to serve as suitable *in vitro* models for establishing the anti-inflammatory effects of stable CM-Alb conjugate. Anti-inflammatory activity of non-cytotoxic dose of CM-Alb has been effectively established using RGE analysis. The significance of using non-cytotoxic dose for reversing the inflammatory response is evident in RGE studies. On exposure to cytotoxic doses, the activated cells up regulated inflammatory markers, indicating that the cytotoxic dose can augment the inflammation caused by cytokine. CM-Alb was found to be beneficial for preserving the disrupted cartilage. CM-Alb may be explored further as potential drug formulation for treatment of inflammatory diseases. Limitations of the study and future prospects are also discussed.

The bibliography section lists the relevant citations.

CHAPTER-1

1. INTRODUCTION

Inflammation is a physiological mechanism of body's immune response to eliminate the disease causing agents along with handling consequences of the disease without causing much deterioration to the native tissue. However, in pathological conditions, inflammation continues to occur without appropriate regulations and monitoring. Disorders due to chronic inflammation are a major cause of morbidity worldwide. Some of the major life-threatening inflammatory disorders are cancer, diabetes, arthritis, cardiovascular diseases (CVDs), neurological diseases, autoimmune diseases etc. The CVDs are one of the most critical and worse disease conditions that contribute to the death of around 17 million people each year worldwide (WHO, 2017). During the initiation and progression of CVD, the inflammatory responses in the endothelial cells (ECs) lining the blood vessel play a major role. Arthritis being a disorder that results in physical disability affecting the joints is expected to affect up to 59.4 million people by 2020 ("Arthritis - an overview | ScienceDirect Topics," n.d.). The major cell type that plays an important role during arthritis is chondrocytes (ChCs) which is the only cell type present in the joint cartilage. Both ECs and ChCs respond to inflammatory cytokines produced in the local milieu and up regulate many other inflammatory proteins that are expressed on the cell membrane or that are released into the extracellular space which act synergistically. The inflammatory cells thus continue to remain dysfunctional and cause morbidity.

Inflammatory diseases are currently treated using drugs, commonly known as non-steroidal anti-inflammatory Drugs (NSAIDs). The major function of these drugs is regulating the production of inflammatory cytokines which are responsible for progression of the disease. These drugs act as inhibitors of either the receptors of cytokines or inhibit the signaling pathways. But administration of these drugs also cause major problems to the biological system by affecting other normal cells and produce several side effects. Therefore, development of new drugs with fewer side effects is a major research interest. Since joints are

avascular, the orally administered drugs hardly reach the inflammatory cells and therefore, local delivery of drug using carrier molecule is another major area of research.

Several research efforts paved way to the identification of a natural molecule curcumin (CM). It is a molecule derived from rhizomes of turmeric plant - *Curcuma longa* (L). CM has been used from olden days owing to its immense therapeutic properties in curing various diseases, and more clearly for topical wound healing. Modern scientific research has identified that CM possess several valuable properties: for e.g. it is anti-angiogenic, anti-cancerous, anti-oxidant, and anti-inflammatory in nature. However, a major concern is poor bioavailability of the molecule because of its poor solubility in aqueous medium and rapid metabolism. Chemically it is a highly hydrophobic molecule and it is very difficult to dissolve in aqueous medium. Hydrolytic breakdown of the curcumin into inactive metabolic products is another major concern. Several researchers made efforts to address the shortcomings of this molecule by incorporating it in liposomes, micelles, polymers, proteins etc. which can serve as drug carriers, both for increasing solubility and stability(Prasad et al., 2014a). The lack of controlled drug release and potential risks associated with toxicity of the drug carrier has been major concerns that limited the clinical use of modified drug forms.

Albumin (Alb) present in the circulating blood is a natural drug carrier. It serves as an ideal drug carrier as it contains specific ligand binding sites which improves the solubility of drugs thereby acting as a solubilizing agent (“Albumin,” n.d.) (Naveen et al., 2016). Several insoluble drugs can bind to Alb because of its ligand binding potential. A novel method demonstrating the increased solubility of CM by conjugation to bovine serum albumin (BSA) has been described in the literature(Thomas et al., 2014a). Studies have shown that conjugating CM with BSA has increased the bioavailability of CM in anticancer treatments in animal model (Aravind and Krishnan, 2016a). Even though studies have shown that Alb improves the bioavailability of CM, the anti-inflammatory property of the CM bound to human serum albumin (HSA) has not been explored. In order to achieve effective anti-inflammatory activity, it is important

to show that in the bound form it is stable, i.e. the hydrolytic degradation is prevented. The Alb conjugated CM administered through oral route cannot reach the target site of action, because Alb will get metabolized in the intestine and the drug which gets freed will undergo hydrolytic degradation. Therefore, design of the drug carrier that increase solubility and stability using Alb should be such that it can be essentially administered through intravenous (IV) route. For IV administration, Alb in native conformation is the most ideal drug delivery molecule. It is important to achieve high concentration of CM in soluble form upon conjugation to Alb, so that without having to administer large quantities of Alb, sufficient amount of CM could be delivered to the target cells. Therefore, other than establishing stability, it is important to prove high solubility of the drug molecule upon conjugation. Another requirement is that the bound molecule should get transported into the cell cytoplasm for biological action. If the conformation of Alb is altered during drug conjugation, the Alb may not be recognized by the cell membrane receptors and drug transportation may be affected. Therefore, the focus of this study is to establish high solubility, stability and transport across the cell membrane, before the anti-inflammatory property can be studied. Also, CM is a highly cytotoxic drug and hence the anticancer activity has been widely explored. The cytotoxicity can also result in the release of inflammatory cytokines from the affected cells. Thus it will be difficult to study the anti-inflammatory property if the drug causes cytotoxic action. Therefore, another objective of the study shall be the identification of non-toxic dose of the drug so that the anti-inflammatory property may be studied reliably using specific cells.

To establish the anti-inflammatory property of conjugated CM at the cellular level, most ideal method is to use primary cultures of target cells. In the case of CVD, the target cell is EC and in the case of arthritis, the target cell is ChC. For *in vitro* evaluation, ECs isolated from human umbilical cord vein and ChCs differentiated from human Adipose Derived Mesenchymal Stem Cells (hADMSCs) could be used. Both sources are readily available for isolation of cells and conditions of *in vitro* culture and maintenance of these cells are already established by various investigators and therefore, both systems may be suitable

for the purpose. Both cell types are known to respond to inflammatory cytokines and relative gene expressions of marker proteins may be studied to establish an inflammatory cell phenotype. Once the inflammatory cell culture model is confirmed, the effect of soluble and non-toxic CM conjugate could be studied by quantifying the inflammatory marker gene expressions.

The ECs are an important structural and functional component of blood vessels and help in maintaining the integrity of vessel walls. When these cells are exposed to harmful and toxic components they begin to show the consequences by secreting certain cytokine molecules or by expressing cell adhesion molecules on their surface. This sudden change in the cell behavior is referred to as the endothelial dysfunction and it occurs when the cells are under stress. These stress conditions contributes to the development of atherosclerosis which is marked by the deposition of cholesterol molecules beneath the wall of blood vessels. Slowly as the disease progresses, the blood vessel narrows and hardens resulting in obstructed blood flow making the target tissue which was being supplied with this same vessel devoid of oxygen and nutrients. As time passes the tissue begins to deteriorate and integrity gets destabilized. So, all these events involve immense role played by various cells of the immune system as well as inflammatory mediators, which are either produced by the ECs itself or those acting on ECs to initiate inflammation.

Even though inflammatory EC model creation in animals is hard to achieve, an inflammatory model joint has been widely used for testing various new drugs. In order to identify a safe concentration of CM-Alb to be used in animal models, an *in vitro* study needs to be carried out using ChCs. ChCs are the only type of cells present in cartilage. Cartilage is the elastic tissue present at the joints protecting the ends of bones against rubbing. Cartilage consists of an extracellular matrix (ECM) in which ChCs are embedded. The degradation of cartilage leads to the development of arthritis which is accompanied with inflammation of joints. The ChCs maintain homeostasis between synthesis and degradation of the matrix. Whenever homeostasis is disturbed, it leads to progression of inflammation leading to pathological conditions. Many factors contribute to the development of arthritis leading to physical inability and

associated trauma. Various proteolytic enzymes and cytokines come into play to disintegrate the tissue cartilage matrix.

So the effect of such inflammatory cytokines on ECs and ChCs and the effect of anti-inflammatory drug could be reliably studied by quantifying relative gene expression of inflammatory marker proteins. Rabbits are suitable animal models for studying cartilage disintegration due to their large joint size among all other small animals and almost comparable cartilage thickness to humans. The inflammatory response to cytokine and anti-inflammatory effect to the drug could be studied at the cellular and molecular level.

1.1. Inflammation

Inflammation is the fundamental mechanism of removing dead and damaged tissues and restoring the homeostasis of body back to normal("Robbins Basic Pathology - 10th Edition," n.d.).It is a complex process involving biochemical reactions that help in reducing the deleterious effects of inflammatory conditions. As per Celsus, there are four cardinal signs of inflammation: rubor (redness), calor (heat), tumor (swelling), dolor (pain); a fifth sign function laesa (loss of function) was added by Rudolf Virchow("Robbins Basic Pathology - 10th Edition," n.d.). The stimuli for inflammation can be dead tissues, physical and chemical agents, pathogens, foreign bodies, immune system etc. The response elicited varies with the nature of stimuli which involves inducers, sensors, mediators and target tissues (Okin and Medzhitov, 2012). Based on the extent to which the inflammatory process persists, it can be acute or chronic. Acute inflammation is characterized by rapid onset of the disease and lasts for a short duration. Chronic inflammation is characterized by delayed onset of the disease and lasts for a prolonged period of time. Blood vessels and leukocytes are two most important components involved in progression of the inflammatory conditions. These are the major sources for releasing cytokines. Cytokines serve as mediators of inflammation by either promoting inflammation as inflammatory molecules or by suppressing inflammation as anti-inflammatory molecules. These molecules are responsible for interaction between blood vessels and cells as well as cell to cell interactions (Hautz et al., 2012). Neutrophils are the first type of cells that migrate to the infected site during acute inflammation. As the

inflammation proceeds to chronic stage neutrophils get replaced by macrophages which perform the role of phagocytic removal of debris from the infected part of the tissue. So in overall it all depends on the type and nature of stimuli that determines the mediators and cells involved in the inflammatory process.

1.2. Inflammatory diseases

Inflammation and disease both terms appear to be interconnected with each other. One is followed by the other in a sequential manner indicating a strong link between both. A disease condition that involves inflammation at any point of disease progression is said to be an inflammatory disease. Few examples of inflammatory diseases are type 2 diabetes, CVDs, obesity, cancer, neurodegenerative diseases, autoimmune diseases etc (Scrivo et al., 2011). If inflammatory conditions were not there, diseases will go on progressing without showing any sign of manifestation. So when the homeostasis between healthy and diseased condition gets disturbed, signs of inflammation begins to appear indicating the need of monitoring and treatment of the pathogenic condition. Among various inflammatory conditions, CVDs are one of the major types affecting the circulatory system. CVDs include peripheral vascular diseases (PVDs), stroke and atherosclerosis. All these conditions are characterized by partially or completely arrested blood flow to the target tissues. These are also known as lifestyle diseases. Restricted blood flow leads to lack of oxygen and nutrients to the tissue as well as waste removal from the dead and damaged tissue also gets interrupted. Arteries are more often affected by this. It is marked by deposition of a core of fat which consists of necrotic tissue, lipid molecules, calcium deposits, foam cells and inflammatory cells (Falk, 2006). ECs play important role in protecting the blood vessels against attacks by cholesterol molecules. These cells maintain the intactness of the vessel walls by preventing the entry and attachment of lipid particles across the endothelial layer. The peculiarity of this condition is that it continues to affect the vascular tree without giving a single hint to the individual and finally when the situation worsens symptoms start appearing. The disease can remain asymptomatic for years. CVDs if left untreated can cause fatal life threatening consequences.

Another major disease is arthritis which is marked by inflammation of the cartilaginous tissue present at the joints. It is a major causative of movement impairment and sometimes may lead to mental depression also. The initial events involve cartilage injury whose causes vary, followed by the action of inflammatory cytokines which worsen the situation. ChCs help in the secretion of ECM that provides the required strength for cartilage. These cells are embedded in the matrix in specialized pouches called lacunae. But once there is an insult to the tissue, matrix undergoes biochemical and pathophysiological changes that disrupts the integrity of cartilage. Being a localized disease it serves as a perfect *in vivo* model for preclinical evaluation of anti-inflammatory property of drugs. Development of an arthritic model is quiet easy. The major problem associated with the treatment of such pathological conditions is the non-availability of efficient drugs that could cure the disease completely.

1.3. Endothelial dysfunction in CVDs

ECs line the entire circulatory tree of biological system forming a protective covering for the blood vessels that includes major arteries, veins and capillaries. Normal endothelium is responsible for maintaining proper vascular tone, cell growth and proliferation, angiogenesis, platelet leukocyte interactions etc (Rajendran et al., 2013).The damaged vascular endothelium is responsible for pathogenesis and progression of disease conditions such as stroke, heart disease, diabetes, insulin resistance, chronic kidney failure, tumor growth, metastasis, venous thrombosis and severe viral infectious diseases (Rajendran et al., 2013). CVDs are one of the major threats to human health. The risks for CVDs are categorized into modifiable and non-modifiable factors. Smoking, uncontrolled elevated cholesterol levels, diabetes, family history of plaque deposition are some of the major modifiable risk factors for CVDs (Hadi et al., 2005).Aging and male sex are non-modifiable risk factors (Tousoulis et al., 2006). Moreover death of vascular cells has been demonstrated in atherosclerotic plaque formation cases with respect to aging, which paves way for increasing the vulnerability of development of CVDs in old age people (Minamino et al., 2004). Diabetic and obese patients are at a higher risk for developing endothelial dysfunction due to high blood glucose levels and intake of high fat rich diet

which damages the normal ECs lining the blood vessels (Hadi et al., 2005). Inflammation has an important role in progress of endothelial dysfunction. Rather it's a balance between the action of pro-inflammatory and anti-inflammatory cytokines that help in maintaining the homeostasis.

Upon action of inflammatory cytokines, ECs express cell adhesion molecules such as vascular cell adhesion molecules (VCAM), which promotes the adhesion of leukocytes to the ECs. The pro-inflammatory cytokines tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β)play an important role in enhancing the expression of adhesion molecules; prevents the nitric oxide production by ECs thereby causing reduced dilation of blood vessels; production of superoxide ions; results in insufficient blood flow leading to higher chances of developing myocardial infarction (Zhang, 2008). Increased resistance to blood flow during hypertensive conditions in cases especially diabetic mellitus results in EC dysfunction further intensifying the overall inflammatory effect on blood vessels by upregulation of cell adhesion molecules stimulated byinterleukin-6 (IL-6) (Savoia et al., 2011).

1.4. Leukocyte - platelet - EC interaction in CVDs

Platelets are known as the firebugs of vessel wall (May et al., 2008). Platelet dependent leukocyte migration to the injured endothelium is a characteristic feature of atherosclerotic plaque progression. The damaged endothelium is a major source for platelet recruitment to the injured site. This occurs via secretions from Weibel-Palade bodies (Von Willibrand factor-vWF) of endothelium (Cerletti et al., 2010). The initial phase of atherosclerosis is the recruitment of leukocytes (especially monocytes/macrophages) to the site of endothelial injury. This recruitment occurs via the chemokines secreted by damaged ECs as well as by the recruited leukocytes which further enhance the overall interaction of cells at the site. Upon reaching the injured site, these cells interact with the ECs via cell specific adhesion proteins expressed by both cell types. This helps in the slowing down of leukocytes in flowing blood, arresting their fast movement, and helping their transmigration across the vessel wall into interstitial place. All these cell to cell interactions are carried out via selectin

molecules which are the typical cell adhesion molecules expressed by these cells facilitating initial rolling of leukocytes on ECs. These leukocytes express many cell surface molecules which are known as integrins which help in the facilitating cell to cell interactions (Huo and Ley, 2001; Tousoulis et al., 2006).

1.5. Arthritis

Arthritis is an inflammatory disorder affecting the joints of skeletal system. It is the degenerative disease of cartilaginous tissue that protects the ends of bones from friction. The disease is marked by intense pain, stiffness and swelling in joints. Smoking, older age, bone deformities, joint injuries by accidents, obesity, alcohol consumption etc are few major risk factors for development of arthritis (Teramoto et al., 2013). According to Arthritic Foundation, an US based non-profit organization, arthritis can be classified into three major types based on cause of the disease: (1) Osteoarthritis (caused due to aging or injury), (2) Inflammatory arthritis (caused due to autoimmune factors or deposition of crystals) and (3) Septic arthritis (caused due to bacterial infections).

The pathological progression of the disease is characterized by certain structural changes such as initial thickening of articular cartilage, enhanced metabolic activity by ChCs in order to compensate for the cartilage loss, increased turnover of the subchondral bone. ChCs are the major cell types that maintain the integrity of cartilage by playing an essential role in remodeling of degraded cartilaginous matrix. The enzymes such as matrix metalloproteinases (MMPs) produced by ChCs are responsible for the homeostasis between synthesis and degradation of cartilaginous matrix. Inflammatory and anti-inflammatory cytokines act in such a way to manage and regulate the arthritic progression at early stages of the disease. This process is regulated by the action of major inflammatory cytokines such as IL-6, IL-1β, TNF-α, IL-15, IL-17, 1L-18 and anti-inflammatory cytokines IL-10, IL-4, IL-13 (Wojdasiewicz et al., 2014).

1.6. Pharmacological intervention for arthritis and CVDs

Due to advances in in-depth understanding of pathological progression of diseases, it has been possible to develop new therapeutic approaches for the treatment of CVDs. Pharmacological interventions such as angiotensin-converting enzyme (ACE) inhibitors, statins, insulin sensitizers, and L-arginine, folates or tetrahydrobiopterin (BH4) have been shown to protect the endothelium (Rajendran et al., 2013). Risks associated with use of such drugs are liver damage, rhabdomyolysis, cancer, gastrointestinal haemorrhage, etc (World Health Organization, 2007).

Treatment of arthritis by pharmacological routes majorly includes the administration of drugs that can help in alleviating pain in the affected joints. Analgesics, Non-steroidal anti-inflammatory drugs (NSAIDs), Disease modifying anti-rheumatic drugs (DMARDs) are some of the major and most effective anti-arthritic category of drugs used for therapeutic prevention of arthritis. The drugs prescribed for arthritic patients depend on the stage of progression of the disease which range from mild to moderate to severe arthritic conditions (Kahlenberg and Fox, 2011).

1.7. New anti-inflammatory drugs and its clinical translation

Development of new anti-inflammatory drugs that can also promote cell proliferation at a regulated rate can help in restoring the integrity of damaged and inflamed body tissues. Even though anti-inflammatory drugs have improved the life-expentency of patients, it is not free from side-effects that may sometimes lead to life threatening complications. Few drugs such as indomethacin which is a NSAID, used for treating osteoarthritis has shown to cause deterioration in hip joints in some cases and it has been also accounted that the drug in combination with TNF-α might be responsible for such concerns (Caramés et al., 2008). Drugs that are used as anti-TNF-α, act by suppressing the immune system but at the same time carry the risk of bacterial infections. DMARDs have been found to cause nausea, diarrhea, allergic reactions, retinal toxicity, bowel perforations etc (Kahlenberg and Fox, 2011). Apart from the side effects, cost of these drugs also projects a major problem for treatment. In such

cases production of new drugs from naturally available raw materials in cost effective manner with minimum side-effects can serve as an alternative remedy for therapeutic approach. One of the significant issue and limitation encountered for development of anti-atherosclerotic drugs is lack of proper pathophysiological disease model (Orekhov et al., 2015). As *in vivo* models are difficult to develop for atherosclerosis, approaches are being carried out to use cell culture system and ex vivo systems (blood serum) for testing the effect of drugs for the disease. Attempts have been carried out to develop atherosclerotic models either by providing cholesterol rich diet or by genetic manipulations but both cases fail to develop lesions and associated molecular and biochemical complications similar to that of humans (Emini Veseli et al., 2017).

1.8. ECs and ChCs as *in vitro* models for inflammation studies

For studying anti-inflammatory action of drugs, inflammatory disease models have to be chosen. In the previous sections role of ECs and ChCs in specific disease conditions have been explained. ECs respond to various physical and chemical stimuli which helps in maintaining blood vessel homeostasis and also it serves as a major source for the synthesis and release of vasodilators and vasoconstrictors, pro-coagulants and anti-coagulants, inflammatory and anti-inflammatory, fibrinolytics and anti-fibrinolytics, oxidants and anti-oxidants, and many others (Esper et al., 2006). They have the immense potential to respond to inflammatory stimuli and produce several key inflammatory molecules which can be studied while testing the anti-inflammatory action of drugs. ECs derived from human umbilical cord have shown to respond when treated with TNF-α, IL-1β, IL-6 and when activated by these molecules, they tend to express inflammatory markers such as VCAM-1, ICAM-1,Cox-2, Endothelin-1(ET-1), Monocyte chemo-attractant protein MCP-1 etc (Onat et al., 2011; Szmitko et al., 2003).

Inflammation in cartilage causes degradation of the matrix by matrix MMPs, this action is contradicted by tissue inhibitor of metalloprotease-1(TIMP-1).TNF-α and IL-1β have shown to regulate the expression of TIMPs, MMPs,cyclooxygenase-2 (COX-2), IL-8 etc which serve as important

inflammatory marker in cartilage degradation produced by ChCs (Johnson et al., 2016; Wojdasiewicz et al., 2014). So, evaluation of the effect of inflammation suppressor on these molecules can help in elucidating the role of anti-inflammatory drugs (Li et al., 2017; Nakashima et al., 2012). Since both cell types are easily grown in cultures and are good responders to inflammation, these cells may be ideal candidates for studying anti-inflammatory property of drugs. Also the regulated expression of markers in response to inducers and inhibitors of inflammation may be studied and quantified using quantitative real time polymerase chain reaction (qRT-PCR). Thus the evaluation protocol may be developed to validate the effect of a new drug form using primary cell cultures of both human ECs and ChCs using well described cell biology experiments. These experiments may become handy to identify the mechanism of action and also to identify the dose range which is non-toxic and anti-inflammatory.

1.9. CM as a drug molecule and its limitations

Natural products are considered to be safe source for developing anti-inflammatory drugs as they are proven to be effective in traditional medicine. Onion bulb powder, beet juice powder, wheat seedlings powder, garlic bulbs powder etc upon given as anti-atherogenic agents have been shown to reduce the plaque formation from 10 to 70% (Orekhov et al., 2015). However, such medicines are not used in modern medicine as these crude preparations are not scientifically studied or their mechanism of action is not well described. CM is one such natural product derived from turmeric known as the golden spice of India which has been traditionally used in ayurveda for alleviating various ailments. Therapeutic applications of CM are many owing to its indefinite action on repair and healing of disturbed biological system. It is anti-cancerous, anti-angiogenic, anti-oxidant, inhibits scarring, promotes wound healing and anti-inflammatory in action. Several inflammatory, cancerous, skin, cardiovascular, neurodegenerative diseases have been treated with CM with good safety and its non toxic levels have been proven in clinical trials (Subash C. Gupta et al., 2012). Due to its wide effectiveness, the molecule has been purified to even the level of pharmacopoeia grade purity and is commercially available (Sigma

Chemicals, USA). Also the biochemical mechanisms of its various actions have been very well described in the literature.

The molecule regulates the pathophysiological conditions by modulating signaling molecules and transcription factors, enzymes, protein kinases etc (Subash C Gupta et al., 2012).The recognized actions of CM are:inhibition of COX, inhibition of prostaglandin synthesis, inhibition of cytokine expressions, nuclear factor-kB (NF-kB) inhibition, inhibition of platelet aggregation, stabilization of lysosomal enzymes, release of hormones, inhibition of MCP-1etc (Kohli et al., 2005). Literature suggests that despite of having so many therapeutic properties, the use of this molecule in standard therapy has been a failure because of its low bioavailability and poor absorption and fast metabolic degradation of CM (Prasad et al., 2014a). However, attempts have been made to improve the bioavailability of CM by preparing complex of CM with metals, nanoparticles of CM with silicon, gold, piperidine, amino acids etc (Priyadarsini and Priyadarsini, 2014). These modifications were hardly found suitable for therapeutic application of the drug due to problems associated with nanoparticle clearance from the body, poor release of drug and its function, toxicity issues materials used for producing nanoparticles etc. Therefore, search for a safe drug delivery vehicle has been a continuous effort to improve the bioavailability of this highly potent drug with multiple well-recognized actions.

1.10. Albumin as a drug carrier

Albumin is the most abundant plasma protein with a molecular weight of 66kDa.The protein has specific domains for binding with ligands. It is biodegradable, non-toxic and non-immunogenic in nature and is a natural drug carrier in blood (Naveen et al., 2016).It has been demonstrated that conjugating CM with human serum albumin (Alb) by simple electrostatic forces can enhance the solubility of CM by >100 times and also prevents the drug degradation (Thomas et al., 2014a). The CM-Alb has been demonstrated to possess effective anti-cancer property confirming that albumin conjugation does not affect the therapeutic property of the molecule (Aravind and Krishnan, 2016).

I.11. Gap Area

Anti-inflammatory property of a water soluble formulation of CM has not been studied. The anti-inflammatory property of CM has been reported mostly by adding the drug dissolved in organic solvents such as dimethyl sulphoxide. Upon adding to aqueous medium at physiological pH, CM degrades to inactive chemical forms. So, identification of the drug effect using a stable formulation has major significance. Also, whether the added drug is transported across the cell membrane efficiently and how much time is required for endocytosis etc are not identified. While the cytotoxic dose of conjugated CM is known, the same dose cannot be used as anti-inflammatory molecule as cells shall not undergo death upon treatment; therefore, there is a need to identify non-cytotoxic but anti-inflammatory dose. Most studies use cell lines; there is a need to explore anti-inflammatory effect of soluble CM on clinically relevant primary cells. For studying the anti-inflammatory role, the cells have to undergo transformation to inflammatory phenotype with marked up regulation of inflammatory cytokines. No systematic study has been conducted using the actual physiological target cells to understand the effect of CM on inflammatory marker gene expressions. No inflammatory joint model has been used to study the effect of CM in regulating the inflammatory response to cytokines at the tissue level.

1.12. Definition of the problem

Inflammation is a leading cause for several diseases. Among them CVDs and arthritis are two major conditions with mortality and/or morbidity. Currently available drugs which mainly include NSAIDs carry potential side effects affecting other normal physiology of the body. So efforts to bring new drugs with potent anti-inflammatory action into clinics are of great relevance in healthcare. Several plant-derived molecules have been identified to impart anti-inflammatory properties. But many of them are not available in the pure form or its cytotoxic effects are not estimated. However, CM has been well-studied in its pure form, several drug actions including anti-inflammatory property has been well established. The major problem in its clinical use is poor aqueous solubility, lack of absorption from the stomach, and metabolic breakdown into inactive molecules. Out of several strategies explored to improve the bioavailability,

improved solubility of CM when conjugated with BSA and its anti-cancerous property has been well established. Therefore, studying the effect of highly soluble CM produced by conjugating with Alb needs much attention. Since the study results may have much clinical relevance, a relevant study system has to be employed for understanding the effects so that the clinical translation is feasible. Both ECs and ChCs may be considered as the actual target cells in physiology and pathology; therefore, these cells could be used as *in vitro* models to establish anti-inflammatory action of CM-Alb. Alb being a natural drug carrier in blood plasma, the potential of translating the knowledge to clinical use is high. Therefore, preclinical evaluation using inflammatory joint model in rabbit may be valuable to establish the anti-inflammatory action of CM-Alb. Since there is high demand of albumin at the arthritic joints, CM-Alb may be injected to the intra-articular space safely.

1.13. Hypothesis

Anti-inflammatory effect of water soluble CM may be studied *in vitro* using TNF-α induced human endothelial cells/chondrocytes in culture, by quantifying relative gene expressions of specific markers; and *in vivo* using inflammation induced knee joints in rabbits upon local administration of soluble and stable CM-Alb conjugate.

1.14. Objectives

In order to prove the above mentioned hypothesis, 6 major objectives were formulated along with sub-objectives as given below:

1. To establish solubility and stability of soluble CM-Alb conjugate at physiological pH
    - To prepare CM-Alb conjugate and its characterisation by FTIR
    - To determine solubility visually
    - To determine solubility using UV-Visible spectroscopy
    - To determine aqueous stability by UV-Visible spectroscopy
    - To determine storage stability by FTIR and HPLC
2. To establish endocytosis and minimally toxic and safe concentration of CM-Alb for testing anti-inflammatory role in characterised human ECs and ChCs

- To develop characterised cultures of human umbilical vein endothelial cells (ECs) using in-house standardized protocols
- To develop characterised cultures of differentiated ChCs from hADMSCs
- To determine time/ dose dependent cytotoxic concentrations of CM-Alb to ECs & ChCs by standard MTT Assay
- To establish transportation of FITC-conjugated CM-Alb into the cells by qualitative analysis – Immunofluorescence detection microscopically; quantitative analysis – Flow cytometry

3. To establish inflammatory *in vitro* models in ECs and ChCs upon stimulation with cytokines and quantifying relative gene expression of specific markers
    - To standardise inflammatory conditions in ECs and ChCs using inflammatory cytokines at different dose and time period of exposure
    - To determine the relative gene expressions of inflammatory markers in ECs & ChCs using qRTPCR
    - To select the most appropriate cytokine/dose/duration to achieve reasonable upregulation of selected markers
4. To study the anti-inflammatory effect and dose range of CM-Alb conjugate using *in vitro* stimulated cultures of EC and ChCs
    - To treat the inflammation induced cells with different dose of CM-Alb
    - To determine the relative gene expressions of inflammatory markers in terms of their up/down regulation
    - To determine the expression of inflammatory markers at protein level by immunostaining and western blot analysis in ECs
5. To develop an *in vivo* inflammatory model in rabbit by intra-articular injection of TNF-α in knee joint
    - To standardise inflammatory conditions in rabbit knee joint
    - To determine the extent of inflammation by H&E staining
6. To establish the reversal of inflammatory changes upon intra-articular administration of different formulations comprising CM-Alb
    - To subject animals to different treatment strategies

- To determine the relative gene expression of inflammatory markers in animal tissue
- To determine the histomorphology of cell alignment in treated tissuesin comparison with normal tissue
- To determine changes in ECM by histochemical and immunohistochemical analysis
- To determine inflammatory changes in terms of blood parameters.

CHAPTER-2

2. LITERATURE REVIEW

The current literature related to the study topic was reviewed thoroughly in order to plan and conduct the experiments abiding to modern scientific principles and technologies. This chapter briefly explains basics and current knowledge on inflammation leading to atherosclerosis and arthritis. Also the events involved in progression of inflammation, molecular/ biochemical pathways involved and the current therapeutic approaches are outlined. The selection of *in vitro* cell culture models, *in vivo* animal model and various test methods are correlated to the current knowledge available in literature. The inflammatory response elicited by these cells upon action of inflammatory cytokines has also been reviewed. Drug development strategies and the shortcomings of currently available anti-inflammatory therapeutics have also been reviewed briefly in this chapter. Properties of CM, limiting its clinical use as an anti-inflammatory drug molecule and different methods explored to improve its bioavailability have been discussed. Methods for inducing inflammatory response in animal models have also been reviewed in this section.

2.1. Current concepts in atherosclerosis initiation

Atherosclerosis is one of the major causes of CVDs and is known to be initiated by inflammation of the arterial blood vessel walls. A typical blood vessel consists of 3 layers: (i) inner most layer tunica intima (endothelium, sub-endothelial layer and internal elastic lamina); (ii) middle layer tunica media (smooth muscle cell layer (SMCs); (iii) external tunica adventitia (connective tissue).

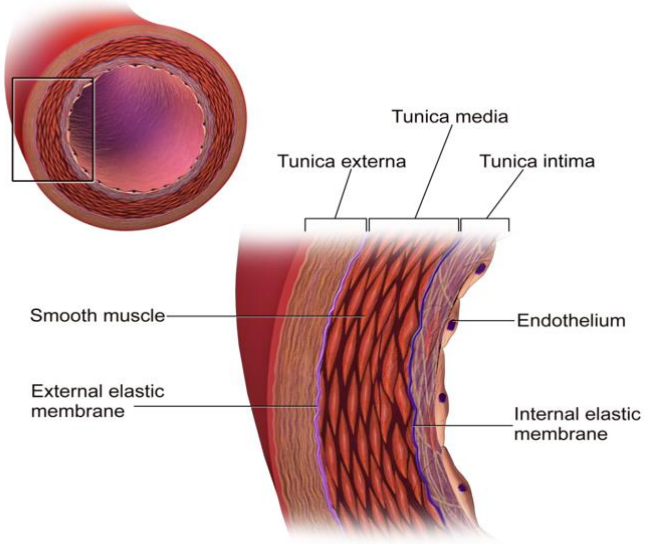


Figure 1: Structure of Arterial wall
(courses.lumenlearning.com/boundless-ap/chapter/blood-vessel-structure-and-function/)

Monocytes, macrophages, lymphocytes, ECs, SMCs are different types of cells involved in the progression of inflammation. The chief characteristic features of the atherosclerotic plaque are EC injury, fibrous cap formation, new matrix formation/degradation, necrotic core formation and its rupture. So the development of atherosclerotic plaque can be divided into three major steps: (i) initiation and formation of fatty streaks; (ii) monocyte recruitment and foam cell formation and (iii) plaque progression (Linton et al., 2000).

2.1.1 Initiation and formation of fatty streaks

The atherosclerotic event is initiated by endothelial dysfunction. These cells respond to various stimuli for maintaining the homeostasis of blood vessels. Inflammatory and mechanical stimuli (changes in shear stress/disturbed blood flow) stimulate the signaling pathways in ECs. Along with this, there is change in shape of cells from polygonal to cuboidal morphology with increased death in the affected region. Due to endothelial dysfunction in injured regions, ECs produce lower amounts of nitric oxide (NO) causing lower barrier capacity in cells followed by leakage or penetration of apolipoproteins containing low density lipoproteins (LDL) from the circulating blood leading to their accumulation in interstitial spaces. These proteins serve as stimuli for activation of transcription factor NF-kB which causes

the up-regulation of cell adhesion molecules (VCAM-1, ICAM-1, P-selectin) and cytokine molecules (MCP-1, IL-8). Thus the overall process is triggered by LDL molecules; in contrast to high density lipoprotein (HDL) that protect the ECs by enhancing NO production to maintain integrity of blood vessels.

2.1.2 Monocyte recruitment and foam cell formation

Immediately after endothelial dysfunction, chemokines such as MCP-1 released by activated ECs assist in the recruitment of monocytes to the site. This recruitment procedure involves initial rolling of cells along the ECs, followed by adhesion mediated by cell adhesion molecules expressed by both cell types, finally resulting in the extravasation of cells across the endothelial layer and get converted to macrophages. Along with monocytes, dendritic cells and T-cells also penetrate into the sub endothelial space. Activated T-cells convert macrophages to pro-inflammatory macrophages (M1) and regulatory T cells (T-reg) convert macrophages to anti-inflammatory macrophages (M2). Following this the macrophages internalize apoliporoteins or the so called cholesterol molecules (LDL) via the scavenger receptors present on their surface. The internalized cholesterol molecules are either degraded by lysosomes of the cell, or transported to endoplasmic reticulum and converted to cholesteryl esters which when becomes excess, gets stored as droplets in the cytoplasm forming the characteristic foam cell. Breakdown of phagocytosed cholesterol molecules can reduce inflammation and also vitamin E reduces the free radical formation by LDL (Ross, 2008). Dendritic cells (DCs) play an important role in T cell activation. The exact function of DCs is not known but it seems to play a role in presenting antigen to T cells (Galkina and Ley, 2009). Upon antigen recognition T cell undergoes differentiation to Th1 (Pro-inflammatory), Th2 (anti-inflammatory), T-reg (anti-inflammatory) and Th17 (clearing of extracellular parasites (Ashley et al., 2012).

2.2 Endothelial dysfunction in CVD initiation and progression

Endothelial dysfunction is any injury or insult caused to the endothelium lining the blood vessels. This disrupts the normal functioning of the endothelium affecting the homeostasis and integrity of blood vessels.

2.2.1 Endothelium structure

Endothelium refers to the single layer of ECs that forms a barrier between the circulating blood or lymphatic fluid and the rest of the structures of blood vessel. On average, approximately 10^{13} cells forms the blood vessel lining contributing to almost 1kg of body weight (Galley and Webster, 2004).

2.2.2 Functions of normal Endothelium

i) Transport Function

ECs facilitate transport of molecules from circulation to the sub endothelial space and vice versa for meeting the nutrient requirement of cells constituting blood vessels. Thus they are responsible for maintaining permeability for specific substances. One major function of ECs is transport of glucose via its receptor GLUT-1, which is expressed by ECs in abundant form. Amino acid transport is another major function performed via its specific transporter. y^+ cationic amino acid transporter is the most highly expressed one involved in transport of L-arginine which is the substrate for nitric oxide synthesis. The transport mechanism is categorized as transcellular (caveolae) and paracellular (tight junctions). Caveolin-1present on the inner side of plasma membrane facilitates transport of albumin and type III nitric oxide synthase. Tight junctions transport ions, water across the endothelium. Dysfunction of tight junctions can result in edema.

ii) Vascular tone

Vasodilators and vasoconstrictors released by ECs upon receipt of proper stimuli monitors and regulate the vascular tone and inflammatory conditions of the endothelium. NO and prostacyclin are a potent vasodilator whereas ET-1 is a potent vasoconstrictor. So, the mutual action between NO, prostacyclin and ET-1 maintains the normal vascular tone of endothelium.

iii)Host defense

ECs play important role in host defense as they are the first type of cells coming in contact with pathogenic antigens circulating in blood. ECs elicit host defense by initiating inflammatory responses based on the type of cytokine that acts on them. Chemokines secreted by ECs are responsible for recruitment of immune cells to the site of injury and regulate the inflammation. This is also enhanced by adhesive

molecules expressed by ECs and leukocytes. The overall process is regulated by the interplay between anti and pro inflammatory cytokine molecules.

2.3. Understanding mechanisms using EC culture

Monolayer of ECs was considered useful for understanding biology and pathology of endothelium. Many challenges were faced for obtaining healthy monolayers of ECs for reliable research purposes. Later, it was established that ECs can be isolated by different methods from different sources. The methods for *in vitro* culture of ECs include isolation of cells by proteolytic digestion; to obtain ECs from large vessels of large animals; to obtain ECs from small vessels of small animals; culture and subculture of the cells; isolating cells without proteolytic digestion etc (Ryan, 1984). The isolation and maintenance of ECs in *in vitro* depends on the supplementation of cocktail of growth factors and the coating surface that is provided for their attachment and proliferation (Pratt et al., 1988). Unlike primary fibroblasts or cell lines that attach to bare tissue culture polystyrene (TCPS), ECs do not adhere, spread or proliferate without additional protein substrate coated on the surface.

2.3.1. Use of substrates for healthy EC culture

An attachment surface for growth ECs isolated from adult or neonatal source was found essential (Karasek, 1989). The proteins coated on cell culture plate mimics *in vivo* basal lamina which provides attachment and signaling sites in vessels. Therefore, different proteins present in basal lamina were explored for modifying the TCPS such as fibronectin, Type I and III collagen and were found to promote proliferation of ECs whereas laminin and Type IV collagen stimulates attachment and differentiation of ECs. Gelatin has also been used for *in vitro* culture of ECs (Jiménez et al., 2013). ECs cultured on Type I fibrillar collagen has been used for studying the action of anti-platelet drugs (Chazov et al., 1981). Fibrin is an important substratum for cell growth as it is composed of several growth factors that are responsible for cell growth, attachment and proliferation. The human umbilical vein endothelial cells (HUVECs) was found to remain healthy through several passages as compared to the cells grown on gelatin coated surface (Chennazhi and Krishnan, 2005). Later, it was established that the fibrinonectin that is inherently present in fibrin is responsible EC attachment, proliferation and ECM deposition (Pankajakshan and Krishnan, 2009).

2.3.2. Different sources for EC isolation

i) Neonatal foreskin

Isolation of microvascular ECs from the highly vascular inner segment of neonatal foreskin is a convenient source for EC isolation. The inner segment is detached from the outer segment by dissection, cut into small pieces and treated with 0.3% trypsin EDTA, in phosphate buffered saline for 45 minutes at 37ºC. The ECs from dermis is extracted into the medium using a blunt scalpel blade, the detached cells are pelleted down by centrifugation (800g) and seeded on gelatin coated culture plates (Karasek, 1989).

ii) Internal mammary artery

ECs isolated from internal mammary artery tissue from patients undergoing coronary artery bypass graft (CABG) surgery is another potential source of these cells especially for studying the pathophysiological changes during CVDs. The tissue lumen was incubated with Type I collagenase for 15minutes at 37ºC. The isolated cells were seeded in EC growth media obtained from Lonza (Moss et al., 2007).

iii)Human umbilical cord vein

Human umbilical cord which is usually discarded after birth is an important source for isolation of ECs. Umbilical cord is collected in 0.14M NaCl, 0.004M KCl, 0.001M Phosphate buffer, 0.011M glucose. The vein is cannulated with blunt end cannulas, washed with buffer to remove any blood clots, incubated with 0.2% collagenase (Worthington Biochemical Corp.) for 15 minutes at 37ºC, centrifuged at 250g for 10minutes and seeded in tissue culture plates. (Jaffe et al., 1973)

iv) Lymphatic endothelial cells

Another source for EC isolation is from lymphatic vessels. Lymphatic tissues are supplied with large number of arteries and capillaries. The lymph tissue collected from spleen, thymus and lymph nodes were transferred into RPMI-1640 medium with 20% serum, washed with PBS, minced and digested with 0.25% collagenase/dispase solution for 3h at 37ºC. The digestion mixture is filtered through 30μm pore filter, the cells washed with PBS and seeded in type I collagen coated plates. (Garrafa et al., 2005).

v) Pulmonary endothelial cells

Pulmonary artery is another major source of EC isolation. Bovine pulmonary arteries are collected, washed with sterile PBS, filled with 0.25% collagenase solution and incubated at 37ºC in shaking water bath for 25 minutes. Digestion is stopped by DMEM, centrifuged at 1000g for 10 minutes at 4ºC. The pellet is resuspended in medium and seeded depending on the seeding density obtained (Ryan, 1984).

2.3.3. Characterization of ECs

i) CD31

CD31/ Platelet endothelial cell adhesion molecule-1 (PECAM-1) is an EC surface marker used for characterizing ECs (Moss et al., 2007). In addition to ECs, leukocytes and platelets also express CD31. CD31 is a member of immunoglobulin gene super family. It plays an important role in leukocyte recruitment during inflammatory events by facilitating cell to cell adhesion and by up regulating integrin functions (Sun et al., 1996).

ii) vWF

Synthesis of vWF is a characteristic feature of ECs. Staining ECs for von Willibrand Factor is another way of characterizing ECs (Suh et al., 1999). It is produced in Weibel-Palade bodies. Major function of vWF is interaction with other proteins especially factor VIII.

iii)Acetylated Low density Lipoprotein (AcLDL) uptake assay

Another characteristic feature of ECs is their ability to take up Ac LDL (Bai et al., 2012).

2.4. Inflammatory changes in arthritis

Arthritis is an inflammatory disorder affecting the joints of the living body. Inflammation and associated events have a major role to play in the progression and pathogenesis of the disease. Earlier based on the early symptoms that appear during arthritis, only rheumatoid, psoriatic and crystal induced arthritis were considered as inflammatory arthritis and rest all other types were classified as non-inflammatory diseases. But as the role of inflammation in synovial membrane gained attention, the disease has been considered to be caused due to inflammation.

2.4.1. Synovial tissue inflammation

Inflammation of synovial membrane is one of the characteristic features of development of pathogenesis in arthritis. Presence of synovial cavity marks the synovial joints. And such joints have a protective covering called synovial membrane that provides nutrients and exchange of debris from the joints capsule. The synovial joint consists of an outer fibrous membrane and inner synovial membrane. The synovial membrane is responsible for secretion of pale, thick yellowish synovial fluid. The membrane in turn consists of type A synovial cells and type B fibroblactic cells. The type A clears off debris from the joint and act as macrophage like phagocytic cells. The type B cells are responsible for secretion of hyaluronic acid (HA). Since cartilage is an avascular tissue, it solely relies on the adjacent synovial membrane for meeting its growth and nutrient requirements.

Infiltration of macrophages, formation of new blood vessels, enhanced production of inflammatory cytokines in the affected joints are some of the key features of inflamed synovium (Benito et al., 2005). In normal conditions, high molecular weight HA is not able to permeate through synovium whereas small molecules such as growth factors can easily cross the membrane. But during inflammation, these molecules escape the synovial cavity thereby reducing the lubrication between the articulating joints. The synovial changes in the diseased joint consist of four patterns: hyperplastic; fibrotic; detritus-rich; and inflammatory. Most of these symptoms are manifested at late stages of the disease (Scanzello and Goldring, 2012).

2.4.2. Extracellular matrix degradation

One of the key enzymes involved in disruption and degradation of ECM of cartilage is MMPs. The inflamed synovium is a major source for release of this enzyme. Normal ECM consists of abundant type II collagen and aggrecan molecules. Aggrecan molecules are proteoglycans that help in retention of water molecules in the cartilage. So once the cartilage starts to degrade, the early stages are characterized by decreased production of aggrecan along with shift in the production of collagen from type II to type I. This affects the mechanical strength of the cartilaginous tissue due to changes in the quantity and composition of the tissue. TNF-α, IL-1β, IL-12, IL-15 are some of the pro-inflammatory cytokines that causes

the activation of transcription factor NF-kB which stimulates the expression of MMPs especially MMP-1 and MMP-13. It has been observed that clustering of ChCs in cartilage which is an early arthritic event is accompanied with enhanced expression of MMP-13 thus showing that it can serve as an ideal biomarker to determine the intensity of disease progression. There is also enhanced formation of advanced glycation end products (AGEs) that forms crosslinking with collagen fibrils and caused increased stiffness in the cartilage. This combination of decreased aggrecan, increased type II collagen, increases MMP-13 and elevates the levels of AGEs, contributing to increased stiffness and decreased flexibility at the joints resulting in decreased range of motion and impaired motility. As the disease progresses there is thinning of the cartilage due to enzymatic action and the weight of the body slowly starts shifting to the subchondral bone lying below the cartilage thereby resulting in formation of osteophytes due to rubbing of bony surfaces against each other (Maldonado and Nam, 2013).

2.4.3. Stages of arthritic progression

Arthritis proceeds through a sequence of events starting from early to late stage. There are mainly four stages depending on the severity of disease progression (van der Kraan and van den Berg, 2012):

i) Initiation stage

During the initial stage, there is hypertrophy of ChCs and they get actively involved in synthesis of MMP-13. An important event is the loss of phosphorylation of smad protein. Phosphorylated smad is an essential blocker of hypertrophy of cells.

ii) Progression stage

There is enhanced synthesis of MMPs and aggrecanases. Fragmentation of proteoglycans is observed. These fragments further stimulate the release of cytokines that again promotes ECM degradation. These cytokines serve chemoattractants for migration of macrophages to the inflamed site. During this stage there is up regulation of TGF-β which promotes subchondral bone formation resulting in osteophyte development.

iii)Late stage

During this stage, the damage to cartilage is severe. ChCs present in the surrounding areas of damaged cartilage begin their work in repairing the cartilage.

They are actively involved in repair of ECM by continuously engaged in matrix production. These ChCs are also known as ‘repair cells’ at this stage.

iv) End stage

The end stage is marked by presence of very little cartilage with subchondral bone surface exposed with cysts. The joint has clefts and fissures. ChCs appear as clusters. Calcified osteophytes are observed. The synovial tissue is characterized by infiltrated macrophages. Following these, there is intense pain due to the rubbing of exposed surfaces of both bones in the joints.

2.5. Chondrocyte dysfunction in progressive arthritis

ChCs are embedded as clusters in spaces called as lacunae in the cartilaginous matrix. Young ChCs are small, flat, irregular cells with high proliferative potential. As they mature, the cells become rounded, metabolically active, loses potential to divide actively and get engaged in protein synthesis rapidly. So when they are in a stage of actively dividing they are referred to as chondroblasts and once they get mature and terminally differentiated they are known as ChCs.

ChCs play an important role in maintaining the homeostasis between production and degradation of ECM in cartilage. These cells are responsible for maintaining the turnover of ECM to ensure integrity of cartilage is maintained. The ChCs arrange themselves into clusters and they get engage themselves in producing ECM in order to cope with the drastic mechanical changes that are being carried out in the cartilage owing to disease progression. ChCs during inflammation produces abundant ECM and thereby contributing to enhanced cartilage thickness which is ideally not favorable for joints. During inflammatory conditions, NO is found to be up regulated in ChCs and this promotes apoptosis of ChCs (Maldonado and Nam, 2013). Normal functions of cartilage include production of hyluronan, glycoproteins, aggrecans, collagen etc (Akkiraju and Nohe, 2015). But when they get inflamed they become the sources for proteolytic enzymes: MMPs and aggrecanases which results in degradation of ECM. It has been said in literature that ChCs upon inflammation, change their phenotype and get converted to terminally differentiated calcified hypertrophic cells. Characteristic feature of hypertrophic chondrocytes is synthesis of type X collagen(van der Kraan and van den Berg, 2012).

2.6. Development of ChCs from mesenchymal stem cells (MSCs)

Stem cells are characterized by its ability of self-renewal and its property of multilineage differentiation. Stem cells that are used for regenerative medicine should meet certain criteria such as (i) it should be abundantly available; (ii) can be obtained by minimum invasive procedure; (iii) can be differentiated into multiple lineage; (iv) can be effectively transplanted either to the same or different patient (Bunnell et al., 2008).

MSCs have been used as an important source for deriving many cell types for tissue engineering and regenerative medicine. Two most commonly dependable sources for these MSCs are bone marrow and adipose tissue. The multipotent property of these cells makes them convenient to be used as source for differentiating into many other cell types. However bone marrow derived MSCs are not preferred for chondrogenic differentiation as such differentiated ChCs tend to express more of type X collagen which is characteristic feature of hypertrophic ChCs (Brown et al., 2014).

2.6.1. Differentiation of MSCs to ChCs

MSCs have high proliferative potential and their easy availability makes them a good source for ChC differentiation. *In vitro* differentiation of MSCs to ChCs has been achieved through the use of TGF-β (Pelttari et al., 2008). The differentiation potential of MSCs depends on source of tissue, age, sex, passaging etc. Age factor has a great influence on the differentiation potential of MSCs. Adipose derived MSCs from elderly donors such as whose age is above 60, have shown less proliferative potential and the rate of osteo and chondrogenic differentiation is also reduced whereas at the same time adipogenic differentiation is not so dependent on age factor. MSCs derived from adipose tissue have shown to exhibit greater potential to differentiate into ChCs (Stromps et al., 2014).

There are many proofs in literature that suggests that adipose tissue is rich in stem cells with multipotency. Histological analysis of subcutaneous adipose layer from patients with progressive osseous heteroplasia has shown the presence of osteoblasts, ChCs and adipocytes confirming the trilineage potential of stem cells present in the adipose tissue (Gimble et al., 2007).

2.6.2. Characterization of ChCs

The differentiated ChCs can be characterized by type II, IX collagen, aggrecan, decorin, biglycan (Pelttari et al., 2008). Collagen, proteoglycans and glycosaminoglycans (GAGs) are gold standards for characterizing ChCs. These can be determined at day 7 and day 14 of cell isolation. Picrosirius red stains collagen, alcian blue stains proteoglycans/GAGs (Oseni et al., 2013).

2.7. Biochemical pathways in inflammatory responses at cellular level

As discussed in the above sections, inflammation is multifactorial process regulated by the correlated actions of both pro and anti-inflammatory cytokines which act on different types of cells initiating biochemical pathways for either enhancing or minimizing the inflammatory responses. Cytokines such as TNF-α and IL-1 mainly function by activating p38 mitogen-activated protein kinase (p38MAPK) or via NF-kB mediated signaling pathways. IL-6 mediate and induce inflammatory pathway via its receptor and a signal transducing protein gp130 resulting in activation of Janus kinase 1 (JAK1) (Tousoulis et al., 2016).

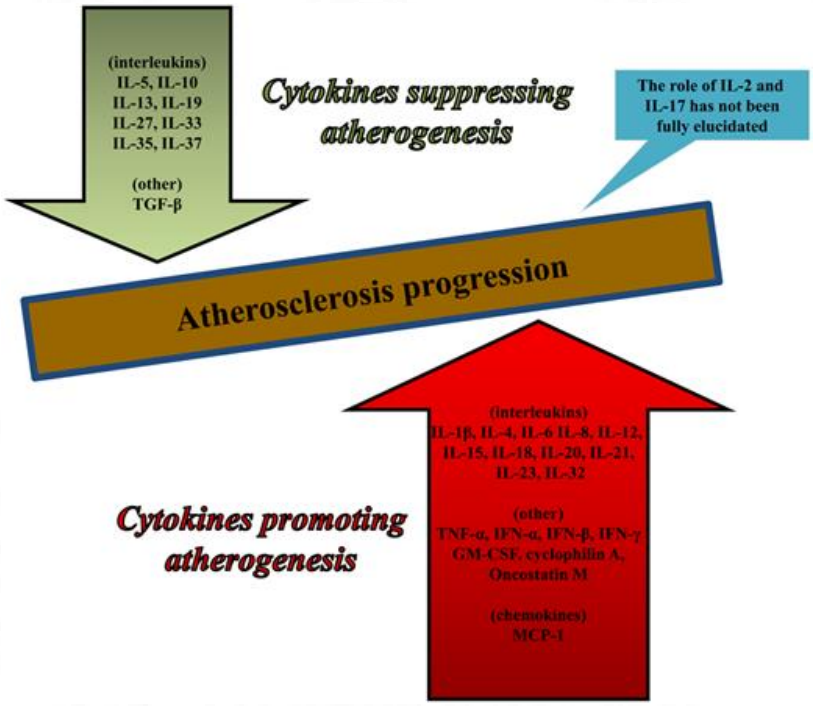


Figure 2: Cytokines involved in atherosclerosis progression *(Tousoulis et al., 2016).*

2.8. Inflammatory response to cytokines in cells

2.8.1 Endothelin-1

Oxidized LDL induces ET-1 expression in ECs thereby promoting EC and SMC proliferation (Galley and Webster, 2004). ET-1 is a vasoconstrictor synthesized by body in three isoforms: ET-1, ET-2, and ET-3. However ECs produce ET-1 only. In response to inflammatory stimuli, TNF-α up regulates the ET-1 expression. ET_A and ET_{B2} are the two types of receptors for ET-1 expressed by SMCs and ET_{B1} is expressed on ECs. Binding of ET-1 on SMCs causes influx of Ca^{2+} causing vasoconstriction whereas binding of ET-1 on ECs cause release of NO resulting in vasodilation. So during endothelial dysfunction, there is upregulation of endothelin recpetors on SMCs and downregulation of endothelin receptors on ECs resulting in vasoconstriction (Sandoo et al., 2010).

2.8.2 Cyclooxygenase-2 (COX-2)

Cyclooxygenases are an important class of enzymes that are involved in catalytic conversion of arachidonic acid to eicosanoids (prostaglandins). Phospholipase enzyme present in the plasma membrane converts phospholipids into arachidonic acid. Arachidonic acid can undergo either cyclooxygenase pathway, or lipooxygenase pathway, or cytochrome p-450 pathway. Prostaglandins are important mediators in controlling various processes of immune system such as inflammation; cancer etc. Two most important isoforms of COX are COX-1 and COX-2. COX-1 is expressed constitutively by almost all tissues in biological system and is responsible for production of protective prostaglandins such as prostacyclin whereas COX-2 is expressed by cells in response to physiological stimuli. These stimuli include interleukins, interferons, TNF, IL-1β, Platelet activating factor, ET-1, Lipopolysaccharides etc(Williams et al., 1999). The mechanism of action of prostaglandins (PGE_2) depends on the tissue in which it is produced. When it is synthesized by COX-1 in gastric mucosa it has a cytoprotective role but when it is produced by the synovial linings by COX-2 it has pro-inflammatory role (Mengle-Gaw and Schwartz, 2002).

2.8.3 NF-kB

NF-kB plays significant role in stimulating the expression of several pro-inflammatory cytokines thus regulating signaling pathways in inflammatory process. The stimuli for activation of NF-kB are based on two pathways: canonical and alternative pathways. The 'canonical pathway' involves nuclear factor activation via cytokines such as IL-1 and TNF-α. The 'alternate pathway' involves TNF-family cytokines such as lymphotoxin-β, CD40 ligand, B cell activating factor etc excluding TNF-α(Lawrence, 2009). NF-kB is located in cytoplasm in an inactive state in bound form with inhibitors of kB (IkB). There are 3 forms of inhibitors: IkBα (transient activation), IkBβ (sustained activation) and IkBε. These inhibitors are cleaved from nuclear factor with the help of phosphorylation via kinases of IkB (IKK). The IKK consists of 3 sub-units: IKKα, IKKβ (kinase subunits) and IKKγ (regulatory subunit). IKKβ is required for activation of canonical pathway and IKKα is required for activation of alternative pathway. Phosphorylation of IkB by IKK activation releases the NF-kB free which then gets translocated to nucleus. It has been found that NF-kB upregulates the expression of cell adhesion molecules VCAM-1, ICAM-1, MMP-13, promotes leukocyte migration to the inflamed site thus justifying the role played by nuclear factor in inflammation (Tak and Firestein, 2001).

2.8.4 VCAM-1

At the time of inflammation, inflammatory stimuli induce ECs resulting in expression of more numbers of cell adhesion molecules (VCAM-1) on their surface. VCAM-1 facilitates the adhesion of leukocytes on to ECs and helps in the arresting the movement of these cells in the circulation thereby promoting the extravasation/transendothelial migration of leukocytes through the EC junctions into the interstitial space (sub endothelial) below the endothelial cell layer. VCAM-1 expression is induced by stimuli such as ROS, high glucose, cholesterol, turbulent shear stress, TNF-α, IL-1β etc. The firm adhesion between EC's VCAM-1 occurs with its corresponding ligand integrin molecules expressed by the counter cells (Cook-Mills et al., 2011). Activation of VCAM-1 via its interaction with integrin ligand causes stimulation of NADPH oxidase in cell membrane which results in

production of ROS. ROS in turn activates MMPs that leads to degradation of extracellular matrix (Cook-Mills, 2002).

2.8.5 MCP-1

MCP-1 (CCL2) is a chemokine/chemoattractant that plays an important role in trafficking or recruitment of monocytes/macrophages to the target site. MCP-1 is a member of C-C chemokine family. There are four variants of this molecule: MCP-1-4. It is expressed by the action of cytokines or growth factors or oxidative stress. TNF-α, IL-6 and IL-1β have shown to upregulate MCP-1 in cells especially during inflammation (Deshmane et al., 2009). MCP-1 also has been found to promote proliferation of macrophages. This has been experimentally proven in MCP-1 deficient mice in which macrophage proliferation was found to be decreased (Cranford et al., 2016).

2.8.6 MMP-13

MMPs are zinc dependent proteolytic enzymes that are actively involved in degradation of ECM. There are almost 28 MMPs identified till now. They play a significant role in tissue remodeling during tissue repair. Depending on the substrate on which enzyme acts, MMPs are categorized as collagenases, gelatinases, stromelysins etc. The balance between MMPs and TIMP is responsible for maintaining the homeostasis between ECM production and degradation (Liu and Khalil, 2017). Of 28 MMPs identified so far, MMP1, 2, 8, 13 &14 are collagenolytic in function. These are produced by ChCs during arthritic progression under the influence of pro-inflammatory cytokines and perform the function of breakdown of collagen fibers in cartilage (Murphy and Lee, 2005). IL-6, IL-1β and TNF-α have found to play significant role during inflammation in up regulating MMPs. MMP-1 (interstitial collagenase) breaks down type I,II,III collagen; MMP-2 (gelatinase A) breaks type IV collagen; MMP-3 (stromelysin-1) breaks down type II, III, IV, IX and X, fibronectin, laminin, elastin; MMP-8 (neutrophil collagenase) is involved in clearance of Neutrophils from the inflammatory site; MMP-9 (gelatinase B) involved in death of hypertrophic ChCs; MMP-13 is the most abundant type of proteolytic enzyme present in arthritic cartilage and is involved in the breakdown of type II

collagen which is the most important collagen type present in ECM of cartilage; MMP-14 is involved in aggrecan degradation (Rose and Kooyman, 2016).

2.8.7 TIMP-1

TIMPs are the inhibitors of MMPs. Four homologous forms of TIMP are TIMP1-4 (Liu and Khalil, 2017). These are secreted molecules but they reside on cell surface (Murphy and Lee, 2005). The proinflamatory cytokines IL-6, IL-1β and TNF-α are responsible for downregulation of TIMPs (Qu et al., 2016). TIMPs play important role in tissue remodeling and growth. TIMP inhibits MMPs by forming high affinity non-covalent complexes with MMPs. This occurs when TIMP binds to the active site of MMP thus affecting the catalytic activity of the enzyme. TIMP-1 has an inhibitory effect on soluble MMPs whereas TIMP-2,3 has inhibitory effect on membrane bound MMPs. TIMPs also has been shown to promote cell proliferation in tissue repair so might be indirectly contributing to the production of ECM in damaged tissue (Bokarewa et al., 2005).

2.8.8 IL-8

IL-8 (CXCL8) is a chemokine that performs the function of leukocyte recruitment to the inflammation site. It is released by leukocytes under the stimulation of IL-1 or TNF-α (Long et al., 2016). IL-8 has been found to stimulate the release of MMPs from ChCs in arthritic condition. It also has shown to promote hypertrophy and differentiation of ChCs in inflamed joints (Takahashi et al., 2015).

2.9. Basic principles of controlling inflammation

2.9.1. Action of anti-inflammatory drugs

NSAIDs are a major category of anti-inflammatory drugs that are being used to treat inflammatory disorders. Way back in 1900, aspirin was discovered and used as a potent anti-inflammatory drug. Later, antipyrine, phenacetin, acetaminophen (paracetamol), phenylbutazone, fenamates, indomethacin and naproxen were discovered; sharing almost similar actions as of aspirin. They were initially known as aspirin like drugs and later were classified as NSAIDs. These drugs exhibit similar pattern of therapeutic actions, such as they help in reducing swelling, redness, pain and fever during inflammation (Vane and Botting, 1998).

2.9.2. Effect on COX enzyme

NSAIDs act via inhibiting the production of prostaglandins by blocking the action of Cox enzymes. As prostaglandins are important intermediators of inflammation, reduced production of these molecules can serve the purpose of reducing the rate of inflammatory processes. There are two sites in COX enzyme. The cyclooxygenase site that cyclizes arachidonic acid and adds a hydroperoxy group whereas the other site is the peroxidase site where hydroperoxy group is reduced to hydroxyl group to form prostaglandins. The drugs mostly act on cyclooxygenase site and have no effect on the peroxidase site (Mengle-Gaw and Schwartz, 2002). The enzymatically active site of the enzyme is a hydrophobic pocket that is blocked by these drugs. Tyrosine 385 and serine 530 are at the tip of the active site of COX-1. So aspirin acetylates serine 530 and prevents binding of arachidonic acid to the enzyme. COX-2's active site is also blocked by similar mechanism and the only difference between two enzymes is that the active site of COX-2 is slightly bigger compared to COX-1 due to the difference in arrangements of amino acids in the active site. So in order to block the COX-2 active site more bulky inhibitor molecules are required(Vane and Botting, 1998).

2.9.3. Side effects of anti-inflammatory drugs

A general side effect of these drugs is that they act as anti-thrombotic agents. These side effects are due to the fact that when NSAIDs come into action, they block COX-2 thereby reducing the extent of inflammation but at the same time they also inhibit COX-1 because of which the cytoprotective action of COX-1 is also affected. So drugs are developed in such a way that they have more selectivity toward COX-2 compared to COX-1 so that the side effects can be minimized. Meloxicam and Nimesulide are some such drugs used for treatment for arthritis and reducing inflammatory pain respectively and has more affinity towards COX-2 thereby exerting minimum side effects (Vane and Botting, 1998). Also by making drugs that have different conformation helps in selectively inhibiting COX-2. For example, rofecoxib and celecoxib are quite larger than normal NSAIDs that cannot fit into COX-1 thereby has less side effects as it cannot block the action of COX-1 (Mengle-Gaw and Schwartz, 2002).

2.10. Strategies for development of new drugs

2.10.1. Anti-inflammatory drugs

The development of anti-inflammatory drugs is an important therapeutic approach for treating inflammatory disorders. As per the recent advancement on understanding the concepts of inflammation, the anti-inflammatory drugs fall into two different categories: (1) broad spectrum drugs and (2) cytokine targeted drugs (Tousoulis et al., 2016). Blocking ET_A receptors can cause vasodilation in atherosclerotic patients facilitating unobstructed blood flow to some extent (Sandoo et al., 2010). Anti-inflammatory drugs such as NSAIDs act by effectively blocking COX activity (Williams et al., 1999). Anti-inflammatory drugs against NF-kB are also being developed (Lawrence, 2009).

2.10.2 Inhibitors of MMPs

MMP inhibitors which are commonly known as TIMPs are being developed synthetically. Attempts have been made to develop antibody fragments, specific to MMPs active site and this has been proven to be more effective in blocking the enzymatic action compared to chemical inhibitors that are developed (Murphy and Lee, 2005).

2.10.3. Hormone replacement therapy

Studies have shown that hormone replacement therapy by administering estrogen in combination with progesterone in post-menopausal women improves endothelial function by promoting vasodilation. It also has shown to reduce the levels of plasminogen activator inhibitor in circulation thereby reducing the risk of thrombus formation (Widlansky et al., 2003).

2.10.4. Inhibition of Angiotensin converting enzyme (ACE) and Angiotensin II

Clinical trials in patients with CVDs have shown that the use of ACE inhibitors can enhance endothelial function to a normal level. Angiotensin II has the ability to up regulate nicotinamide adenine dinucleotide phosphate (NADP) oxidase that is responsible for increasing the levels of ROS and downregulating the level of NO. So in such patients when ACE is administered there is decreased production of ROS as well as it can cause breakdown of bradykinin molecule that is responsible for enhanced production of NO (Widlansky et al., 2003).

2.10.5 TNF-α Inhibitors

As of now there are three inhibitors of TNF-α that are currently marketed for treating inflammatory disorders. Etanercept, infliximab and adalimumab are the currently available ones and two other TNF inhibitors, certolizumab pegol and golimumab are in the development process. They have been effectively used in rheumatoid arthritis and inflammatory bowel disease. There are basically two forms of TNF-α present in biological system: soluble form (17kDa) and membrane bound form (26kDa). The membrane bound form has to be detached from membrane with the help of TNF-α converting enzyme (TACE). So it is possible to bring into clinical use, the TACE inhibitors can reduce inflammation and associated events to a much lower level; however, as of now there are no TACE inhibitors available in the market (Cazzola et al., 2012).

2.10.6 NF-kB Inhibitors

Developing inhibitors of IKK can prevent the translocation of NF-kB. BMS-345541 is a potent IKK inhibitor that exhibits the ability to inhibit nuclear factor activation. Another strategy is the development of NF-kB decoy oligonucleotides. These decoy oligonucleotides are double stranded DNA oligonucleotides that bind to the consensus sequence of the transcription factor by competitively inhibiting the binding of nuclear factor to its binding site on the respective gene. The only disadvantage of developing these inhibitors is that there are increased chances of developing immune suppression upon its administration (Cazzola et al., 2012).

2.11 Methods to quantify inflammatory/anti-inflammatory response in cells

2.11.1 Specific markers expressed during inflammatory disorders

Some of the important biomarkers expressed during inflammatory diseases involving vascular system are C-reactive protein, fibrinogen, plasminogen activator inhibitor, soluble CD40ligand, myeloid related protein, adiponectin, interleukin-18, matrix-metalloproteinase 9, and MCP-1 produced by ECs in response to lipoproteins that assists in recruiting monocytes and macrophages to the target site is an. The CD40L and its receptor is up regulated in CVD patients. Its major function is to stimulate the synthesis of cell adhesion molecules on the surface of cells. It also has the ability to induce pro-thrombotic effects in blood vessels. Adiponectin is an

important anti-inflammatory molecule that is responsible for increasing insulin sensitivity, reducing plasma triglycerides, increasing HDL molecules, decreasing expression of cell adhesion molecules etc. and it is seen that adiponectin is found to be reduced in obese patients compared to lean people. Even though many such biomarkers are available for CVD risk assessment, the only recommended markers are LDL, HDL and triglycerides for assessing the development of atherosclerosis (Packard and Libby, 2008).

Tenascin-C (TN-C) is a major ECM component, basically a glycoprotein that is found in elevated levels in synovial fluid during inflammation. This molecule stimulates proinflammatory mediators and contributes to ECM degradation. Biglycan and fibronectin fragments have also been found to be elevated during inflammation thereby showing that they can be used as important biomarkers for determining inflammation in cartilage during arthritis (Maldonado and Nam, 2013).

2.11.2 Selection of suitable method for quantifying inflammation

Research for delineating the mechanism of inflammation and anti-inflammatory response to drugs at the molecular/cellular/tissue level needs to use reliable and effective methods for quantifying biomarkers. Some of the most important methods for quantifying the inflammatory markers are qRTPCR at mRNA level or Enzyme linked immune sorbent assay (ELISA) and Western blot (WB) at the protein level. The ELISA and WB methods which detect release of specific proteins depend on an antibody interaction with a specific antigen expressed by the target cell. The quantification of protein is dependent on an appropriate method for isolation. In the case of cell culture, the target protein may be intracellular or may be released into the medium. So, the quantitative protein detection requires isolation of protein, which can cause lot of uncertainty. Also, this detection can often be complicated by similar competing antigen in the sample. For identification of proteins in the tissues immunohistochemistry is employed. This method becomes more valuable when the specifically detected protein can be correlated to the cell morphology and the subcellular location.

Conversely, polymerase chain reaction is based on amplification of the DNA of target molecule. In the case of real-time PCR, specificity is increased by the use of probes and primers designed to target highly conserved regions of the target genome.

Also mRNA isolation from cells is well standardized and the reaction is initiated using precise quantity. RT-PCR helps in performing detection of specific genes from multiple samples at the same time which further eases out the technical delay faced while opting for other procedures (Freeman et al., 1999)(Hadrich et al., 2011).In a similar experiment where a comparison of ELISA and RT-PCR was used to screen the presence of pathogenic organisms present in patients suffering from influenza virus attack showed that RT-PCR method works with more sensitivity compared to ELISA (Plakokefalos et al., 2000). ELISA carries the disadvantage of protein detection from a small sample of tissues and only limited number of inflammatory/anti-inflammatory markers can be detected which is overcome by RT-PCRthat requires only a small amount of mRNA for detecting a huge network of cytokine panels (Peinnequin et al., 2004).In another study, it was found that while detecting viral pathogens from diseased samples, qRT-PCR showed a sensitivity of >0.99 whereas ELISA showed a sensitivity between 0.5-0.7 (Webster et al., 2017).

2.12 Biochemical pathways of anti-inflammatory action of CM

As previously described CM is the most active element present as one of the three most important constituent of turmeric with potent therapeutic actions. CM was first identified in 1910 by Lampe and Milobedzka. It accomplishes its anti-inflammatory function by downregulating cyclooxygenase, lipooxygenase, inducible nitric oxide synthase (iNOS) enzymes which are responsible for the production of inflammatory cytokines. This downregulation is achieved via inhibition of NF-kB activation. CM is believed to suppress nuclear factor activation by inhibiting the action of IKK. It has also shown to down regulate as well as exert a negative effect on action of IL-6, IL-1β and TNF-α thereby having a regulatory effect on controlling the inflammatory processes(Jurenka, 2009). TNF-α plays an important role for inactivation of transcription factor NF-kB and vice versa (Hewlings and Kalman, 2017). Thus by inhibiting the action of transcription factor various genes that are involved in promoting inflammation can be controlled. Various transcription factors that are involved in activation of TNF are transcription factor ETS, activating transcription factor 2 (ATF2), nuclear factor of activated T-cell transcription factor (NFAT), cAMP response element binding protein (CREB) etc. CM has shown to inhibit these

transcription factors and thereby preventing TNF expression (Aggarwal et al., 2013). So CM being a potent TNF blocker justifies the fact that it is a valuable anti-inflammatory agent with good therapeutic action.

2.13. Various attempts for improving bioavailability of CM

Even though CM has high therapeutic value, its poor bioavailability due to insolubility in aqueous medium and rapid hydrolytic degradation limits its use as a drug molecule. So attempts are being carried out in order to improve its bioavailability so that it can be made available at the target site with maximum efficiency and safety. The low bioavailability of CM was first shown by Wahlstrom and Blennow in a study carried out in Sprague-Dawley rats. The rats were orally administered with 1g CM/Kg body weight but only very little amount was found in plasma which might be due to poor absorption. So various strategies for improving bioavailability of CM are as follows:

2.13.1 Nanocurcumin

Nanoglobule based nanoemulsions have been prepared and has shown improved solubility compared to CM suspension. Enclosing CM in hydrogel nanoparticles also showed enhanced solubility. CM enclosed apotransferrin nanoparticles have also shown enhanced bioavailability. Colloidal nanoparticles of CM known as theracurmin has shown forty fold higher area under curve in blood concentration versus time curve thereby showing that nanoparticles are a way to improve the bioavailability of CM.

2.13.2 Polylactic-co-glycolide acid (PLGA)

CM encapsulated in PLGA particles showed increased uptake of the drug molecule by cells. PLGA-CM has also been encapsulated in Polyethylene glycol (PEG). It has been found that PLGA and PLGA-PEG nanoparticles enhanced the bioavailability by 15.6 and 55.4 fold respectively.

2.13.3 Liposomal encapsulation

Liposomes are considered as one of the best candidates for drug delivery as they help in solubilizing the hydrophobic molecules easily. When rats were given orally liposome encapsulated CM, the rate of absorption was found to be higher. Propylene glycol liposomes encapsulated with CM has been found to show a higher uptake by cells *in vitro*.

2.13.4 Cyclodextrin and Piperine

Cyclodextrin has shown to improve the bioavailability of CM by encapsulating it. The increase in animal skin permeability of cyclodextrin encapsulated CM was about 1.8 fold compared to free CM. Piperine is a naturally available compound obtained from pepper. Attempts have been made to conjugate piperine with CM. This conjugation has shown to improve the bioavailability by protecting the degradation of CM that is by preventing hepatic and gastric glucuronidation. Piperine conjugation also increases the intestinal absorption (Prasad et al., 2014a)**.**

2.14. Alb as drug carrier – advantages

Alb is the most abundant protein present in plasma (35-50g/L) with a molecular weight of 66.5kDa. It is synthesized by liver at a rate of 0.7mg/h/g of liver. Alb exhibits many properties that cause it to be considered for therapeutic applications. Few of its properties are:

(i) It acts as a solubilizing agent for many long chain fatty acids.

(ii) It binds to bilirubin, the breakdown product of heme.

(iii) It easily binds to many therapeutic drugs.

(iv) It binds to metal ions and facilitates in their transportation.

(v) It is responsible for maintaining the osmotic pressure in blood.

(vi) Its breakdown results in release of amino acids that are utilized by the nearby tissues and cells for their growth (Kratz, 2008).

The accumulation of Alb at the inflamed site, easy and abundant availability and biodegradability makes it an ideal candidate for drug conjugation. The accumulation of Alb in the arthritic joints makes it convenient to be used in delivering drugs to the site (Kratz, 2008). Alb based microspheres have been developed to treat inflammation. For treating arthritis, Alb-methotrexate is the only conjugate currently available in market. Alb nanoparticles are also in use. Alb-paclitaxel is the only conjugate available for cancer treatment in market. Alb-docetaxel and Alb-rapamycin are in clinical trials. Due to the accumulation of Alb in tumor tissue it serves as an important drug delivery vehicle in treating tumors. Antibody against TNF-α conjugated with Alb is also in clinical trial for treatment of rheumatoid arthritis (Dusad, 2013). The conjugation of BSA with CM has been shown to

improve its bioavailability and aqueous solubility to 100 times as compared to free CM. This conjugate has been found to be much effective in inhibiting the growth of cancer cell lines. This conjugation not only improved the solubility but also prevented drug degradation (Thomas et al., 2014a). So the above mentioned properties of Alb make it a suitable carrier for drug delivery.

2.15. Animal models for studying inflammatory response

For the purpose of testing the effectiveness of anti-inflammatory drugs, several *in vivo* models have been considered. Edema, granuloma and arthritic models have been suggested widely to assess the activity of anti-inflammatory drugs in *vivo* (Kabir and Ansari, 2018). These models have successfully been used for studying the effect of plant derived products with medicinal values. Carrageenan-induced paw edema in rats, croton oil-induced ear edema, papaya latex, monosodium-urate crystals *Candida albicans* etc induced arthritic models have been preferred for evaluating the actions of anti-inflammatory drugs (Kabir and Ansari, 2018).Mice, rats, rabbits and guinea pigs are the most commonly used small animal models for inducing arthritis; dogs, goats, sheep, pigs and horses are some of the large animal models for arthritic studies (Gregory et al., 2012). Histamine/5-HT-induced paw edema, bradykinin-induced paw edema, dextran-induced paw edema, lipopolysaccharide (lps)-induced paw edema etc are some of the other agents used to create inflammatory models *in vivo* (Patil et al., 2019). The major focuses on such *in vivo* models have always been the assessment of inflammatory mediators. The inhibition of these mediators are determined with the help of such inflammatory models (Phanse, 2012).Cytokines such as TNF-α have also been used to induce inflammation *in vivo* (Seleme et al., 2017).

2.16. Significance of cell transplantation/HA application

Cell based therapies for example utilization of mesenchymal stem cells for suppressing inflammatory conditions can lead to tissue repair and regeneration (Ennis et al., 2013). It has also been reported that a combinatorial therapeutic approach wherein both anti-inflammatory drugs and stem cells are delivered, showing better responses towards bringing down the deleterious effect of inflammatory disease progression (Mashkouri et al., 2016). Cells are responsible for

maintaining the microenvironment of tissues. These microenvironments are often referred to as ECM where these cells survive and replenish the damaged structures. So, considering the transplantation of cells in the affected tissue/organ is one of the ways to compensate for the loss at the native tissue regions. In case of inflamed knee joints, an approach of administering adipose derived stem cells has resulted in a slight reduction in swelling at the joints (Kim and Keating, 2019). This signifies that cells do play an important part in remodelling the inflammatory pathways. In inflammatory conditions like arthritis, stem cells, articular chondrocytes and fibrochondrocytes are the usually preferred cell types that are transplanted into patients for recovery (Yubo et al., 2017). As an alternative strategy, autologous chondrocytes implantation (ACI) performed in patients with defective joints showed much improvement and paved away the joint replacement option (Minas et al., 2010). In certain cases, surgical removal of the injured tissue accompanied with transplantation of chondrocytes have been tried to treat inflammatory joints (Sato et al., 2019). Apart from administration of anti-inflammatory drugs and cell transplantation, approaches have been carried out to use hyaluronic acid (HA) as a lubricating agent at the inflamed joints.

HA is basically a polysaccharide synthesized with the help of hyluronan synthase enzyme and belongs to the group of non-sulfated glycosaminogycans (Masuko et al., 2009). Often, instead of using HA alone, it is being used in a modified form wherein it is either chemically restructured or applied in combination with other substances such as drugs and cells for better and promising outcomes. Use of HA based hydrogels for repair of cartilaginous tissue is often considered to be an effective strategy that yields its beneficial effects of providing a suitable environment for the cells embedded in matrix to survive and synthesize important molecules crucial for tissue regeneration (Li et al., 2019).The recommendation rate of HA by Osteoarthritis Research Society International (OARSI) for arthritic treatment is about 64% (Masuko et al., 2009). Successful efforts have been accomplished in developing cytomodulin - HA for treatment of damaged cartilage along with stem cells that resulted in chondrocyte differentiation in the affected region (Park et al., 2019). It has also been reported that apart from being a mere provider of viscosity to the inflamed joints, HA can initiate the role as anti-inflammatory agent by inhibiting

the activity of MMPs in cartilage damaged due to the action of pro-inflammatory cytokines and also can promote synthesis of ECM molecules (Masuko et al., 2009). The exact molecular mechanism by which HA initiates its action is not well studied. However, there are few experimental data that suggests that HA exerts its action via receptor mediated pathways. CD44 expressed by cell surface especially in ChCs has been the targets for HA and MMPs, thereby both molecules competing for the same receptor which might be the reason for suppressing MMPs (Gupta et al., 2019).

2.17. Methods to assess anti-inflammatory action of drugs in tissues

2.17.1. Analysis of mRNA expressions in tissues

The detection of mRNA expression levels in tissues is one of the most reliable quantitative technique that has been widely employed for detection of inflammatory cytokines (Amsen et al., 2009).One of the finest methods to determine the anti-inflammatory action of drugs *in vivo* is by analyzing the expression of markers in the diseased tissues. Quantifying the genetic profiling of cytokines at definite time intervals can help in monitoring the changes observed which might contribute to assess the extent of progression of the diseases. Gene expression studies have been carried out to assess the progression of cartilage disintegration with respect to ECM components (Salazar-Noratto et al., 2019). Assessments of cartilage specific gene that play an important role in maintaining the integrity of the tissue are usually determined via genetic marker detection (Al-Sabah et al., 2016).

2.17.2. Histopathological assessment

Histopathology is regarded as the gold standard for detecting arthritic progression in animal models. Histological evaluation accompanied with immunohistochemical staining provides a method for assessing the status of the tissue in disease and recovery upon treatment. Collin and co-workers have developed a scoring system based on the morphological appearance of the tissues (Grade 0 – smooth cartilage with no defects; Grade I – limited damage to the superficial zone; Grade II – fibrillations in the deep zones; Grade III – significant loss of cartilage with exposure of the bony surface). A point-based grading system was developed by Mankin et al. In this method, femoral heads are subjected for DNA and carbohydrate determination. The DNA and carbohydrate content are studied by the incorporation of ^{3}H-thymidine and $^{35}SO_4$, respectively. Higher carbohydrate content is correlated

with lower disease progression, even though the same could not be concluded for DNA. From the experimental observation, a new 14-point grading system based on cellular, histochemical, and biomechanical changes was created. This system is known as the Mankin score system. The HHGS identifies ‘cartilage structure’, ‘cell distribution’, ‘Safranin-O staining’ and ‘tidemark integrity’ as separate subitems. The sum of the separate scores ranges from 0 (normal) to 14 (severe OA). The OARSI system assesses the severity and the extent of cartilage surface involvement in the local osteoarthritic process(Kuyinu et al., 2016). The immunohistochemical staining is considered to be valuable to assess up/down regulation of molecular marker expressions at the protein level (de Matos et al., 2010).

CHAPTER 3

3. MATERIALS AND METHODS

The study comprises of *in vitro* and *in vivo* experiments to establish the anti-inflammatory effect of CM-Alb. ECs derived from human umbilical cord and ChCs obtained by differentiation of hADMSCs have been employed to demonstrate the anti-inflammatory effect of soluble CM-Alb *in vitro*. The experimental design involves identification of a safe dose of CM-Alb using cytotoxicity assay, demonstration of cellular uptake of FITC tagged CM-Alb by both cell types, standardising inflammatory conditions in cells, followed by treatment of inflammation induced cells with CM-Alb. The *in vivo* model of cartilage inflammation was created using intra-articular injection of TNF-α in rabbit knee joint. After establishing *in vivo* model of inflammation, animal groups were subjected to treatments with different formulations comprising CM-Alb, followed by assessments at various levels. The detailed protocols for each experiment have been explained in this section.

3.1 Preparation of Curcumin-Albumin (CM-Alb) and FITC tagged CM-Alb conjugate

Conjugate was prepared by adding 5mM CM (Sigma, USA) from stock solution (0.5M CM in Dimethyl Sulphoxide (DMSO-Merck)) with 200mg/ml of human serum albumin (Alb) (Intas Pharmaceutical Ltd India) followed by incubation for 2h (Christina et.al. 2014). Unreacted CM was removed by passing the solution through gel filtration column packed with Sephadex G-50-300 beads (Sigma, USA). Based on molar ratio (A280:A420), fractions were pooled, filtered using 0.22μm syringe filter (Merck, Millipore) and lyophilised (Edwards Modulyo, Edwards, UK) as 1ml aliqoutes. Molar ratio of fractions was measured using UV-Visible spectrophotometer (Hewlett Packard Diode Array 8543, Germany). One aliquot of lyophilized CM-Alb was dissolved in carbonate buffer pH 9.0 and tagged with 50mM Fluorescein isothiocyanate (FITC) (Sigma, USA)) using standard method to track cellular uptake of the conjugate. For tagging pure Alb to demonstrate cellular uptake, the protein was dialyzed against carbonate buffer. Unreacted FITC was removed by gel-filtration on a 5 ml column of Sephadex G-50-300. Fractions with

molar ratio >2.0 (A495:A280) were pooled, sterile filtered (0.22µm) and freeze dried as small aliquots for tracking experiments. The CM from replicate aliquots of conjugate and FITC-tagged conjugate were extracted into 9:1 DMSO-water mixture and quantified based on standard curve plotted using serially diluted CM in DMSO in the range of 1-12 µM. Alb in the conjugate was estimated using Lowry's protein assay. The aliquots were stored at 4°C for future studies. The conjugate was characterised for conjugation and storage stability using Fourier-transform infrared spectroscopy (FTIR) (JASCO FTIR - 4200). The aqueous solubility was analyzed using UV-Visible spectrophotometer. Further the purity and storage stability was analyzed using High performance liquid chromatography - Gel permeation chromatography (HPLC-GPC) system separation based on molecular weight with UV detection system. The HPLC system consisted of Waters HPLC system 600 series pump equipped with 7725 Rheodyne injector and 2487 dual absorbance detector (Waters) at 430 nm. The column used was Waters Styragel HR-2 column with a principle of GPC. The mobile phase used was Dichloromethane (Sigma chemicals, USA) at flow rate of 1.0 ml/min. To extract CM from the conjugate (CM-Alb), the conjugate was dissolved in dichloromethane (mobile phase) to get a required concentration of 20µg/500µl and submitted for analysis.

3.2. Isolation of Human Umbilical Cord Derived Vein Endothelial Cells (HUVEC-ECs)

Endothelial cells (ECs) were isolated from Human umbilical vein using collagenase digestion (Jaffe et al., 1973). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM-F12) (Gibco, Invitrogen, USA) medium with 10% fetal bovine serum (Gibco Invitrogen, USA) and Antibiotic-antimycotic solution (Gibco Invitrogen, USA). The collected umbilical cord from c-section delivery was washed with sterile 1X Hank's Balanced Salt Solution (HBSS) buffer to remove any blood stains. Cannulas were inserted at both the ends of the cord through the vein and the ends. Buffer was pushed through the cannula to flush out the blood from vein and the process was repeated until clear buffer comes out through the other end of the cord. Following this, the cord was incubated with 0.5% collagenase (Gibco Invitrogen, USA) solution at 37°C for 10-12 minutes. After incubation, collagenase action was stopped with serum containing medium. The cell pellet was

obtained by centrifugation of collagenase digested solution at 400g for 6 minutes. For culture of EC, TCPS was coated with fibrin composite as described previously (Chennazhi and Krishnan, 2005). In brief, the dishes were incubated with 5 IU/ml thrombin for 30 min at 37 °C in CO_2 incubator. The solution was then discarded and dishes were coated with a fibrin composite containing 10 mg/ml fibrinogen, 0.2 % gelatin and 50 µg/ml VEGF. The clot was allowed to form for 30 mins by incubating the dishes at 37°C in CO_2 incubator. Following this, the dishes were lyophilised and used for cell culture.

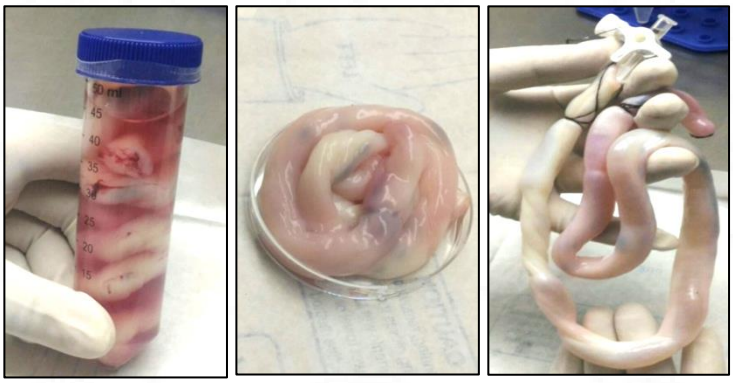


Figure 3*: Isolation of ECs: (a) Human umbilical cord in collection buffer (1X HBSS+ 1mg/ml Glucose), (b) Cord with blood stains removed after washing with HBSS buffer, (c) Collagenase filled cord prior to incubation.*

3.3 Characterization of ECs

Cells from Passage 3 were taken for endothelial cell characterization. The cells were given a wash with 1X HBSS and then fixed in 3.7% formaldehyde (Merck) solution for 30 minutes. 1X Phosphate buffered saline (PBS) wash was given 3 times, 5 minutes each. Then blocking was done using 1% Bovine serum albumin (BSA) (Sigma, USA) solution for 30 minutes, washed with PBS 3 times, 5 minutes each. The cells were incubated with Alexa Fluor tagged CD31 antibody (1:50) (Abcam, UK) for 1h in dark at RT. Washed with PBS 3 times, 5 minutes each. The cells were incubated with 4,6, diamidino-2-phenylindole (DAPI) (Invitrogen, USA) (1:5000) for 3 minutes in dark at RT. Washed with PBS 3 times, 5 minutes each. The images were acquired using Fluorescent microscope (Leica DMIRB, Germany).

Cells from Passage 3 were taken for Von Willibrand Factor (vWF) staining. The cells were given a wash with 1X HBSS and then fixed in 3.7% formaldehyde

solution for 30 minutes. PBS wash was given 3 times, 5 minutes each. Cells were permeabilized using 0.1% Triton-X (Sigma, USA) for 5 minutes. Washed the cells with PBS 3 times, 5 minutes each. Then blocking was done using 1% BSA solution for 30 minutes, washed with PBS 3 times, 5 minutes each. The cells were incubated with vWF antibody (1:100) (Santa Cruz Biotechnology) for 1h in dark at RT. Washed with PBS 3 times, 5 minutes each. The cells were incubated with anti-goat texas red antibody (1:1000) (Abcam, UK) for 1h in dark at RT. Washed with PBS 3 times, 5 minutes each. Then the cells were incubated with DAPI (1:5000) for 3 minutes in dark at RT. Washed with PBS 3 times, 5 minutes each. The images were acquired using Fluorescent microscope (Leica DMIRB, Germany).

Cells from Passage 3 were taken for Acetylated Low Density Lipoprotein (Ac LDL) (Gibco Invitrogen, USA) uptake assay. The cells were incubated with Ac LDL (1:100), for 4h in dark at 37°C. After incubation, 1X HBSS wash was given 3 times, 5 minutes each. The images were acquired using Fluorescent microscope (Leica DMIRB, Germany).

3.4 Determination of non-cytotoxic concentration of conjugate by MTT assay

Non-cytotoxic concentration of CM-Alb conjugate for ECs was determined by MTT Assay. ECs (5000cells/cm^2) were seeded in 24 well culture plates. Once cells attained 70% confluency, CM-Alb conjugate was added in concentrations as follows: 5, 10, 20, 30, 50, 80 and 100µM; incubated for 24h and 48h. After incubation, cells were given a wash with 1X HBSS and MTT Reagent (0.5mg/ml) (Sigma, USA) in medium was added and incubated for 2h in dark at 37°C. Following incubation, 250µl DMSO was added for cell lysis and incubated for 5 minutes in dark at 37°C. The absorbance was measured at 595nm in microplate reader (Bio-Rad, iMark). Cell viability was calculated as follows: Absorbance of Test/Absorbance of Control X 100.

3.5 Endocytosis of FITC tagged conjugate

ECs (5000cells/cm^2) were seeded in 6well culture plates. Upon reaching 70% confluency, FITC tagged CM-Alb (30µM) conjugate and FITC tagged Alb was added and incubated for 8h. The images were acquired using Fluorescent microscope at definite time periods. The cells were trypsinised and pelleted down. Following incubation, the cells were washed with 1X HBSS at 400g for 6 minutes, fixed with

3.7% formaldehyde for 30 minutes, washed with PBS for 3 times at 400g for 6minutes and resuspended in PBS for FACS analysis (Beckman Coulter Life sciences-Cytoflex).

3.6 Effect of conjugate on cell proliferation potential

Cell Proliferation potential was studied using endothelial cells (5000cells/cm^2) and treated with CM-Alb (5µM, 10 µM, and 30µM). The cells were trypsinised and pelleted down. The cells were washed with 1X HBSS at 400g for 6 minutes each. Cells were fixed with 3.7% formaldehyde for 30 minutes, washed with PBS for 3 times at 400g for 6minutes. The cells were incubated with proliferating cell nuclear antigen (PCNA) antibody (1:100) (Santa Cruz Biotechnology) for 1h at RT. The cells were washed with PBS for 3 times at 400g for 6minutes. Following this, the cells were incubated with alexa fluor tagged secondary antibody (1:1000) (Abcam, UK) for 1h at RT in dark. Washed with PBS at 400g for 3 times 6 minutes each and resuspended in PBS for FACS analysis (BD FACS Aria II). The acquired data was analyzed using Flow-jo software (Tree Star Inc., USA).

3.7. Establishing inflammatory endothelial Cells (ECs) phenotype

For standardizing the inflammatory conditions in endothelial cells, optimum concentration of inflammatory proteins required to induce up-regulation of inflammatory markers had to be determined. So for inflammatory conditions, three inflammatory proteins at two different concentrations were selected: TNF-α, IL-6 and IL-1β (all three from Sigma, USA). 5000cells/cm^2 was seeded for the study. Upon attaining 70% confluency, cells were incubated with TNF-α (2ng/ml; 4ng/ml), IL-6 (10ng/ml; 20ng/ml) and IL-1β (10ng/ml; 20ng/ml) for a period of 24h. RNA was isolated using Trizol reagent (In vitrogen, USA) based on the standard protocol. Isolated RNA was quantified spectrophotometrically using Nanodrop (ND 2000; Thermo Scientific, USA). cDNA was synthesized using Orion X cDNA synthesis kit (Origin, India) in a thermal cycler (Master cycler; Eppendorf). qRTPCR was performed in Bio-Rad iQ5 Real time PCR detection system (Bio-Rad, USA) under the following conditions: Enzyme activation, 95°C for 15 minutes; denaturation, 95°C for 30 seconds; annealing, 50°C for 20 seconds; and extension, 72°C for 20 seconds. The reaction was carried out using Orion X 2X Real time PCR mastermix

(Origin Diagnostics, India). Primers (specific for our gene of interest) were procured form Eurogentec, GeneX India Bioscience Pvt.Ltd, India. Glyceraldehyde 3-phophate dehydrogenase (GAPDH) was used as house-keeping gene. Fold change in expression was calculated after normalisation with GAPDH expression using the formula $2^{-\Delta\Delta Ct}$. NF-kB, MCP-1, ET-1, COX-2 and VCAM-1were the inflammatory markers analyzed by Quantitative Real Time polymerase chain reaction (qRT-PCR) in Bio-Rad iQ5 Multicolour Real Time PCR Detection System.

Genes	Amplicon size	Primer Sequence
GAPDH	120bp	FP - GAAATCCCATCACCATCTTCCAGG RP – GAGCCCCAGCCTTCTCCATG
NF-kB	98bp	FP – AGCCCACAAAGCCTTATCA RP – CAATGCCAGTGCCATACAG
MCP-1	120bp	FP - CCGAGAGGCTGAGACTAAC RP – ATGAAGGTGGCTGCTATGA
ET-1	96bp	FP – TGAAGCCATAGCCTCCAC RP-AGTCAGGAACCAGCAGAG
COX-2	86bp	FP - TCATCAACACTGCCTCAATTC RP – CTCTGGATCTGGAACACTGA
VCAM-1	90bp	FP - CCTCCTTAATAATACCTGCCATTG RP - TCTGTGCTTCTACAAGACTATATGA

***Table 1**: List of Primers specific to human genes*

3.8 Assessment of anti-inflammatory effect of CM

The anti-inflammatory property of conjugate was studied using endothelial cells (5000cells/cm^2). Based on the qRTPCR data, TNF-α was selected for inducing inflammation. The cells were incubated with TNF-α (10ng/ml) for 24h. After incubation, the cells were subjected to the different treatment conditions for a period of 24h: CM (5, 10, 20, 30 and 60µM) and CM-Alb (5, 10, 20, 30 and 60µM). RNA was isolated, cDNA was synthesized and qRTPCR was performed (as per the method explained in section 3.7) for determining the expression of the following inflammatory markers: NF-kB, MCP-1, ET-1, COX-2 and VCAM-1.

3.9 Protein level assessment of inflammatory markers

To identify the inflammatory markers at protein level, after 24 exposure of > 70% confluent ECs with TNF-α (10ng ml^{-1}), the activated cells were treated with CM and CM-Alb (5µM) for 24h. Cells were washed and fixed with 3.7% formaldehyde and

were blocked with 1% BSA; cells were permeated with 0.1% Triton X-100. Following this the cells were incubated with primary antibodies: NF-kB, ET-1, COX-2 & VCAM-1 (Abcam, Cambridge, UK) in the dilution of 1:500. After washing, Alexa fluor tagged anti-rabbit antibody (Abcam, Cambridge, UK) in the dilution 1:1000 was used to identify specific protein expressions. Nucleus was stained with 1:5000 DAPI. The fluorescent images were acquired using Fluorescent microscope and LAS camera system (Leica DMIRB, Germany) and the fluorescence intensity was calculated using Image J software by selecting fluorescent cells from different fields (20X) from different different fields.

Protein from cells was isolated using radioimmunoprecipitation buffer (RIPA) (Thermo Scientific, US) along with addition of protease inhibitor (Sigma, USA) by intermittent freeze and thaw mechanism on ice along with a time gap of 10minutes with vortexing for 30seconds each time. Following this, the mixture was centrifuged at 14000g for 30minutes and supernatant was taken for protein estimation by Lowry's method. 20µg of total protein was loaded and resolved through Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) (Bio Rad apparatus). For NF-kB, COX-2 & VCAM-1, 10% resolving gel was used and for ET-1 12% resolving gel was used. Following this, the gel was blotted on to methanol (Merck) activated Polyvinylidene fluoride or polyvinylidene difluoride (PVDF) (Sigma, USA) with blotting conditions of 25V, 0.1A for 60min. Blot was blocked with 3% BSA (Sigma, USA) for 2h at RT, washed with PBS plus 0.1% tween 20 (PBST) buffer. The blot was incubated with respective primary antibodies at dilution of 1:500 at 4ºC overnight with continuous shaking, followed by washing, incubation with Goat anti-rabbit HRP tagged secondary antibody (Abcam, Cambridge, UK). Blot was developed in dark using developer solution containing 3'3' Diaminobenzidine (DAB) (Sigma, USA), Nickel chloride (Sigma, USA) and 0.2% hydrogen peroxide (Sigma, USA). Images were acquired using Geldoc (Alpha Imager Documentation System 2000).

3.10. ADMSC Isolation and characterization

Collection of human adipose tissue was approved by the Institutional Ethics Committee & Institutional Committee for Stem Cell Research of Sree Chitra Tirunal Institute for Medical Sciences & Technology (IEC-IC-SCTIMST) and Department of

plastic surgery, Kerala Institute of Medical Sciences hospital (IEC-KIMS)(IEC-SCT/IEC/1231/June-2018 and IC-SCR/46/March-2017). Informed consent was also obtained from subjects/volunteers undergoing liposuction before collecting the tissue under aseptic condition by the plastic surgeon. The isolation of MSCs from adipose tissue was performed according to the modified protocol published (Zhu et al., 2008). Briefly, the adipose tissue (~10 g) was washed thoroughly with HBSS to remove blood stains and was chopped into~1 mm^3 pieces and in the case of liposuction samples, the chopping section was omitted. Cells were dissociated by treating with1.5 mg/ml using Collagenase NB 4 Standard Grade (SERVA Electrophoresis) at 37 °C with continuous shaking for 45–60 min. The suspension was diluted with an equal volume of serum-containing medium, passed through a 70 µm cell strainer (BD Falcon) and washed by centrifugation. The cell pellet was resuspended in MSC basal medium (BM) consisting of low-glucose dulbecco's modified eagle's medium (DMEM LG; Gibco, USA), supplemented with 10 % fetal bovine serum (FBS; Gibco, USA) and antibiotic–antimycotic (AbAm) solution (Invitrogen, USA). The cells were seeded onto a 25 cm^2 TCPS flask (Nunc, Denmark) and kept at 37 °C and 5 % CO_2; medium change was done at 3 day intervals. When ~ 80-95 % confluence was achieved, the cells were passaged using standard trypsinization protocol. Cells in passages 2 - 4 were used for the MSC markers and also to start differentiation experiments.

The cells from passage 3 were characterized as per the standards laid by the International Society for Cellular Therapy (ISCT) for surface markers by Flow cytometry (BD FACS Aria II) analysis (Positive markers: CD90 PE, BD Pharmingen; CD105 PE, Santa Cruz; CD73 PE, Biolegend and Negative markers: CD14 FITC, Millipore; CD45 FITC, Beckman Coulter). The results were analyzed using Flow-jo software. The cells were also characterised for their multipotency by tri-lineage differentiation (Adipogenic, osteogenic and chondrogenic lineage) using Stem Pro Differentiation kit (Gibco Invitrogen, USA). The trilineage differentiation was confirmed by specific staining for all the three lineages as per the standard staining protocols. The adipogenic lineage was confirmed by Oil red O staining; osteogenic lineage by Alizarin red and chondrogenic lineage by Toluidine blue staining.

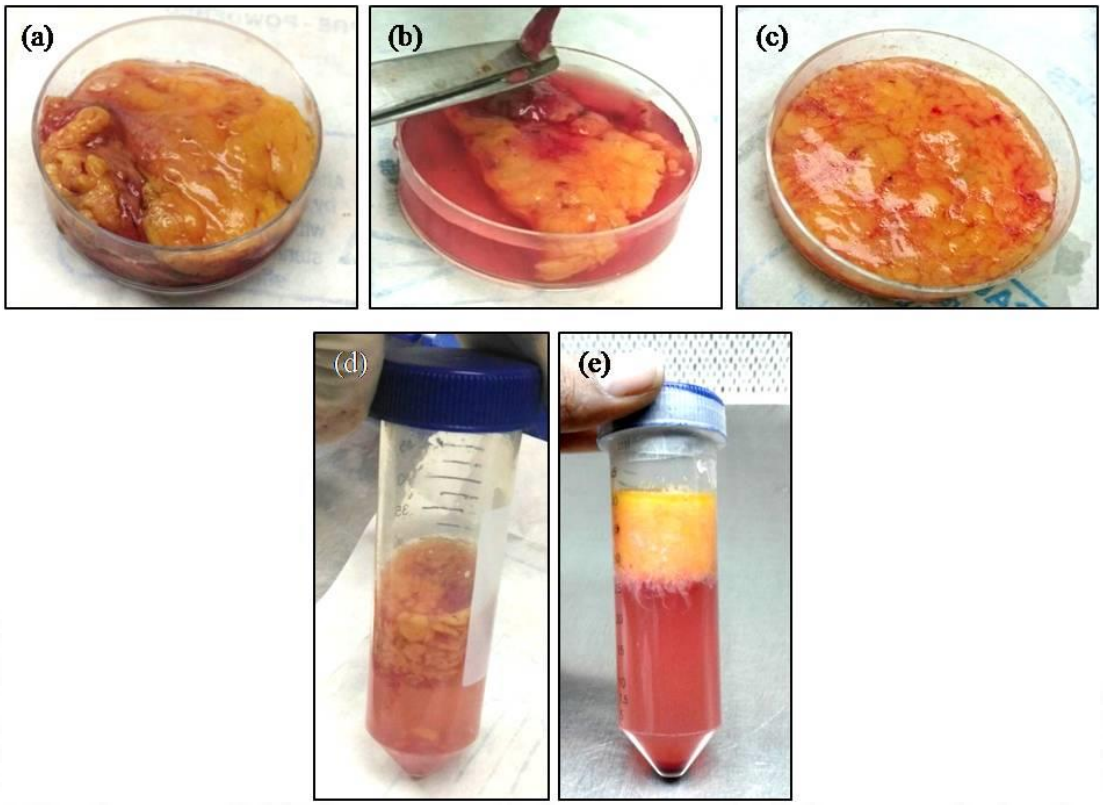


Figure 4*: Isolation of hADMSCs (a) Cardiac adipose tissue (b) Tissue washing and removal of blood vessels (c) Minced tissue (d) Minced tissue with collagenase enzyme for incubation (e) After enzyme digestion the chunk tissue can be seen at the top portion and with cell pellet at the bottom of the tube.*

3.11. Establishing differentiation of ADMSCs to Chondrocytes

hADMSCs from Passage 3 were taken for differentiation studies. The cells were seeded in clumps and incubated for 4h at 37ºC, following which the 10% DMEM LG medium was added. After 24h, chondrogenic supplement medium (Gibco Invitrogen, USA) was added and cultured for 7-14days for chondrocytes differentiation (Stromps et al., 2014). The differentiated cells were characterized using Type II Collagen (Abcam, UK) staining. The staining protocol was performed as mentioned in section 3.3.

3.12 Endocytosis of FITC tagged conjugate by Fluorescent imaging

hADMSCs (5000cells/cm^2) were seeded in 6well culture plates. Upon chondrogenic differentiation, endocytosis assay was carried out as mentioned in section 3.5.

3.13 Effect of conjugate on cell viability and Proliferation

hADMSCs (5000cells/cm^2) were seeded in 6well culture plates. Upon chondrogenic differentiation, cells were subjected to MTT assay (5, 10, 30, 50, 80, 100µM) and PCNA (CM-Alb - 5µM and 10µM) assay as described in sections 3.4 & 3.6 respectively.

3.14. Establishing inflammatory Chondrocytes (ChCs) phenotype

For standardizing the inflammatory conditions in ChCs, optimum concentration of inflammatory protein required to induce up-regulation of inflammatory markers had to be determined. So for inflammatory conditions, TNF-α was selected. 5000cells/cm^2 was seeded for the study. Upon attaining 70% confluency, cells were incubated with TNF-α (2, 4 and 10ng/ml) for a period of 24h. RNA was isolated, cDNA was synthesized and qRTPCR was performed as per the method explained in section 3.7 for determining the expression of the following inflammatory markers: NF-kB, IL-8, MMP-13, COX-2 and TIMP-1.

Genes	Amplicon size	Primer Sequence
GAPDH	120bp	FP - GAAATCCCATCACCATCTTCCAGG RP – GAGCCCCAGCCTTCTCCATG
NF-kB	98bp	FP – AGCCCACAAAGCCTTATCA RP – CAATGCCAGTGCCATACAG
IL-8	92bp	FP – GCAGAGGGTTGTGGAGAA RP- CACTGGCATCTTCACTGATTC
MMP-13	119bp	FP – GACTATGCGTGGCTGGAA RP - TGGTGTGGGAAGTATCATCAA
COX-2	86bp	FP - TCATCAACACTGCCTCAATTC RP – CTCTGGATCTGGAACACTGA
TIMP-1	105bp	FP – GGCTTCACCAAGACCTACA RP – GTCCGTCCACAAGCAATG

***Table 2**: List of Primers specific to human genes*

3.15. Assessment of anti-inflammatory effect of CM in ChCs

The anti-inflammatory property of conjugate was studied using ChCs (5000cells/cm^2). Based on the qRTPCR data, TNF-α was selected for inducing inflammation. The cells were incubated with TNF-α (10ng/ml) for 24h. After incubation, the cells were subjected to different treatment conditions for a period of 24h: CM (7.5, 15µM) and CM-Alb (7.5, 15µM). RNA was isolated, cDNA was

synthesized and qRTPCR was performed as per the method explained in section 3.7 for determining the expression of the following inflammatory markers: NF-kB, IL-8, MMP-13, COX-2 and TIMP-1.

3.16. Estimation of *in vivo* anti-inflammatory action of CM-Alb

For demonstration of anti-inflammatory effect of CM-Alb, the first step was to establish an inflammatory model in rabbit knee joint. Further, the model was treated with the conjugate to identify the anti-inflammatory function of the conjugate.

3.16.1 Development of an inflammatory disease model in Rabbit

The rabbit species used for developing inflammation in knee joint was New Zealand white with approximate weight of 2 to 3 kg coming in the age group of 8 to 12 months. The study was carried out after getting institutional animal ethics committee (IAEC) approval (B-form No. SCT/IAEC-240/AUGUST/2017/94). TNF-α has been known to play an important role in initiation of arthritis accompanied with inflammation. And based on the results obtained in *in vitro* studies, which showed significant up-regulation of inflammatory markers in both ECs and ChCs using TNF-α, this pro-inflammatory marker was used for inducing inflammation and cartilage disintegration in rabbit knee joints. The concentration of TNF-α used in knee joints was 500ng/ml which was obtained after standardization. The medium of administration was sterile normal saline. Animals were divided into 7 groups based on the treatments given to animals. Each group comprised of three animals (n=3). TNF-α was administered into the knee joints for 4 consecutive weeks at 7 days interval time following which treatment was given. In addition to treating with CM-Alb, the inflammation induced tissue was also treated with HA which is practiced in clinics. Another experiment group was treated with ChCs derived from rabbit adipose tissue.

3.16.2 Rabbit ADMSCs isolation and differentiation to chondrocytes (rChCs)

Approximately 20g of adipose tissue was collected surgically from the neck portion of the IAEC approved animal (B-form No. SCT/IAEC-240/AUGUST/2017/94). The collected adipose tissue was subjected to collagenase digestion and rabbit ADMSCs (rADMSCs) were isolated as per the standard protocol described in section 3.10. The cultured cells were characterized for their

multipotency by trilineage differentiation as mentioned in section 3.10. The cells from passage 3 were subjected to chondrocytes differentiation as per the method described in section 3.11. These differentiated chondrocytes (rChCs) were used for further transplantation studies. For treatment strategy, rChCs were transplanted after labelling the cells with PKH26 (Sigma, USA). The differentiated cells ($1X10^{6)}$ were trypsinised, resuspended in 500µl diluent solution which is supplied along with the labelling kit. Following this, 5µl of the red PKH26 was added into the cell suspension and incubated for 1-2minutes. Then the cells were washed with serum containing medium and suspended in 1X HBSS for treatment.

3.16.3 Treatments strategies

Weights of animals (in Kg) were taken on all treatment days starting prior to giving anesthesia. All the 7 groups received TNF-α injection in knee joint for 4 consecutive weeks with a time interval of 7 days. Prior to each injection in the knee joint, the animals were given general anesthesia (5mg/Kg xylazine and 30mg/kg Ketamine). Group I was the sham control which received TNF-α alone. After injection of the inflammatory inducer into the knee joints, the joints were subjected to flexion and extension movement along with gentle massaging so that the injected solution can evenly spread throughout the knee joint. Group II to VII received different treatment strategies on day 14, 21 and 28. Group II received standard anti-inflammatory drug available in the market i.e, methotrexate (1mg/ml) was injected into the knee joints. Group III received CM-Alb (5µM) and rChCs cells ($1X10^{6}$ cells). Group IV received PKH26 labelled rChCs ($1X10^{6}$ cells). Group V received CM-Alb (5µM/ml) and HA (4mg/ml). Group VI received CM-Alb (5µM). Group VII received HA (4mg) in 3 joints and Alb (0.2mg) in 3 joints. The animals were sacrificed on day 35. The treatment strategies are represented in the table given below.

Study groups	**Experiments**	TNF-α Administration/Blood collection	**Type of treatment**	Treatment adminsistration/blood collection	Termination
Group I	TNF-α – 500ng/ml (Sham control)	Day 0, 7, 14, 21	None	Day 14, 21, 28	Day 35
Group II	TNF-α – 500ng/ml	Day 0, 7, 14, 21	Methotrexate (1mg)	Day 14, 21, 28	Day 35
Group III	TNF-α – 500ng/ml	Day 0, 7, 14, 21	CM-Alb (5µM)+cells (rChCs) ($1X10^6$)	Day 14, 21, 28	Day 35
Group IV	TNF-α – 500ng/ml	Day 0, 7, 14, 21	Cells (rChCs) ($1X10^6$)	Day 14, 21, 28	Day 35
Group V	TNF-α – 500ng/ml	Day 0, 7, 14, 21	CM-Alb(5µM)+ HA(4mg)	Day 14, 21, 28	Day 35
Group VI	TNF-α – 500ng/ml	Day 0, 7, 14, 21	CM-Alb (5µM)	Day 14, 21, 28	Day 35
Group VII	TNF-α – 500ng/ml	Day 0, 7, 14, 21	HA(4mg)/Alb (0.2mg)	Day 14, 21, 28	Day 35

***Table 3**: Experimental groups for the in vivo study*

3.16.4. Assessment of anti-inflammatory effect

3.16.4.1 Gross morphological assessment

During the course of treatment, the knee joints were routinely examined for any edema or change in surface temperature by gently touching the joints. Upon termination, the knee joints were excised out using bone cutter. The surface of cartilage was carefully observed for any morphological changes by naked eyes. A small amount of cartilaginous tissue was taken into trizol from the knee joint for mRNA extraction and qRT-PCR analysis. Following this, the joints were put in 10% neutral buffered formalin (NBF) (Merck) and submitted for histological analysis.

3.16.4.2 Distribution of PKH26 Labelled rChCs in knee joint

The knee joints of animal groups that were transplanted with PKH26 labelled cells were subjected to imaging using *In vivo* imaging system (IVIS Spectrum Preclinical in vivo imaging system, Perkin Elmer, USA). The excitation wavelength used was 535nm and the emission was measured at 580nm. Images were then analyzed using Living Image 4.3.1 software. Based on the colour scale obtained, the region of high cell density can be identified. Relative high cell density is depicted by yellow colour and red colour indicated relative low cell density.

3.16.4.3 Analysis of haematological parameter

2ml blood was collected from all animals on all experiment days (7d interval) from the ear vein for analysis of haematological parameters. The collected blood was subjected to haematology analysis (Animal blood cell counter- ABC Vet) to detect any change in the count of different cell types in blood (platelets, RBC, WBC) as well as in haemoglobin (Hb) and hematocrit (HCT). 1ml blood was submitted for analysis of ESR.

3.16.4.4 Histological assessment

After animal sacrifice, the joints were collected in 10% NBF and subjected to decalcification before processing for histological assessment. The joints were placed in nylon bags and incubated in decalcifying solution comprising 12.5% Ethylene Diamine Tetra Acetic acid (EDTA) (Merck) (pH- 7) with pellets of sodium hydroxide (NaOH) (Merck) for complete dissolution of EDTA along with continuous stirring on table top stirrer (York Scientific industries Pvt. Ltd, India). The samples were checked for decalcification by grossing at regular intervals of 1month using needle pricking after. The grossed tissue was again put in decalcifying solution for approximately 2 to 3 months along with continuous stirring. Once the tissues were decalcified, the tissues were subjected to processing, embedding, sectioning, staining and mounting. Tissue processing was carried out in automated tissue processor (Leica ASP 300). Tissue processing consists of following steps: (i) fixation in 10% Neutral buffered formalin (NBF); (ii) dehydration in series of alcohol (70, 80 and 100% - 2 changes); (iii) clearing in xylene (Merck) (2 changes) and (iv) paraffin impregnation. Then the tissues were embedded in paraffin using embedder (Leica EG1150H) to make tissue blocks. Required number of sections for immunohistochemical and other important staining were obtained using rotary microtome (RM2550, Leica, Germany). The sections (5μm) were cut using Leica disposable blade, put in water bath at 45ºC, and carefully placed on albumin coated slides (Starfrost). These slides were used for further staining procedures as described below. In case of Haematoxylin and Eosin staining, autostainer (Leica Autostainer XL) was used and the slides were mounted using mounting media (Leica)with the

help of coverslipper (Leica CV5030).For rest of the staining procedures, staining and mounting was done manually.

i) Haematoxylin and Eosin (H&E) staining

For determining cellular arrangement in the tissue cartilage, the slides with sections were subjected to H&E staining. The obtained slides with sections were subjected to deparaffination by keeping slides at in hot air oven at 60ºC for 1h. The deparaffinised sections were cleared in xylene (2changes), rehydrated in descending series of alcohol (100, 95, 70%), 5min each with 2 changes in 100%. Then the slides were placed in Hematoxylin (Sigma, USA) for 20min, washed in running tap water for 10min, following which the slides were placed in Eosin (Sigma, USA) for 2min and washed in running tap water for 10min.The sections were dehydrated in ascending series of alcohol (70, 95, 100%), 5min each with 2 changes in 100%. Once the staining was done, slides were mounted as explained in above section and images at different magnifications were taken using light microscope (Nikon E 600, Nikon, Japan).

ii) Picrosirius red staining

The presence of collagen in the obtained sections was determined using Picrosirius red staining. The deparaffinised sections were rehydrated and stained for nuclei using haematoxylin as explained in H&E staining in the above section. After washing, the slides were stained with Picrosirius red stain for 1h. The staining solution was prepared by dissolving 0.5g Sirius red (Sigma, USA) in saturated solution of picric acid (Merck) in water. Washed in 2 changes of acidified water and dehydrated in ascending series of alcohol with 2 changes in 100% alcohol and 2 changes in xylene. The stained sections were mounted using the mounting media and allowed to dry overnight. Images at different magnifications were acquired using bright field microscope (Leica DMIRB, Germany).

iii) Alcian blue staining

For detecting the presence of glycosaminoglycans (GAGs), the sections were stained with alcian blue. The deparaffinised slides were rehydrated and stained with haematoxylin as mentioned in the above section and washed. The washed slides were stained with alcian blue for 30min. The staining solution was prepared by dissolving 1g alcian blue (Himedia) in 3% acetic acid (Merck) solution. The stained sections

were washed in running tap water. The sections were dehydrated in alcohol, cleared in xylene, mounted and images were taken as mentioned in the above section.

iii) Immunohistochemistry (MMP-13, TIMP-1)

The deparaffinised tissue sections were pretreated for antigen retrieval using a pressure cooker in 10 mM citrate buffer for 20min. Endogenous peroxidases were quenched with 3% hydrogen peroxide for 20min. After this, the slides were cleared, hydrated and nuclear stained with haematoxylin as mentioned in the above section. For blocking nonspecific binding of antibodies, 5% FBS was used. The tissue slides were stained with MMP-13 (1:1000) and TIMP-1 (1:1000) antibodies (Abcam) by incubating with the respective antibodies overnight at 4ºC. Following which the sections were washed in PBS and then incubated with horseradish peroxidise (HRP) conjugated anti-rabbit immunoglobulin (Abcam) for 2h at RT, washed with PBS, incubated with DAB and washed with PBS. Following this, the sections were dehydrated, cleared mounted as mentioned in the above section and examined under light microscope.

3.16.4.5 qRTPCR analysis for inflammatory and anti-inflammatory marker expression

The cartilage tissue samples that were collected in trizol from the knee joints of animals at the time of sacrifice were subjected to RNA isolation, cDNA synthesis and qRT-PCR was carried out as per the protocol explained in section 3.7. The inflammatory markers analyzed were NF-kB, IL-8, MMP-13, COX-2, MMP-13 and anti-inflammatory marker analyzed was TIMP-1.

Genes	Amplicon size	Primer Sequence
GAPDH	88	FP – TTTAACTCTGGCAAAGTG RP - TGGAATCATACTGGAACAT
NF-kB	123	FP – CAAGAAGTCCACAAACAC RP – ACCGATATGTCCTCTTTC
IL-8	133	FP – GCTAAGAATACTGGAATTGT RP – TAGGATGTTGGCTGATAC
MMP-13	84	FP – CAGTAACGAGGATGATGA RP - GGATTCAGAGGATGGTAG
COX-2	145	FP – AACATCGTCAATAGCATTC RP - AGTAGGAGAGGTTAGAGAA
TIMP-1	111	FP – CTCCAGAAGTCAATCATAC RP - TGTAGACAAACCGGATAT

***Table 4**: List of Rabbit specific Primers*

3.17 Statistical analysis

All qualitative and quantitative experiments were carried out in triplicates (n=3). All quantitative data is represented as mean ± SD (Standard deviation). Statistical significance was calculated using two tailed t-test in to determine differences among individual groups. $P < 0.05$ was considered to be statistically significant for all experiments. $P < 0.05$ (*); $P < 0.01$ (**); $P < 0.001$ (***).

CHAPTER 4

4. RESULTS

This chapter presents the results obtained from *in vitro* and *in vivo* experiments. The major illustrations comprise: spectral properties of the conjugate; proof of conjugate endocytosis into EC/ChC; establishing non-toxic/safe dose that can be treated with EC/ChC for inducing anti-inflammatory effect; graphical representations of relative gene expressions marking the effect of conjugate on inflammatory response in EC/chondrocytes and images of stained tissue sections demonstrating anti-inflammatory effect on inflammatory rabbit knee model.

4.1 Physicochemical properties of CM-Alb conjugate

4.1.1 FTIR of CM-Alb conjugate

FTIR spectra of lyophilized CM-Alb conjugate showed specific peaks for CM and Alb in (Fig 5). The interaction between CM and Alb can be inferred from the specific peak shift that is observed in the conjugate spectra. The spectra showed CH_2 (2961.16 cm^{-1}), C=O (1628.59 cm^{-1}), C-O (1027.87cm^{-1}), OH (3297.68cm^{-1}) depicting the specific peaks for CM and NH_2 (3297.68cm^{-1}) depicting specific peak for Alb. So the presence of specific peaks along with slight peak shift confirms the interaction of both molecules to form the conjugate.

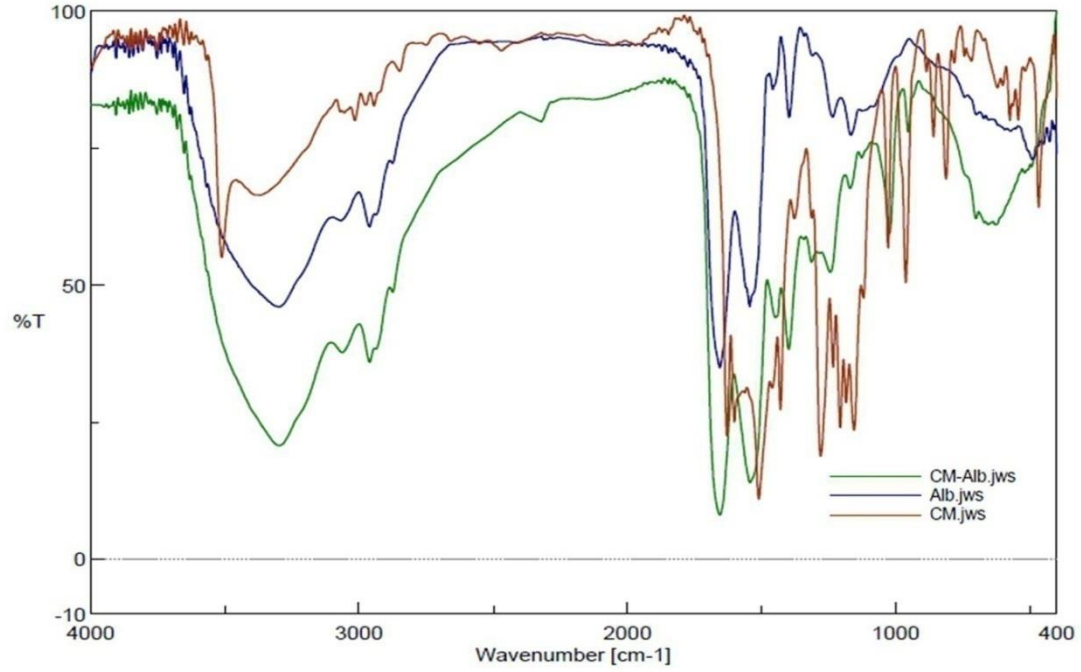


Figure 5: Representative tracing of FTIR spectra. *The spectral ovarlays represent free CM (red); Alb (blue); and CM-Alb (green) conjugate.Characteristic spectral peaks of free CM is evident in CM-Alb conjugate.*

4.1.2 Yield and recovery of CM and Alb

From the initial quantities of raw materials used (CM-12.88mg; Alb-1000mg), there was a recovery of 85.5% CM and 35.8% Alb with a total yield of 11mg CM and 359mg Alb. Pooled total volume of fractions was 14.3ml. The concentration per ml for CM and Alb were 0.77mg/ml and 25.1mg/ml respectively.

Raw material ID	Quantity (mg) added	Volume of reaction mixture	volume (ml) of pure conjugate	Concn (mg) ml^{-1}	Total yield (mg)	Recovery (%)
C78246	12.88	70µl	14.3	0.77	11	85.5
Albumin	1000	5000 µl	14.3	25.1	359	35.8

Table 5: *Tabular representaion of yield and recovery of CM & Alb. Initial concentrations of CM and Alb used for preparing the standard drug formulation; total volume of reaction mixture was 5070*µl*; pooled volume of the purified conjugate; concentration of reactant(mg)recovered; and % recovered from the added reactants.*

4.1.3 Proof of Purity

Gel permeation chromatography analysis of unconjugated CM (raw material) showed a major peak with retention at 9.11 min which corresponds to ~ 94% of dimethoxycurcumin (DMC) in raw material and a minor peak of other impurities at 10.67.Extraction and analysis of CM in the bound CM-Alb showed only the main peak at 9.14, proving that only the DMC binds to Alb. More affinity of DMC towards albumin may be attributed to the higher hydrophobicity of it as compared to other chemical forms.

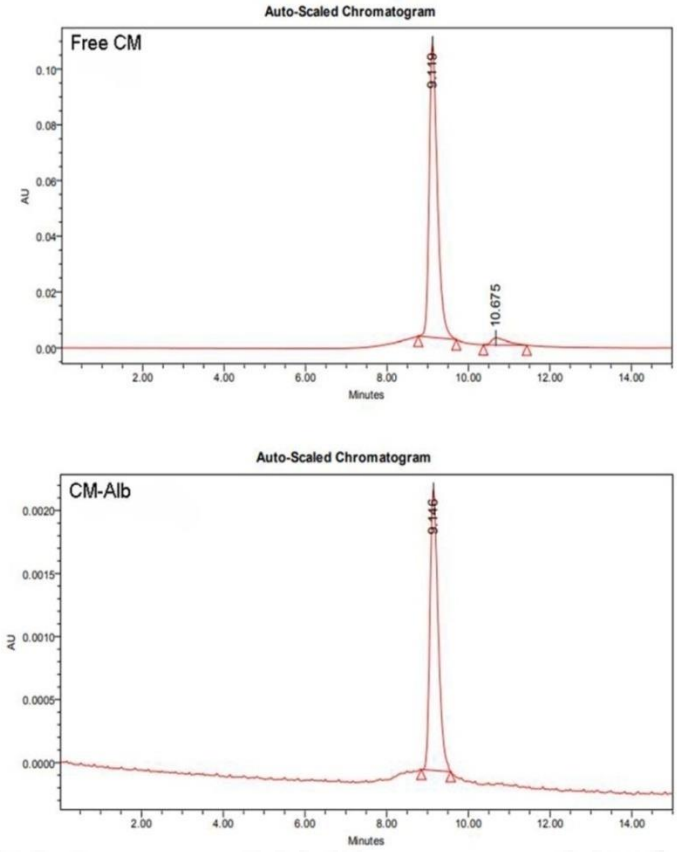


Figure 6: HPLC chromatogram of free and conjugated CM.*(a) Elution profile of CM extracted from raw material and (b) Elution profile of CM extracted from CM-Alb conjugate.*

4.1.4 Solubility of Free CM and CM-Alb conjugate

The solubility of free CM is shown in Fig 7a. The raw material solubility in water was less than 12µM; after 11 µM the linearity of absorbance was lost. However, in DMSO, linearity was evident till 80 µM (Fig7b). In the conjugate, 0.2±0.05 mg CM was bound to 40±5mg Alb. The lyophilized powder of conjugate containing 0.2 mg CM (Fig7c) dissolved within 1-2 min in 0.1 ml water; i.e. 2mg ml^{-1}. Therefore, the conjugation increased the CM solubility from 12µM to 5.4mM giving very strong yellow color of CM (Fig7d) in the dissolved conjugate. The concentration dependent decrease in the characteristic yellow color is seen upon serial dilution of the concentrate.

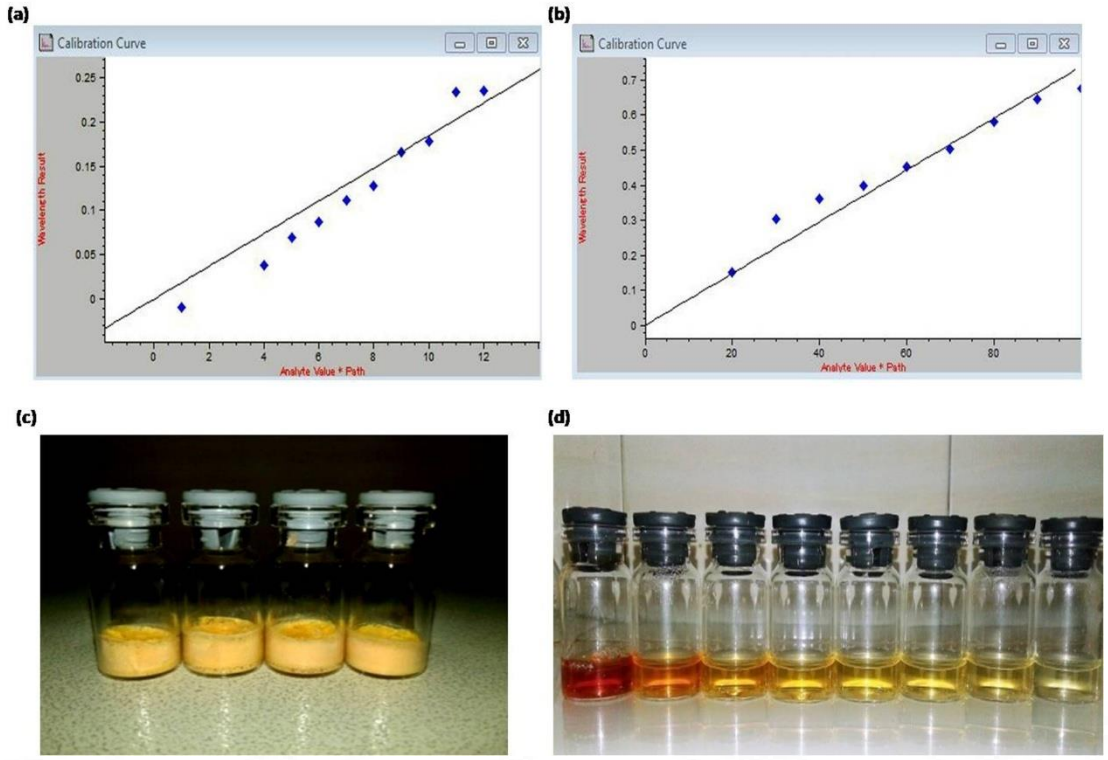


Figure 7: Illustration of difference in solubility of CM and CM-Alb. *(a) Dependence of A420 on solute concentration in water; b, dependence of A420 on solute concentration in DMSO; (c) Appearance of Lyophilised CM-Alb conjugate; (d) Graded color of serially diluted CM-Alb. Repeatability was established in>3 replicate experiments.*

4.1.5 Spectral comparison of fresh and stored CM-Alb

The FTIR spectra of CM-Alb conjugate reconstituted from lyophilized samples stored for 6 months (Fig 8) showed the presence of specific peaks: OH, NH_2 ($3276.7cm^{-1}$), CH_2 ($2962.1cm^{-1}$), C=O ($1646.3cm^{-1}$) and C-O ($1168.2cm^{-1}$) corresponding to the specific functional groups present in CM and Alb as similar to that observed for fresh CM-Alb conjugate. This similar pattern of peaks observed for fresh and stored conjugate samples demonstrates > 6 months storage stability of CM conjugated to Alb and stored in lyophilized state. The exposure to aqueous medium during the conjugation process has not caused metabolic degradation to CM.

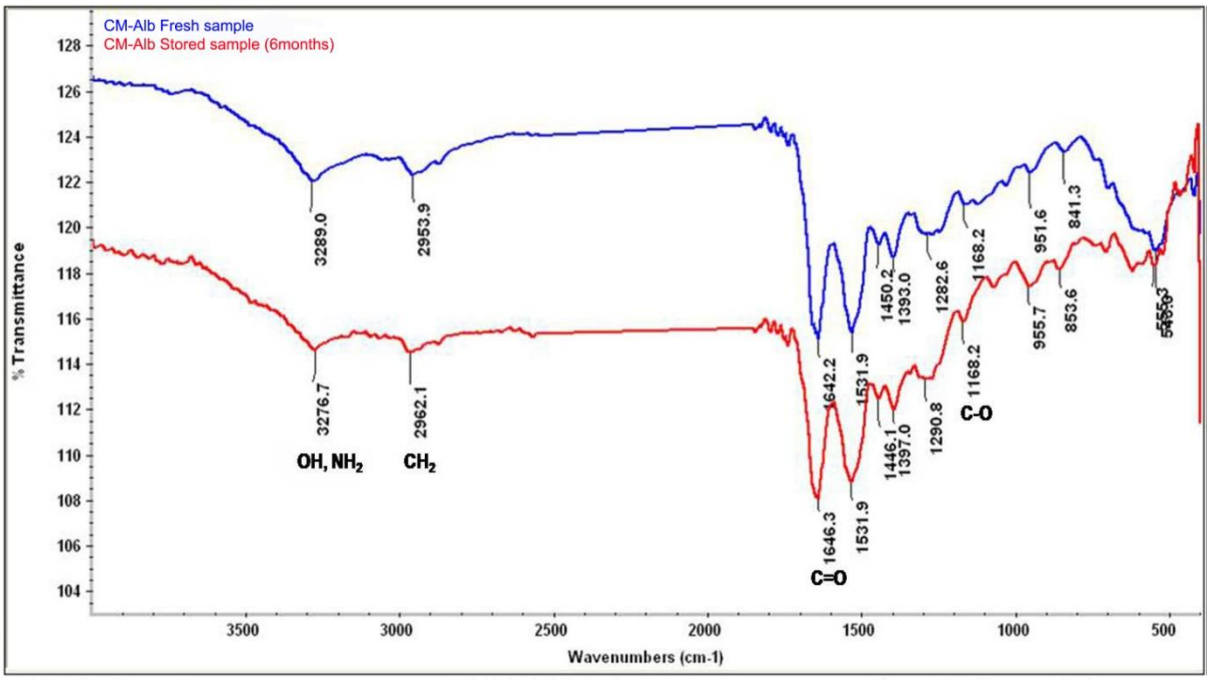


Figure 8: FTIR Spectral recordings of fresh and stored CM-Alb. *Representative FTIR spectra for fresh CM-Alb conjugate (blue tracing) and stored for 6months (red tracing).*

4.1.6 UV-Vis Spectral Comparison of dissolved CM & CM-Alb

The lyophilized sample upon reconstitution and storage showed good stability as compared to free CM. The instability of free CM is evident in the UV-Vis spectral recording of stored 12.0µM sample. Gradual but quick hydrolytic degradation of free CM stored for 1h, 2h and 24h as compared to the fresh diluted solution (Fig 9a-d) is evident. On the contrary, lyophilized CM-Alb reconstituted in water and diluted to 12µM was found stable for 24h with minimal change in the absorbance at 420nm (Fig9a-d). The observation confirms better stability of conjugated CM.

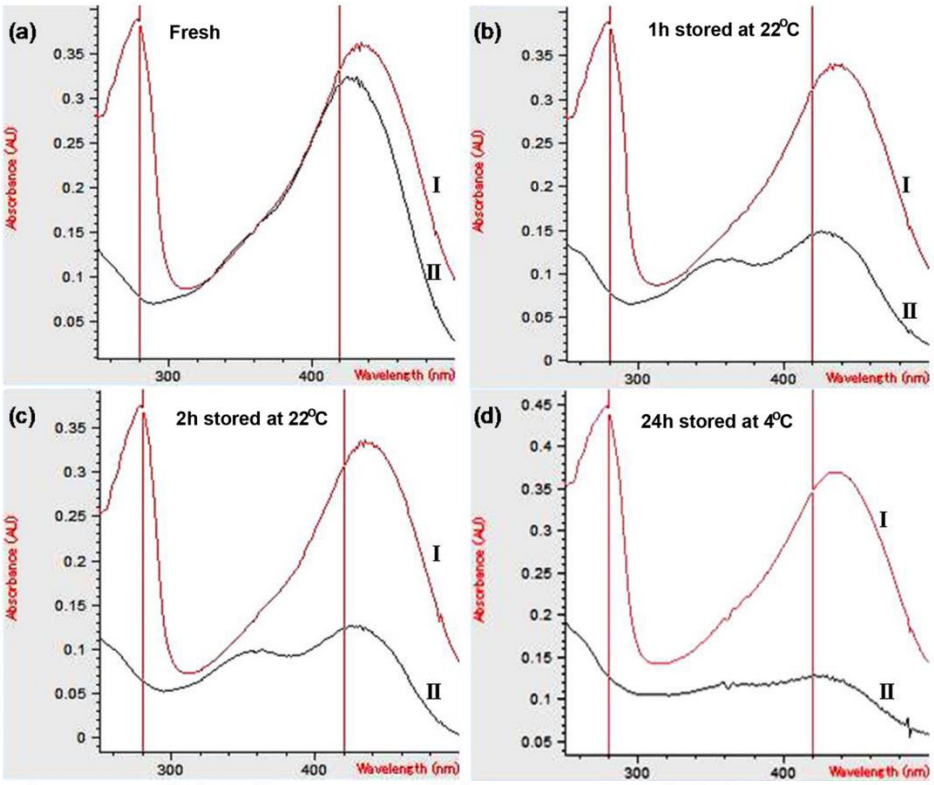


Figure 9: Effect of storage of free CM and CM-Alb dissolved in water.*(a) to (d), Comparison of UV-Vis spectra of dilute solution of CM-Alb (Sample I) after 1h, 2h and 24h of storing; and spectra of dilute solution of free CM (Sample II). a, fresh samples; b, 1h stored samples; c, 2h stored samples; and d, 24h stored samples.*

4.1.7 Comparison of HPLC of fresh and stored CM-Alb

The HPLC elution profile of freshly prepared CM and > 6months stored sample are comparable (Fig 10 a&b). The major component is eluting at ~9.2min in both cases; therefore, no hydolytic breakdown occurs upon lyophilizatio, storage or dissolving the conjugate back in water after storage.

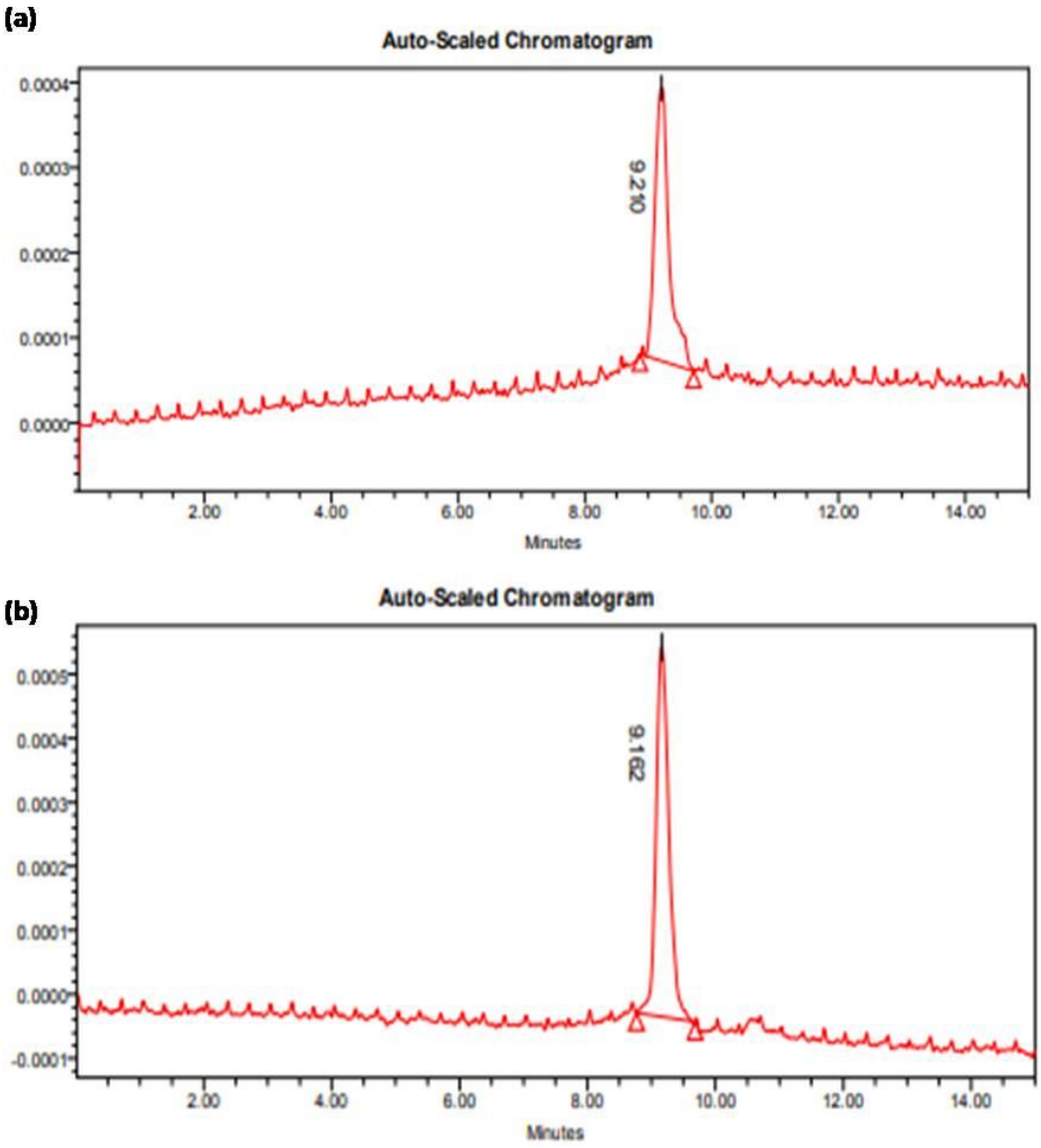


***Figure 10*:Comparison of HPLC elution profile of fresh & stored CM-Alb.** *a, Elution profile of curcumin extracted from fresh CM-Alb conjugate; b, Elution profile of CM extracted from lyophilized CM-Alb stored for 6months.*

4.2 Characteristics of isolated ECs

The isolated EC cells showed their typical cobble stone morphology (Fig 11a) in cell culture system. The isolated cells showed uptake of AcLDL (Fig 11b) confirming the characteristic endothelial mophology in monolayer culture. The ECs were positive for both CD31 and vWF. The membrane protein CD31(Fig 11c) is stained distinctly on membrane of ECs. The vWF (Fig 11d) presentt in Weibel Palade bodies are distictly observedin the cytoplasm of ECs.

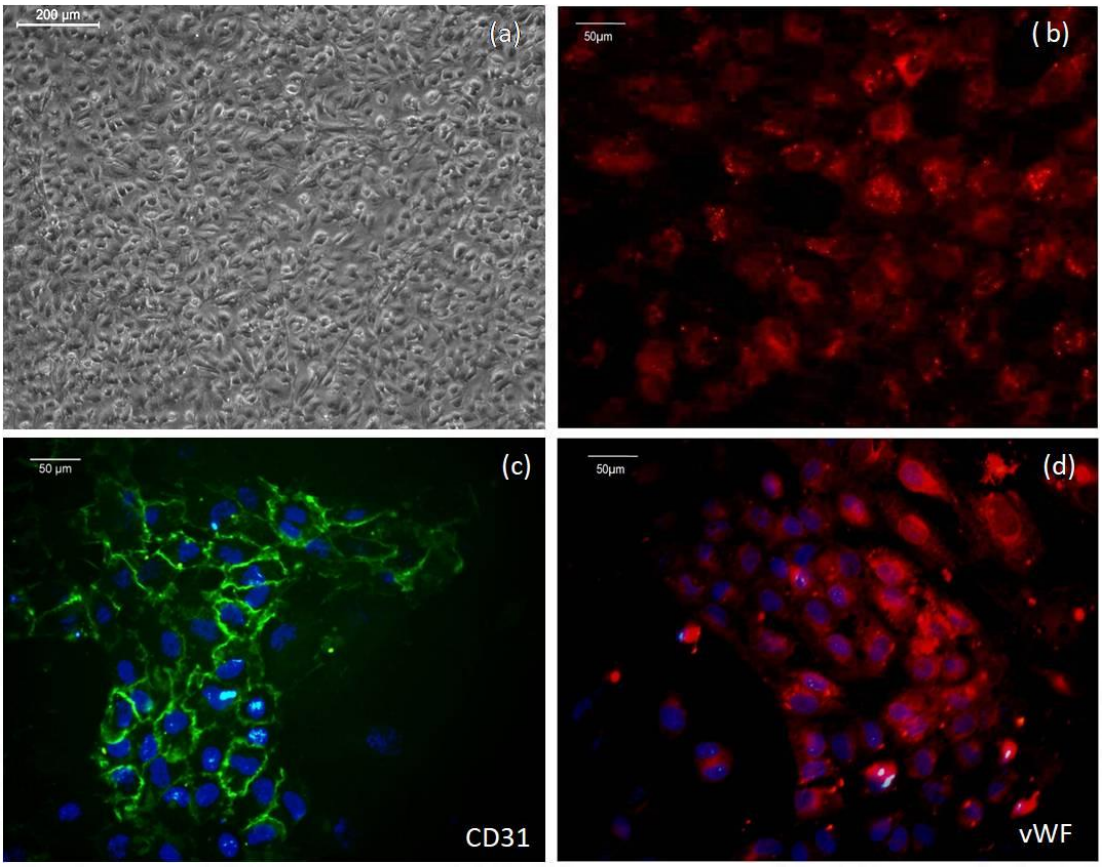


Figure 11: Images establishing characteristics of ECs.*(a) Confluent monolayer in 3rd passage showing cobble stone morphology (10X), (b) AcLDL taken up by ECs seen in red (40X), (c) Immuno stained green color of CD31 on EC membrane; blue-DAPI (40X) (d) immunostained vWF in red-vWF; blue-DAPI (40X)*

4.3 Cytoxic effect of CM on ECs

The ECs responded to CM-Alb in both dose-dependent and time-dependent manner. Exposure of CM-Alb conjugate to ECs at low concentrations (10 to 30µM) resulted in >90% viability (Fig 12a). However, upon exposure to concentrations > 50µM, significant reduction in cell viability was observed. There is significant difference in viability between 24h and 48h exposure at all concentrations; the latter producing more nonviable cells. On the other hand, ECs exposed to free CM, showed much lower cytotoxic effect, as compared to the effect of CM-Alb, at 10µM to 30µM concentrations (Fig 12b). At high concentrations (50 to 100µM) free CM also showed significant reduction in cell viability (6 to 66%). The cytotoxic effect was not remarkably or consistently upon increasing the exposure period with free CM from 24h to 48h.

The CM-Alb conjugate as 5µM, 10µM or 30µM did not cause any effect on EC proliferation (Fig 12c). More than 99% ECs were found to be proliferating even after incubating the conjugate for 24h with 10µM, 20µM and 30µM of CM in the conjugated form. Since the cell viability and proliferation were not affected significantly by 10µM, 20µM and 30µM of the conjugated CM, these concentrations may be used for testing the anti-inflammatory effect in ECs.

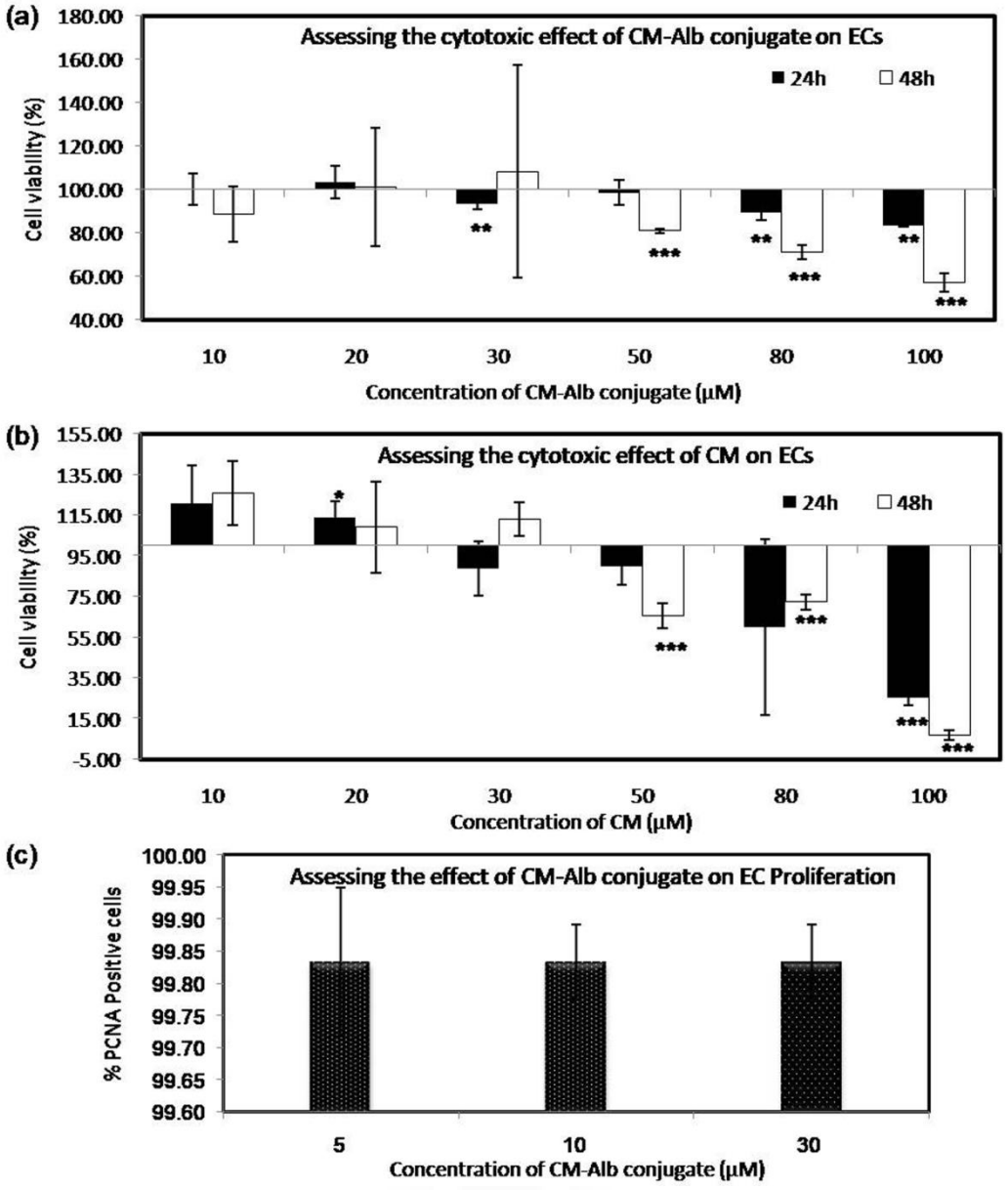


Figure 12: Graphical representation of cytotoxic response of EC to CM. *(a) Compiled data of exposure time-dependent dose reponse of CM-Alb on ECs; (b),*

compiled data of time dependent and dose response of free CM on ECs; (c)dose response of conjugated CM on EC proliferation quantified by flow cytometry of PCNA stained cells,after 24h incubation with the conjugate.The percentage viability, is represented relative to the untreated control cells considered as 100% viability. Values are represented as Mean±SD; n=3.P< 0.05 (*); P< 0.01 (**); P < 0.001 (***).

4.4 Endocytosis of CM-Alb conjugate by ECs

The endocytosis of FITC tagged Alb by ECs was clearly observed under fluorescence microscope (Fig 13a) in a similar way as seen in the case of endocytosis of FITC tagged CM-Alb conjugate (Fig 13b) upon incubation for 8h. Mean fluorescence intensity (MFI) quantifies significant uptake of both albumin and CM-Alb (Fig 13c). There was significant increase in the uptake of FITC tagged conjugate (> 150000 MFI) compared to uptake of FITC tagged native Albumin (>60000 MFI). Both the microscopic imaging and flow cytometry analysis clearly showed that the cells in culture have the ability to transport albumin to the cytoplasm.

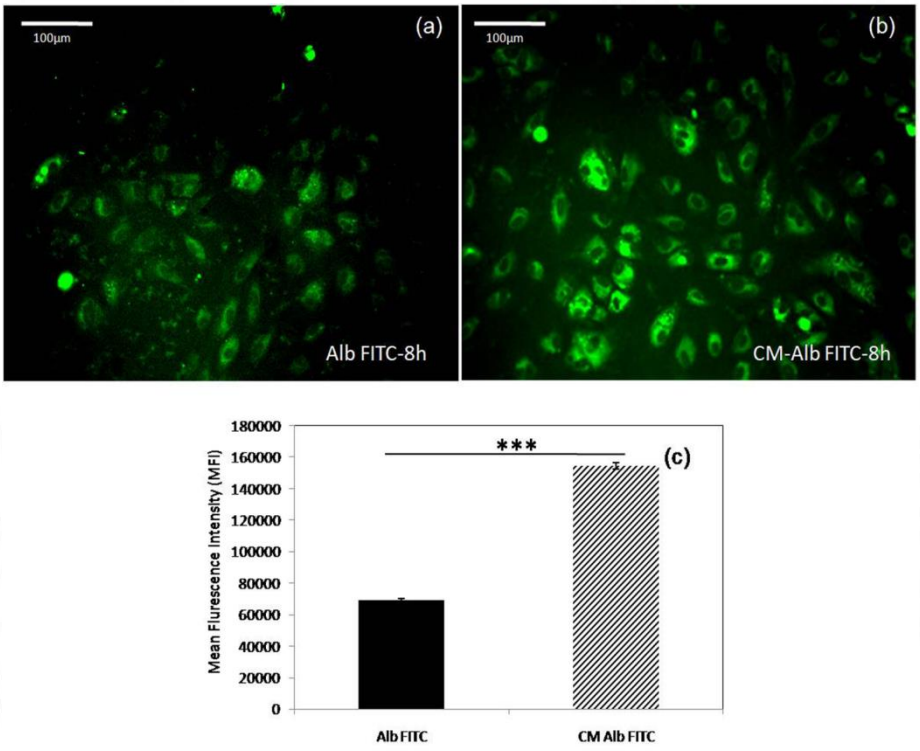


Figure 13: Data demonstrating endocytosis of CM-Alb conjugate by EC. *(a) fluorescence micrograph showing uptake of FITC tagged Alb (20X); and (b) fluorecence micrograph of FITC-tagged CM-Alb by ECs- both at 30µM concentration upon 8h incubation (20X); (c) Quantification of FITC conjugated albumin and CM-Alb conjugate based on MFI measured using flow cytometer.*

Values are represented as Mean±SD; n=3.P< 0.05 (*);P< 0.01 (**); P< 0.001 (***).

4.5 Inflammatory and anti-inflammatory responses in EC

4.5.1 Inflammatory response of EC to TNF-α, IL-6 and IL-1β

Cytokines TNF-α (2ng/ml) and IL-6 (10ng/ml), but not IL-1β (10ng/ml), incubated with ECs showed a time- and dose- dependent effect in the relative expressions of inflammatory markers (Fig 14). Also a higher dose of these inflammation inducers (TNF-α-4ng/ml), IL-6-20ng/ml), IL-1β-20ng/ml) were given for a period of 24h in order to standardize inflammatory conditions in ECs. Upon 8h(Fig 14a) induction with inflammatory inducer, TNF-α (2ng/ml) showed up-regulation of all the inflammatory markers (NF-kB, MCP-1, ET-1, COX-2, VCAM-1) compared to control (normal cells). However, compared to control there was no significant increase in gene expressions of any of the inflammatory markers. Upon 24h (Fig 14a) induction with 2ng/ml also, there was no upregulation of the inflammatory markers. But at a higher dose of TNFα (4ng/ml), upon 24h induction all the tested inflammatory markers in ECs were up-regulated. TNFα (4ng/ml) treatment resulted in significant increase in COX-2 (1.98) expression compared to control; MCP-1(6.71) and VCAM-1(6.61) showed highly significant increase in gene expressions relative to control. Upon IL-6 (10ng/ml) treatment for a period of 8h (Fig 14b), except ET-1, all other four inflammatory markers showed upregulation and in particular significant increase in gene expression was found for COX-2 (1.46). Upon 24h induction (Fig 14b) with the same concentration of IL-6, there was no upregulation observed in any of the inflammatory markers. Upon IL-6 (20ng/ml) treatment for a period of 24h (Fig 14b) showed upregulation in terms of gene expression of all the five inflammatory markers but compared to control, significant increase in gene expression was found only for NF-kB (1.82) and MCP-1 (1.34). Upon IL-1β (10ng/ml) treatment for a period of 8h (Fig 14c), it was found that there is no significant upregulation of any of the inflammatory markers in terms of gene expression. Upon 24h induction with IL-1β at same concentration, failed to upregulate the expression of inflammatory markers. Upon 24h induction with IL-1β (20ng/ml) also, all the inflammatory markers except ET-1 (0.67) and COX-2 (0.74) were found to be upregulated compared to control. However for IL-1β (20ng/ml)

treated ECs for a period of 24h, there was no significant increase in gene expression for the upregulated inflammatory markers. Therefore, based on time and dose dependent expression of inflammatory markers subjected to different concentrations of inflammatory inducers for different time periods, TNF-α was found to be contributing significantly to the over expression of inflammatory markers (MCP-1, COX-2 and VCAM-1) upon 24h incubation with ECs. This in turn makes TNF-α, a suitable inflammatory inducer for further studies.

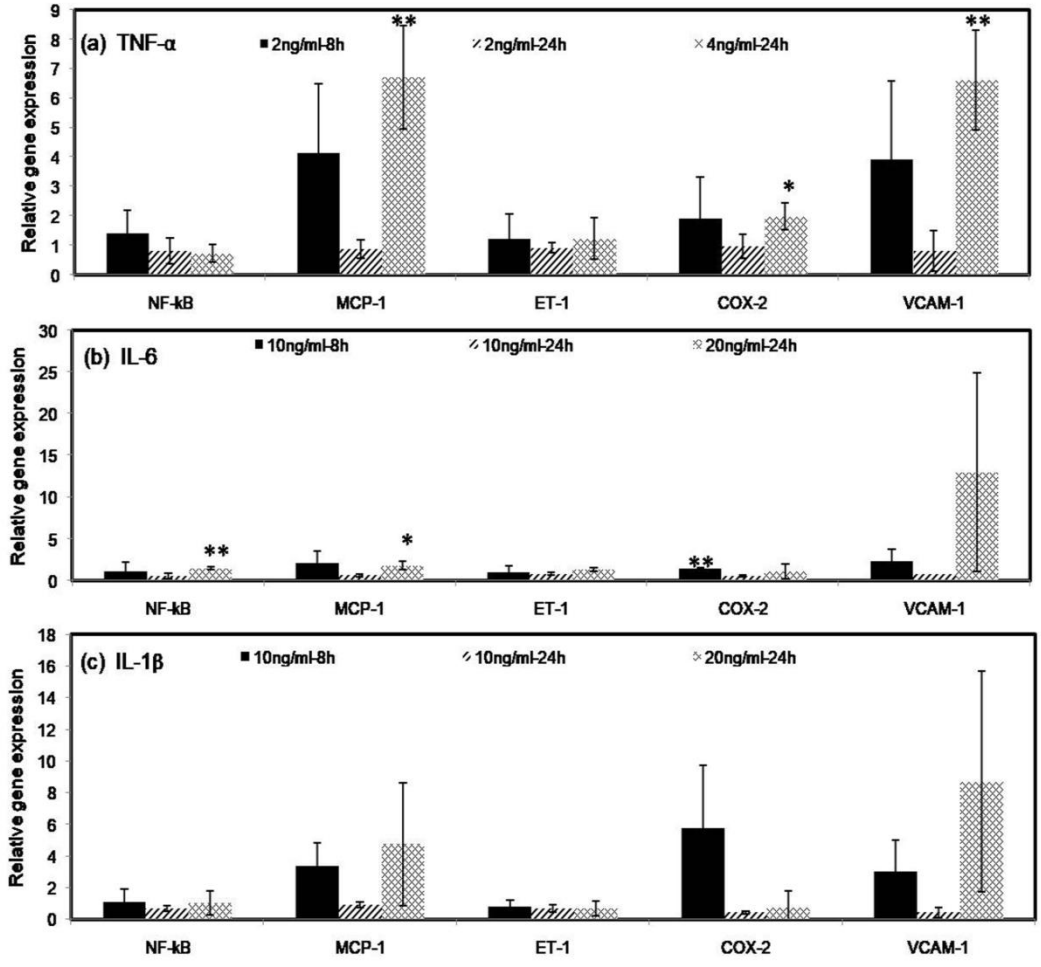


Figure 14: Effect of Cytokines on relative expressions of inflammatory markers in ECs.*(a) Response to low dose of TNF-α (2ng/ml) exposure with ECs for a period of 8h, 24h and high dose (4ng/ml) for a period of 24h; (b)Response to low dose of IL-6 (10ng/ml) exposure with ECs for a period of 8h, 24h and high dose (20ng/ml)for a period of 24h;(c)Response to low dose IL-1β (10ng/ml) exposure with EC for a period of 8h, 24h and high dose (20ng/ml)for a period of 24hfor 2 different time periods 8h and 24h respectively. Values are represented as Mean±SD; n=3.P < 0.05 (*); P < 0.01 (**); P < 0.001 (***).Fold change is quantified relative to GAPDH*

expression using the $2^{-\Delta\Delta Ct}$ method, upon normalization with EC grown in normal culture conditions.

4.5.2 Effect of CM-Alb on inflammation induced ECs

The anti-inflammatory effects of CM-Alb conjugate on ECs induced with TNF-α ($10ngml^{-1}$), treated for a period of 24h are represented graphically (Fig 15). The expressions of NF-kB (Fig 15a), MCP-1 (Fig 15b), ET-1 (Fig 15c) and VCAM-1 (Fig 15e) were found to be significantly reduced when treated with CM-Alb conjugate at 5, 10, 20µM concentrations whereas at 30 and 60µM, most of the markers were up-regulated. In case of COX-2, even though the gene expression levels were found to be lowered compared to the TNF-α treated control, there was no significant downregulation observed. For NF-kB, MCP-1 and ET-1, CM-Alb and free CM at 30µM and 60µM showed a slight increase in gene expressions of inflammatory markers compared to their corresponding lower doses (Fig 15a to 15c). For ET-1 and VCAM-1, free CM at lower doses showed significant reduction in gene expression whereas the level of significance was not so much observed for NF-kB and MCP-1. However, for COX-2 (Fig 15d), both conjugate and free CM showed no significant effect in terms of lowering the expression at lower and higher doses. These results show that CM-Alb at lower doses (5 to 20µM) are able to down-regulate the expressions of inflammatory markers whereas owing to its cytotoxic effect, at higher concentrations (30 and 60µM), the inflammatory regulation is not much evident. Free CM also controlled the expression of inflammatory markers but the effect of CM-Alb seems to be higher.

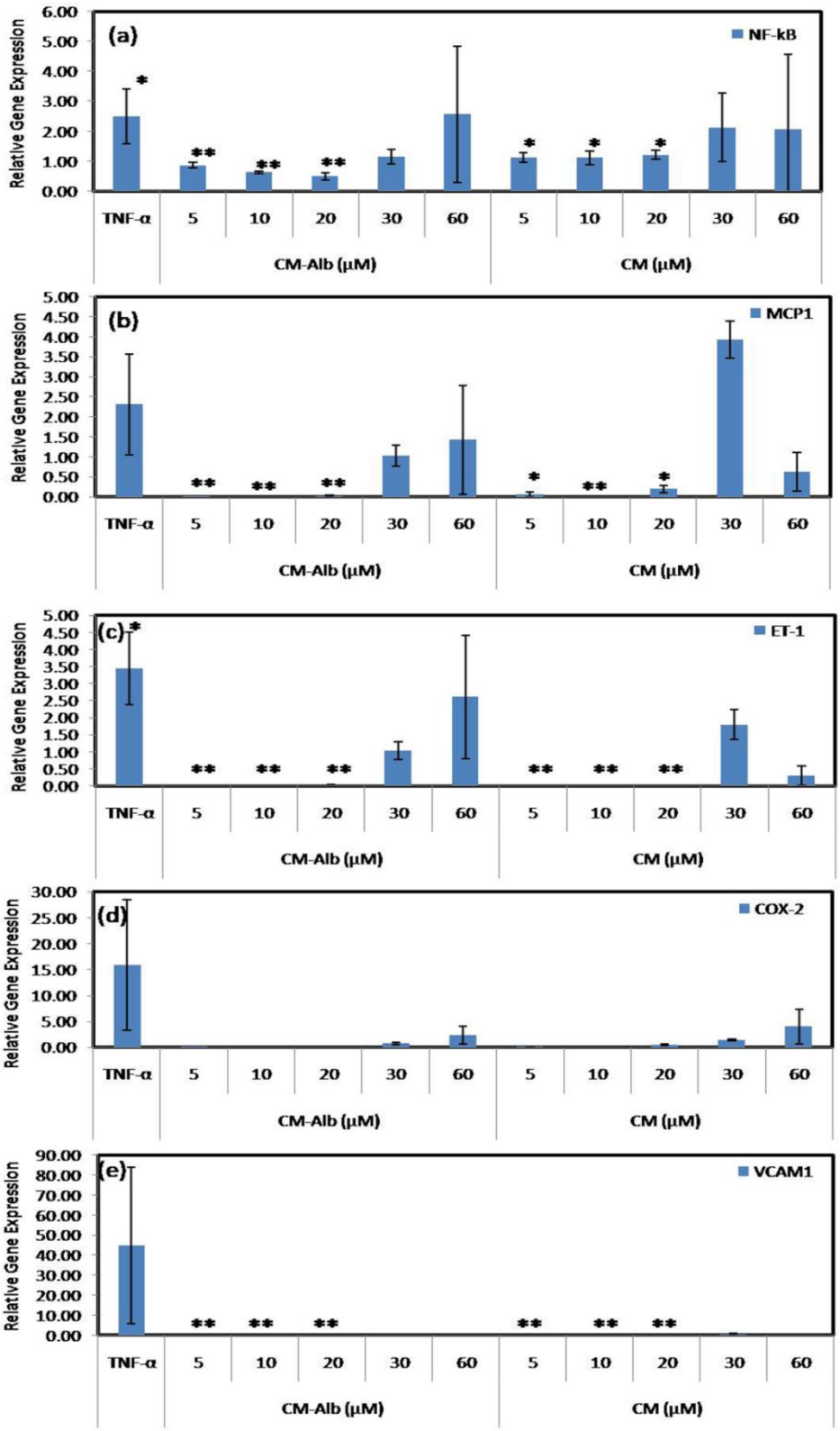


Figure 15: Effect of CM on inflammatory markers in EC at mRNA level. *Relative gene expression w.r.t control EC and EC treated with TNF-1α(10ng ml^{-1}) for*

24h,estimated using qRTPCR for demonstrating effect of CM-Alb and free CM on(a) NF-kB; (b) MCP-1; (c) ET-1; (d) COX-2; and (e)VCAM-1 expressions. Data represent dose response of CM-Alb upon treating with EC preexposed to TNF-1α for 24h, followed by incubation with added conjugate at different cocentrations for additional 24h. Values are represented as Mean±SD; n=3.P<0.05 (*); P< 0.01 (**); P < 0.001 (***).Fold change is quantified relative to GAPDH expression using the $2^{-\Delta\Delta Ct}$ *method, upon normalization with EC grown in normal culture conditions.*

4.6 Expression of inflammatory markers at protein level

Confirming the results seen in qRTPCR, activated ECs immunostatined positive for all the four inflammatory markers NF-kB, ET-1, COX-2 and VCAM-1 (Fig 16a to d). It is seen that control EC, CM-treated and CM-Alb treated ECs were stained positive for inflammatory markers. Upon quantification of fluorescence intensity TNF-α activated ECs showed significantly higher expression of markers NF-kB, ET-1 and COX-2 compared to control ECs (Fig 16f). Also, it is seen that on treating the TNF-α treated ECs with CM-Alb and CM, the frequency/intensity immunostained EC has decreased considerably. The finding in immunostaining is confirmed in Western Blot analysis; specific bands for ET-1 (24kDa), COX-2 (above 100kDa),NF-kB (60Kda) and VCAM-1 (100kDa) can be seen (Fig 16e). The respective protein band intensity is higher in TNF-α activated ECs as compared to untreated control EC and the CM/CM-Alb treated cells. The protein down-regulation was detected by treating TNF-α activated ECs with 5uM CM-Alb/ free CM only because the RT-PCR results was promising at this dose.

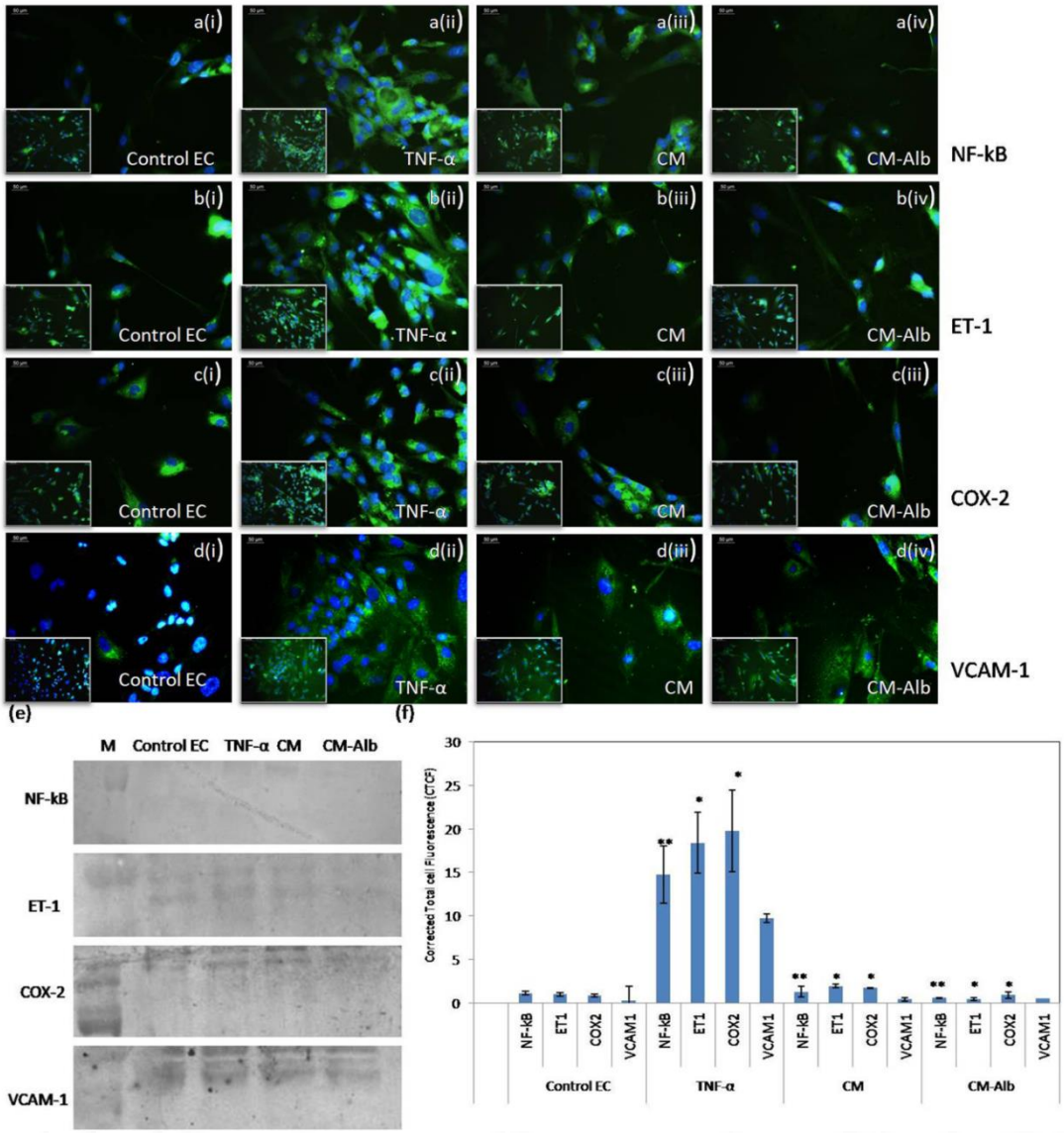


Figure 16: Effect of CM on inflammatory markers in EC at protein level.*(i) Normal EC culture (control); (ii) EC treated with TNF-α; (iii) EC treated with TNF-α+CM; (iv) cells treated with TNF-α+CM-Alb. a (i-iv), cells stained for NF-kB; b(i-iv) cells stained for ET-1; c(i-iv) cells stained for COX-2; d(i-iv) cells stained for VCAM-1. Green fluorescence represents cell cytoplasm and nucleus is represented by blue colour stained with DAPI. (e) Western blot images for NF-kB (60kDa), ET-1 (24kDa), COX-2 (above 100kDa) and VCAM-1 (100kDa); Lane 1-M-Marker, Lane 2-Protein lysate from medium treated cells, Lane 3-protein lysate from TNF-α treated cells, Lane 4-protein lysate from TNF-α+CM treated cells, Lane 5-protein lysate from TNF-α+CM-Alb treated cells. (f) quantified inflammatory marker expression in ECs upon different treatment conditions.*

4.7 Characteristics of isolated hDAMSCs

The isolated hADMSCs were characterised for their stem cell properties by flow cytometry and trilineage differentiation (Fig 17). Upon analyis, it was found that >90% cells in the culture were positive for the MSC markers CD105, CD90 and >36% positive for the MSC marker CD73. Only <2% cells were positive for HSC markers. Thus the mesenchymal nature of the grown stem cells was confirmed. Furthermore, the isolated cells upon inducion with specific media for chondrogenic lineage, adipogenic lineage and osteogenic lineage showed positive staining. The hADMSCs differentiated into chondrogenic lineage showed the presence of purple coloured proteoglycans which are released by chondrocytes confirming the chondrogenic differentiation (Fig 17f).The cells differentiated into adipogenic lineage showed presence of red coloured triglycerides confirming upon staining with Oil Red O confirming adipogenic lineage (Fig 17g). The cells differentiated into osteogenic lineage showed the presence of red calcium deposits confirming the osteogenic differentiation (Fig 17h). The positivity in MSC markers and trilineage differentiation proves the stemness of isolated hADMSCs.

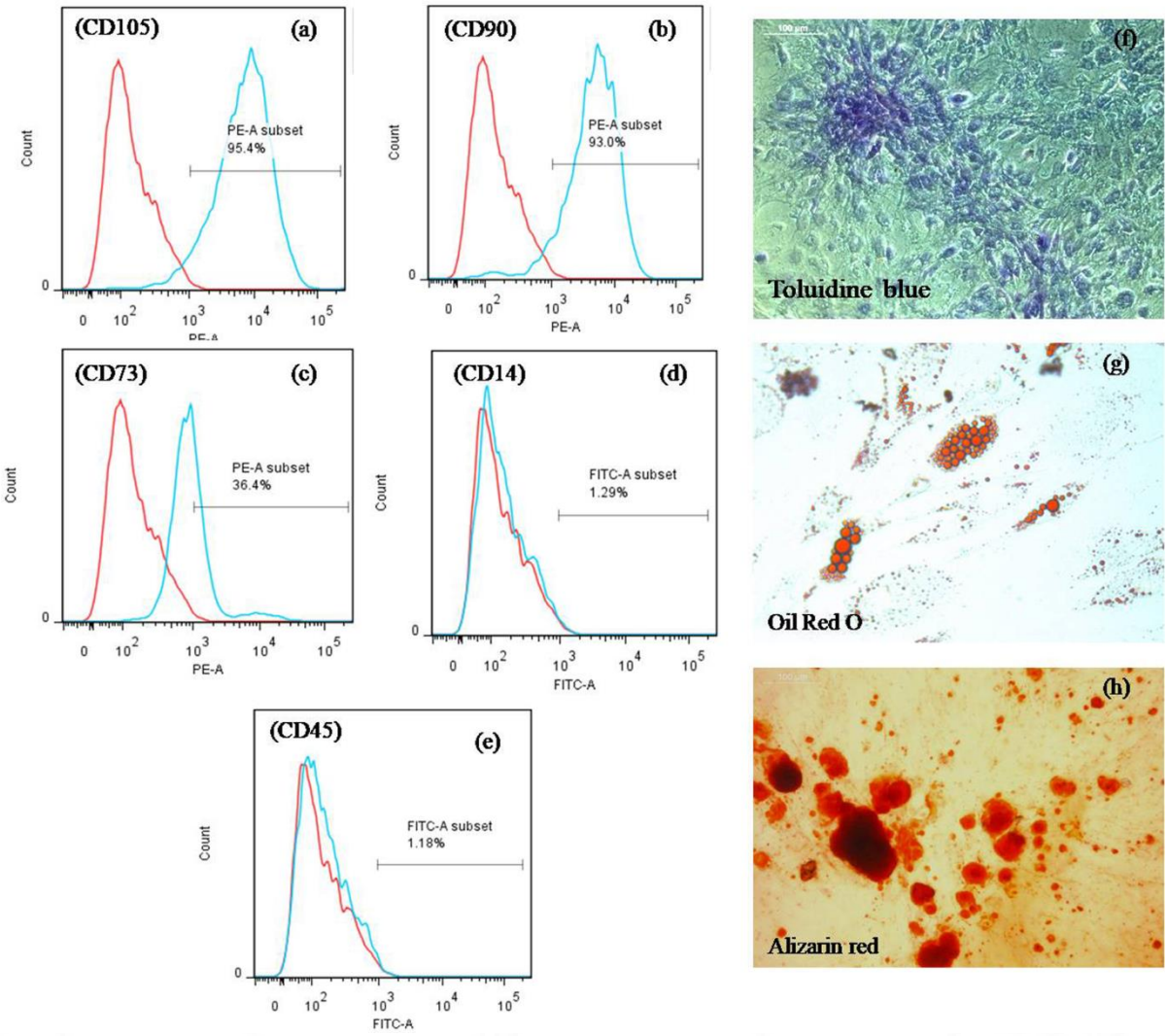


Figure 17: Characteristic CD marker expressions and multipotency ofhADMSCs.*Quantified surface marker expressions of hADMSCs by flow cytometry: (a) CD105,(b) CD90 & (c) CD73 represent Mesenchymal stem cell (MSC) markers; (d) CD14 & (e) CD45 represent Haematopoietic stem cell (HSC) markers. Bright fluorescent images represent trilinegae differentiation corresponding to (f) Chondrogenic differentiation stained with Toluidine blue (20X), (g) adipogenic differentiation stained with Oil Red O (20X)& (h) osteogenic differentiation stained with Alizarin red (20X).*

4.8. Characteristics of ChCs

Spindle shaped hADMSCs can be seen in Fig 18a. These cells when subjected to induction with chondrogenic differentiation medium resulted in chondrocytes (ChCs) with typical rounded morphology which is evident from Fig 18b & c. These differentiated cells were again confirmed for their chondrogenic nature by immunostaining with Type II Collagen. Fig 19e & f shows positivity for type II

collagen (green fluorescence) which can be seen as depositions in the form of extracellular matrix secreted from the cells, at the same time the non-differentiated cells (Fig 18d) were hardly stained positive.

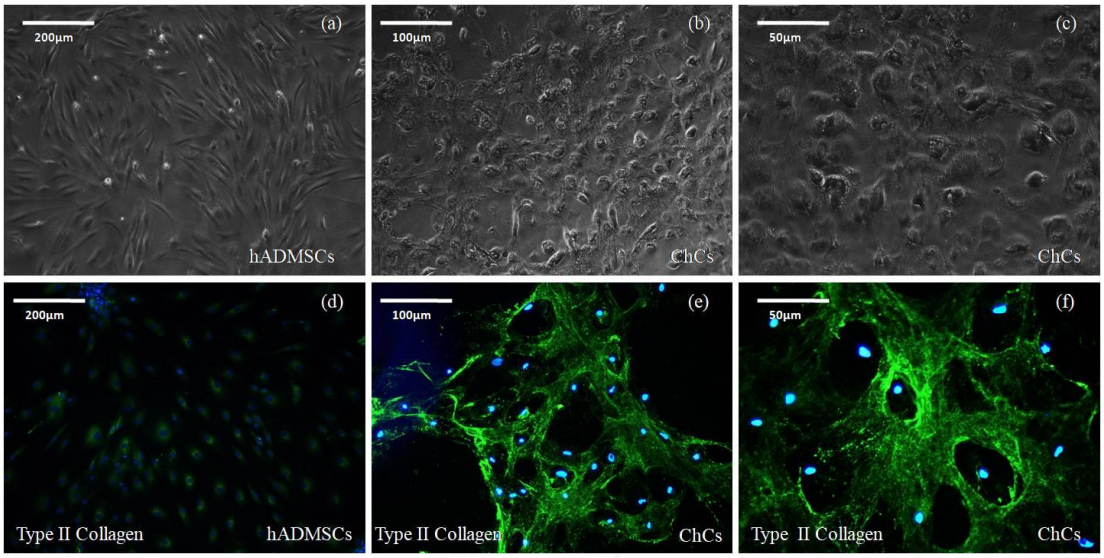


Figure 18:Images proving differentiation of hADMSCs into chondrocytes. *(a),Micrographs of isolated hADMSCs showing their typical spindle shaped morphology (10X); (b)&(c),Morphology of hADMSCs differentiated to ChCs (20X&40X respectively); (d), Type II Collagen immunostainedcontrol hADMSCs (10X); (e) & (f),micrographs of Type II Collagen immunostained ChCs (20X&40X respectively;green-Type II Collagen; blue-DAPI.*

4.9 Cytotoxic response of ChC to CM-Alb conjugate

The ChCs responded to CM-Alb in both dose-dependent and time-dependent manner (Fig 19a). Upon 24h exposure of CM-Alb conjugate to ChCs at a range of concentrations, (5 to 100µM), the ChCs showed cell viability above 60% with highly significant reduction in cell viability at 100µM concentration showing highest toxicity to the cells. However, free CM at concentration of 10 to 100µM, showed viability in the range of 40 to 60% only on treatment for a period of 24h with significant reductions observed at 30 & 50µM. Upon 48h exposure of conjugate to the ChCs, there was above 57 to 70% cell viability observed with highly significant reduction at 100µM as compared to 10µM. When ChCs were exposed to free CM for a period of 48h, all concentrations (10 to 100µM) showed significant reduction in cell viability (51 to 66%). Even though, upon 48h exposure, there was not much difference observed in cell viability on comparison between conjugate treated and

free CM treated, the reduction in cell viability in terms of significance was very high in free CM treated compared to the control cells for concentrations 10 to 100µM.

The cell proliferation assay showed that varying concentrations (lower doses) of conjugate (5, 10µM) did not affect the cell proliferation significantly (Fig 19c). Cell proliferation in ChCs was found to be around 18% after incubating the cells with conjugate for 24h irrespective of the concentration of conjugate. Thus it shows that concentration of conjugate as low as 5 to 10µM have no much influence on cell proliferation in ChCs.

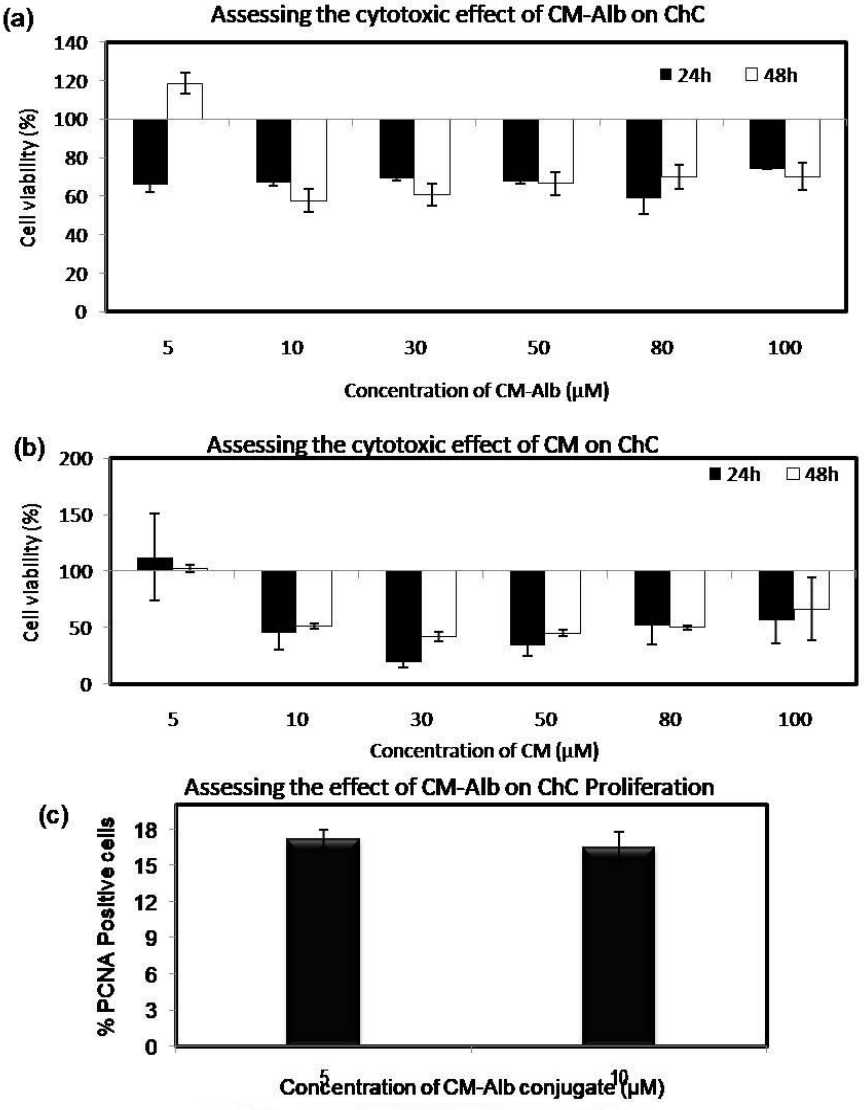


Figure 19: Graphical representation of cytotoxic response of ChC to CM*. (a) & (b) cell viability assay in ChCs treated with CM-Alb and free CM for different time periods 24h and 48h respectively; (c) proliferation assay by flow cytometric analysis*

to determine the effect of conjugate at 5 & 10µM concentrations upon 24h incubation with the conjugate. Values are represented as Mean±SD; n=3.P < 0.05 (*); P < 0.01 (**); P < 0.001 (***).

4.10. Endocytosis of CM-Alb into ChCs

The endocytosis of FITC tagged Alb and FITC tagged CM-Alb conjugate into ChCs can be observed from the microscopic images (Fig 20 a & b respectively) upon incubation for 8h. The quantified mean fluorescence intensity (MFI) confirmed uptake of both Alb and CM-Alb (Fig 20c). The uptake of FITC tagged conjugate (>50000 MFI) was low as compared to uptake of FITC tagged Alb (> 150000 MFI).Both the microscopic image and quantified MFI showed that the cells have the tendency to uptake albumin *in vitro* which can be closely correlated to specific interactions of Alb with its receptors present on the cell surface.

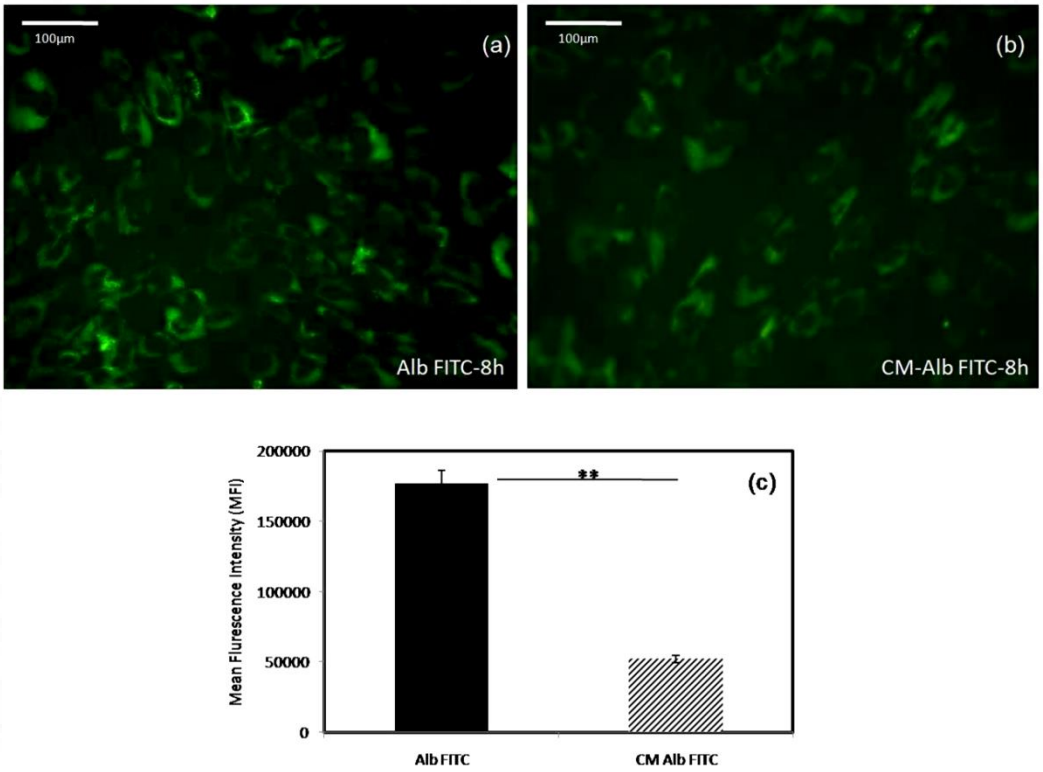


Figure 20: Illustration of endocytosis of CM-Alb conjugate into ChC. *Fluorescence micrographs representing the endocytosis of (a) FITC tagged native Alb (20X); (b), FITC-tagged CM-Alb conjugate by ChCs incubated with 30 µM concentration for 8h (20X); (c) compiled data on quatified MFI of endocytosis;values are represented as Mean±SD; n=3.P < 0.05 (*); P < 0.01 (**); P < 0.001 (***).*

4.11. Response of ChC to inflammatory cytokines

4.11.1 Inflammatory marker expression in ChCs upon induction with TNF-α

The expression of inflammatoary markers in ChCs upon induction with TNF-α for a period of 24h showed dose dependency (Fig 21). All the four inflammatory markers (MMP13, COX-2, NF-kB, IL-8) were up-regulated when treated with all the three concentrations of TNF-α, as compared to control cells grown in normal medium. However, two markers namely NF-kB and IL-8showed significant increase in fold change in terms of relative gene expressions. Upon treatment with 2ng/ml of TNF-α (Fig 21a), significant up-regulation in terms of relative gene expression was observed for NF-kB (15.83) and IL-8 (6.24). Upon treatment with 4ng/ml of TNF-α (Fig 21b), up-regulation in terms of relative gene expression observed for NF-kB was (13.2). Upon treatment with 10ng/ml of TNF-α (Fig 21c), relative gene expression was up-regulation highly significant for NF-kB (80.17) and IL-8 (50.92). So based on these, since the fold changes for NF-kB and IL-8 was found to be significantly high when treated with 10ng/ml of TNF-α, the same was used for further studies.

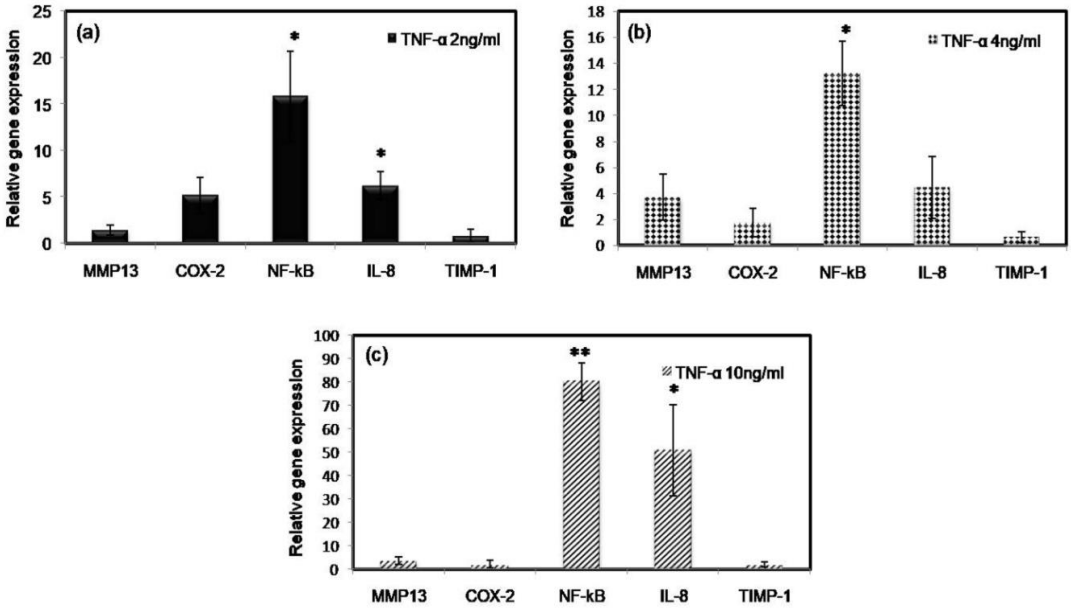


Figure 21: Graphical representation of the dose dependent effect of TNF-α in ChCs*. The concentrations of TNF-α added into the culture medium were (a),2ng/ml ;(b), 4ng/ml;(c), 10ng/ml and incubated for a period of 24h.Values are represented as Mean±SD; n=3.P < 0.05 (*); P < 0.01 (**); P < 0.001 (***). Fold change is*

quantified relative to GAPDH expression using the $2^{-\Delta\Delta Ct}$ *method, upon normalization with ChC grown in normal culture conditions.*

4.11.2 Effect ofCM-Alb conjugate in TNF-α treated ChCs

Effect of CM-Alb conjugate and free CM was determined using qRT-PCR in terms of relative gene expressions of inflammatory markers (Fig 22). The expression of MMP-13 (Fig 22a), was found to be reduced at all treatment conditions. However, when treated with CM-Alb at concentration of 7.5 & 15μM significant reduction in relative gene expression of around 0.08 & 0.02 respectively. Upon treatment with free CM, at concentration of 7.5μM there was significant reduction (0.16) in MMP-13 expression. In case of COX-2 (Fig 22b), there was reduction in gene expression in all treatment groups especially when treated with CM-Alb conjugate at concentration of 7.5 & 15μM compared to their corresponding doses of free CM. In case of NF-kB (Fig 22c), when treated with CM-Alb, there was down-regulation observed at concentrations of 15μM, compared to free CM at the same concentration. In case of IL-8 (Fig 22d), CM-Alb at concentration of 15μM (0.2) showed significant reduction in gene expression compared to free CM. In case of TIMP-1, CM-Alb conjugate at concentration of 7.5μM showed significant upregulation (34) in terms of relative gene expression. Free CM at concentrations of 7.5 & 15μM also showed significant upregulation for TIMP-1. For all the inflammatory markers except IL-8, free CM was found to upregulate the expression compared to their corresponding doses of CM-Alb.

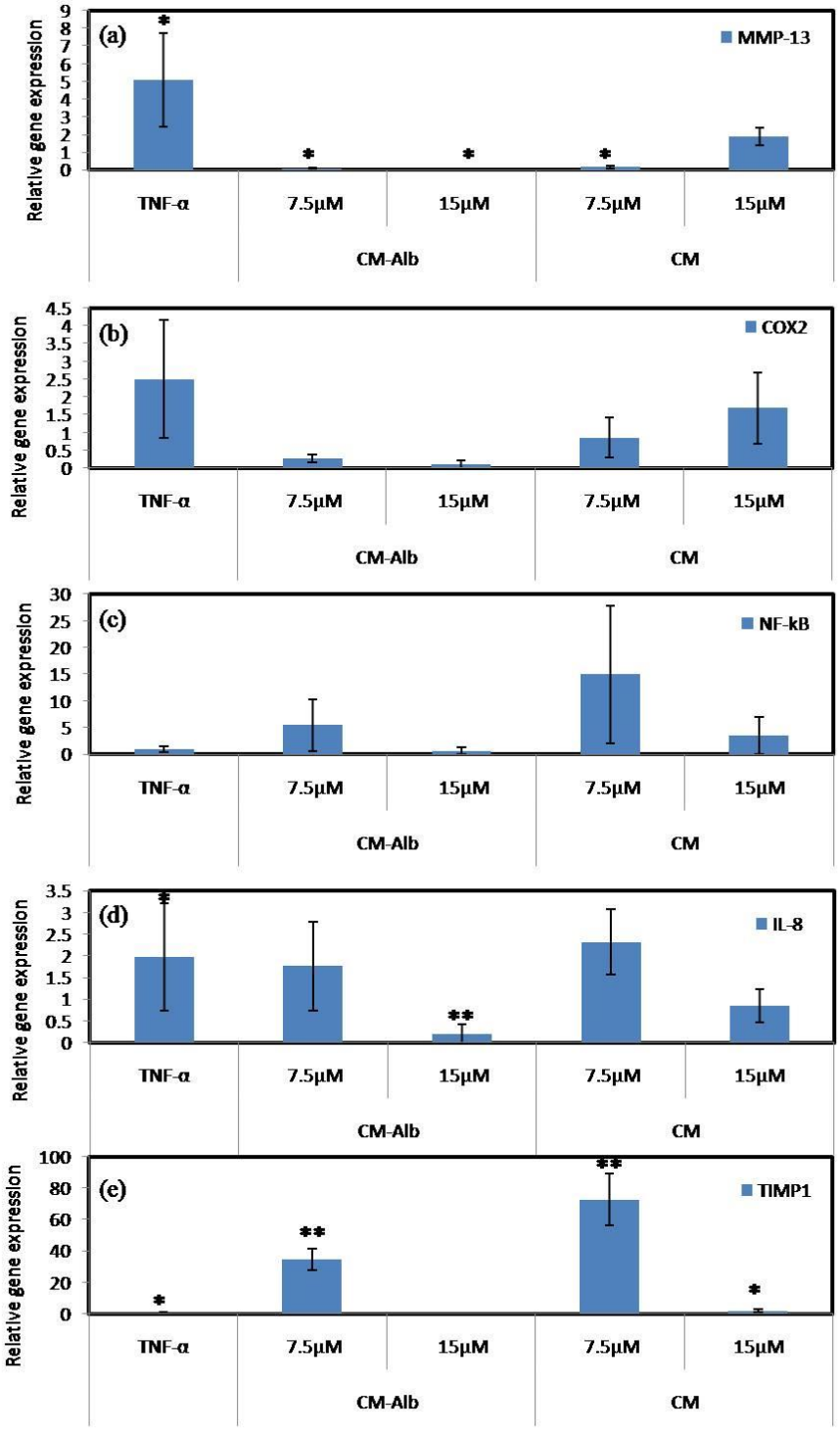


Figure 22: Effect of CM on inflammation induced ChC*. Graphical representation of relative gene expressions demonstrating the dose response of CM-Alb and free CM in 10ng/ml TNF-α treated ChCs on inflammatory markers; (a) MMP-13; (b) COX-2;(c) NF-kB; (d) IL-8;and (e)TIMP-1. Values are represented as Mean±SD; n=3.P < 0.05 (*); P < 0.01 (**); P < 0.001 (***).Fold change is quantified relative*

to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method, upon normalization with ChC grown in normal culture conditions.

4.12. Proving inflammation model in rabbit knee joint

The protocol followed (as mentioned in section 3.15.3) for induction of inflammation in rabbit knee joint using TNF-α is represented in Fig 23 a to d. The animals upon administration of TNF-α, there were no immediate symptoms observed. However, when the animals were observed for any symptoms after a period of 24h, there was a slight rise in temperature near the surface of knee compared to rest of the body parts. A mild level of swelling was also seen adjacent to the knee joint which might be due to edema formation. Upon touching the knees, the animals were showing a tendency to withdraw their legs backward which can be correlated with pain associated with the inflamed joints.

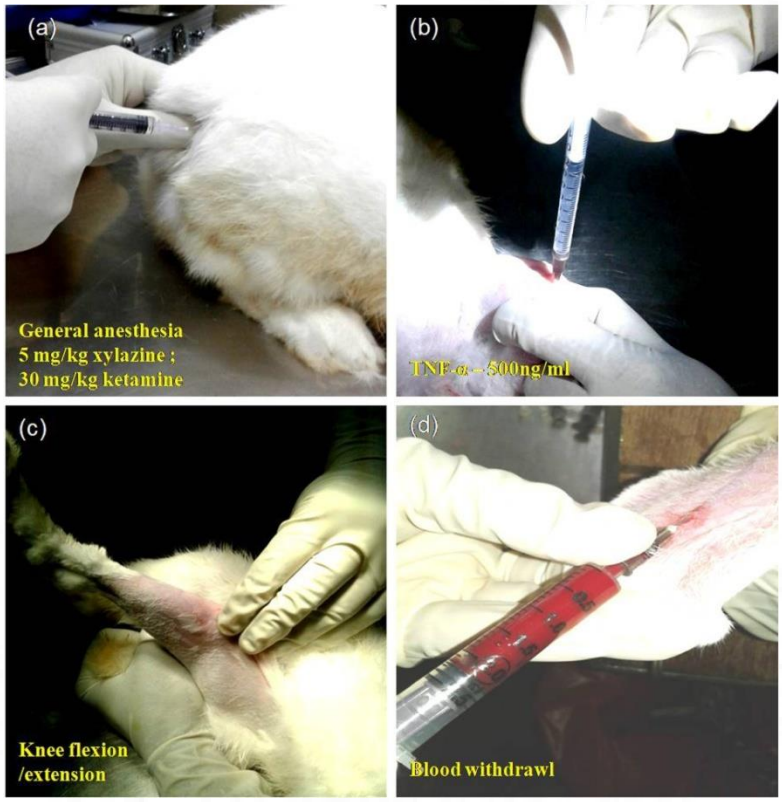


Figure 23: Representative images illustrating the experiment*. (a) to (d; in vivo induction of inflammation in rabbit knee joints (a) administration of general anesthesia; (b) administartion of TNF-α into the synovial cavity (c) flexion and*

extension movement of knee joint post administration (d) blood withdrawl from rabbit ear vein.

H& E stained images of knee joints are represented in Fig 24 a to i. Normal control (NC) showed columnar arrangement of chondrocytes (Fig 24 a to c). There was no difference observed in collagenase treated animal, the cartilage was normal without any abnormalities in terms of cellular arrangement and observed intact membrane covering (Fig 24 d to f).The stained sections of TNF-α injected sites showed chondrocytes scattered throughout the matrix (Fig 24 g to i), with disrupted outer membrane of cartilage (Fig 24h), infiltration of lymphocytes in large quantities (Fig 24i) and isolated islands of diapedesis (Fig 24i). Based on the histological assessment, for further experiments, for inducing inflammation in rabbit knee joints, TNF-α (500ng/ml) was used.

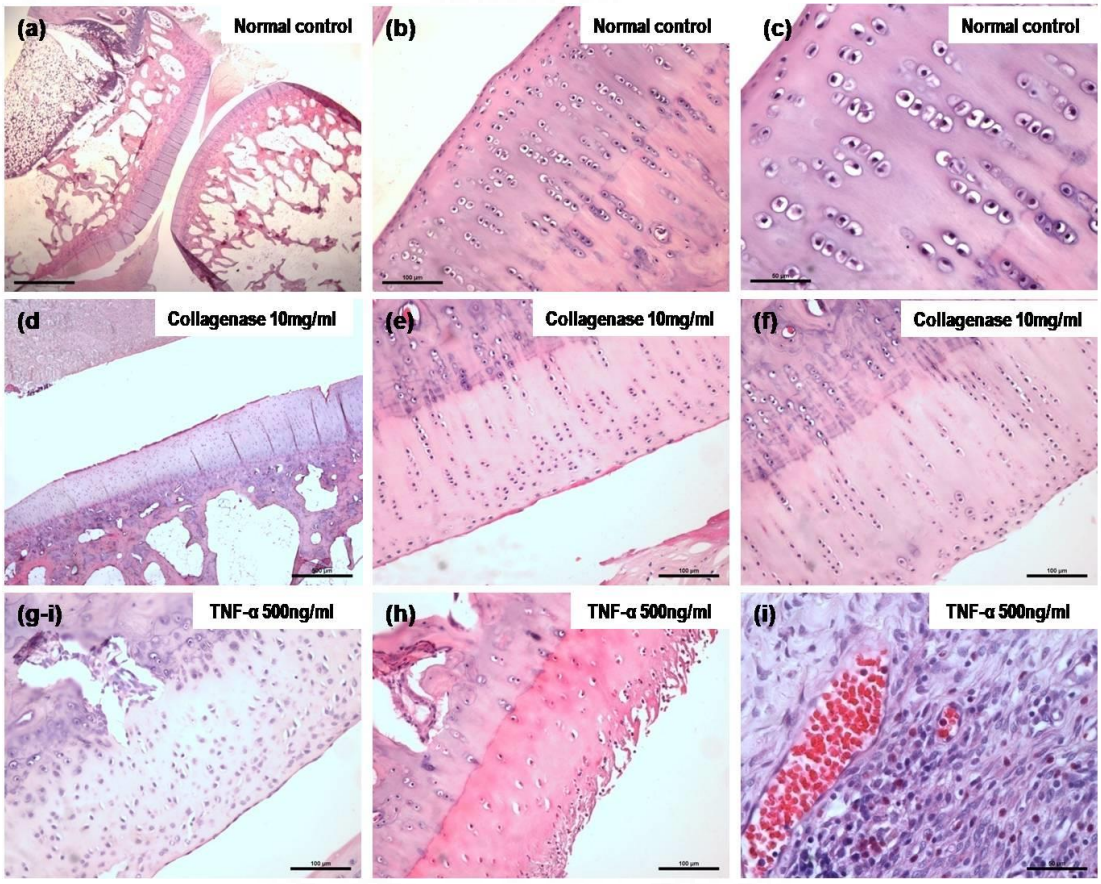


Figure 24: *Representative images of H&E stained sections of knee cartilage (a to c) Normal Control (NC); (d to f) Induced Control (IC) treated with collagenase; (g to i) Induced Control (IC)– treated with TNF-α.*

4.13. Preparation of rChCs for transplantation

The isolated rADMSCs were characterised for their stem cell properties by trilineage differentiation (Fig 25). The isolated cells upon inducion with specific media for osteogenic lineage, adipogenic lineage and chondrogenic lineage showed positive staining. The cells differentiated into osteogenic lineage showed the presence of red calcium deposits confirming the osteogenic differentiation (Fig 25a). The cells differentiated into adipogenic lineage showed presence of red coloured triglycerides confirming upon staining with Oil Red O confirming adipogenic lineage (Fig 25b). The rADMSCs differentiated into chondrogenic lineage showed the presence of purple coloured proteoglycans which are released by chondrocyes confirming the chondrogenic differentiation (Fig 25c). The positivity in trilineage differentiation proves the stemness of isolated rADMSCs.

Spindle shaped rADMSCs can be seen in Fig 25d. These cells when subjected to induction with chondrogenic differentiation medium resulted in cells (rChCs) with typical rounded morphology which can be seen in Fig 25e&f. Therefore, the toluidine blue staining along with the rounded morphology confirms the chondrogenic differentiation of isolated rADMSCs. The staining showed that rADMSCs (Fig 25g) and differentiated rChCs (Fig 25h) are positive for PKH26 dye and this confirms that these cells are able to uptake the dye

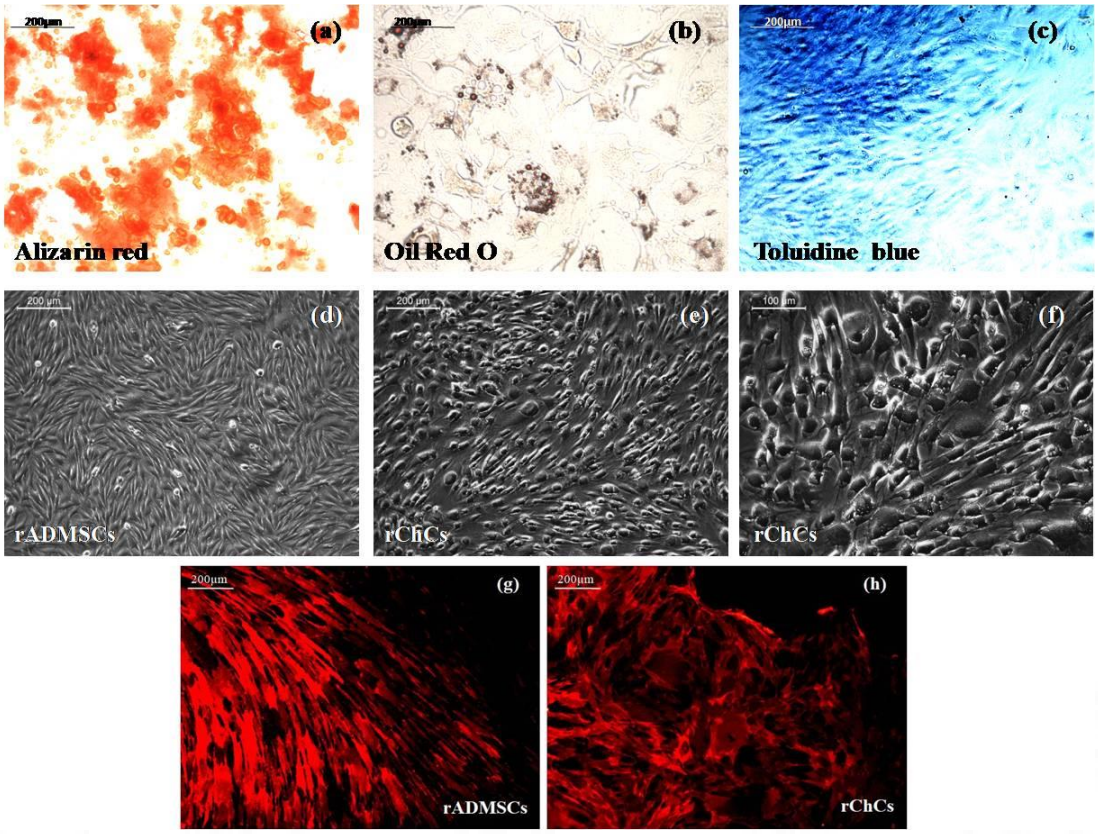


Figure 25: *Micrographs depicting properties of cells prepared for transplantation (a)-(c) Confirming multipotency of rADMSCs by osteogenic, adipogenic and chondogenic differentiation; (d) rADMSCs; (e);& (f) rChCs differentiated from rADMSCs. (g)PKH26 stainedrADMSCs (10X); and (h) rChCs stained with pkh26 (10X).*

4.14 Comparison of inflammatory response in tissues

4.14.1 Gross morphological analysis

Upon sacrifice of the animals on termination day, the appearance of knees was evaluated with naked eyes (Fig 26). There were no visible changes on the surface of knee in any of the sites. There were no diseased alterations observed on the surface after exposure of cartilage. There were no distinguishable differences among the different treatment group's tissues. So, TNF-α induced inflammatory changes may not have persisted till 35th day. There was no sacrifice done before 35th day; therefore, it is not possible to conclude if there was no visible change at all or if the change subsided with time.

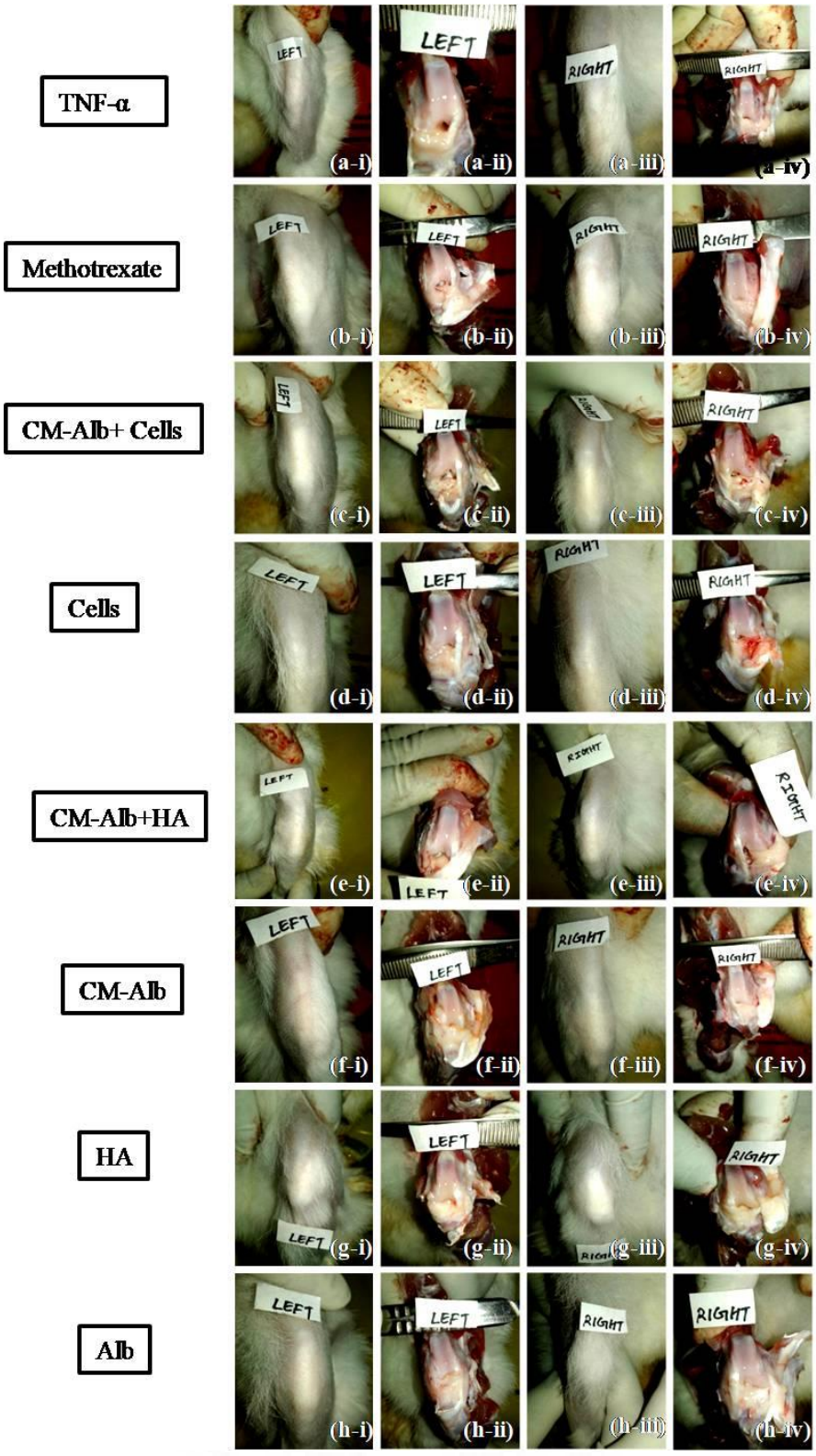


Figure 26: Representative gross images of knee joints from each experimental group. *The skin covered knee joints and carilage exposed joints are shown from bothe left and right side of the same animal. (a, i-iv) TNF-α-induced ; (b, i-iv)TNF-*

α-induced&Methotrexate-treated; (c, i-iv)TNF-α-induced &CM-Alb+ChC-treated; (d, i-iv) TNF-α-induced &ChC-treated; (e, i-iv)TNF-α-induced& CM-Alb+HA-treated; (f, i-iv) TNF-α-induced &CM-Alb-treated; (g, i-iv) TNF-α-induced &HA-treated;(h, i-iv) andTNF-α-induced&Alb-treated respectively. Images in column (i) and (iii) represent superficial observation on the skin for clinical symptoms; column (ii) and (iv) reprresent corresponding images after removing the overlying skin exposing the cartilege tissue.(n=6).

4.14.2. Proving the survival of transplanted cells in the joints

The *in vivo* imaging of knee joints injected with PKH26showed the presence of tagged cells (Fig 27). The yellow colour signifies the presence of highly intense region locating PKH26 tagged cells. Autofluorescence is seen as dark red colour in the controls. Therefore, the *in vivo* imaging suggests that rChCs are retained in the joints and the cells have migrated from the site of injection.

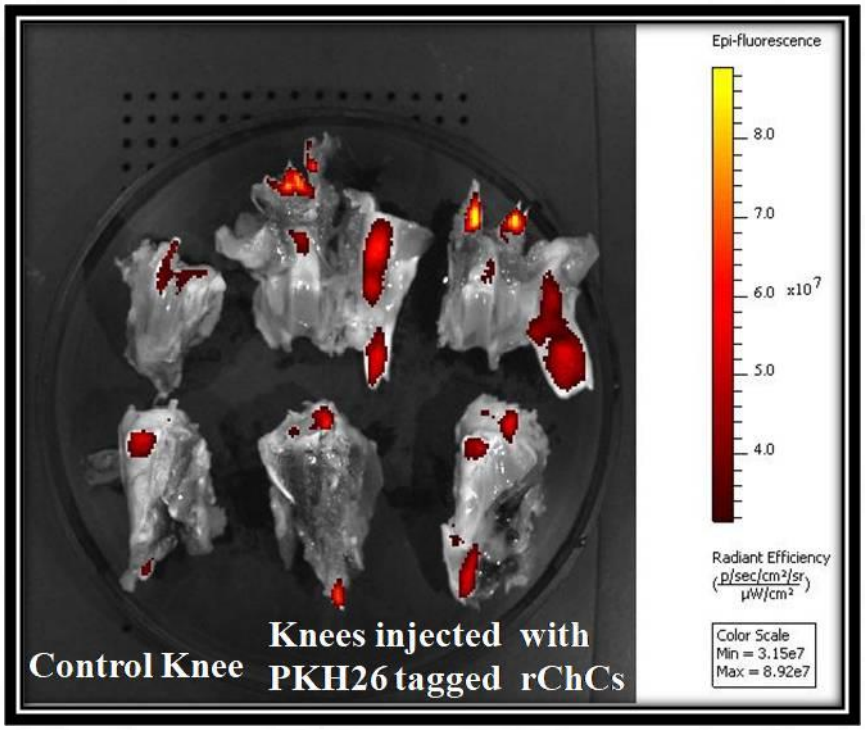


Figure 27: IVIS images of knee joints injected with PKH26 tagged rChCs*. Representative images of control (without cells) and treated knee joints (administered with PKH26 tagged cells).*

4.14.3. In vivo regulation of inflammatory markers

Upon induction with TNF-α, MMP-13 (Fig 28a) and IL-8 (Fig 28c) were found to be upregulated *in vivo*. The MMP-13 expression was significnatly increased upon TNF-α induction compared to the control group. All the treatment groups

(Methotrexate, CM-Alb+cells, cells, CM-Alb+HA, CM-Alb and Alb) except HA treatedtissues showed significnat reduction in the relative gene expression of MMP-13. Of which the lowest fold change was found to be for the tissues that recieved methotrexate treatment (0.03), followed by cells (0.182), CM-Alb+HA (0.183), CM-Alb+cells (0.49), Alb (0.51), CM-Alb (0.55) and HA (2.78). IL-8 was downregulated in all treated tissues as compared to TNF-α induced tissues. There was no upregulation COX-2 in the TNF-α induced tissues or other treated tissues except in the control Alb and HA injected tissues. Therefore, these 2 molecules appears to have induced synergistic effect with TNF-α, upregulating COX-2. Similarly upregulation of NF-kB was was also not achieved by TNF-α induction; however, Alb & HA injections seemed to have upregulated the NF-kB. It is likely that the induced inflammatory changes subsided by 35th day, but Alb and HA have adversely affected the reversal of both COX-2 and NF-kB expressions. TIMP-1 was also not found quiescent upon induction with TNF-α but was upregulated upon tretament with CM-Alb+cells, CM-Alb, Alb and HA. From the gene expression analysis, the only valuable observation that MMP-1 and IL-8 expressions persisted in the induced tissues till 35th day; but other markers subsided with time. Only if the tissues harvested more frequently, like 7d or 15 d is analyzed, it may be concluded if other markers are upregulated in the disease model. Therefore, protein level expressions of markers are more valuable to assess the effect of induction and treatments in the the tissues.

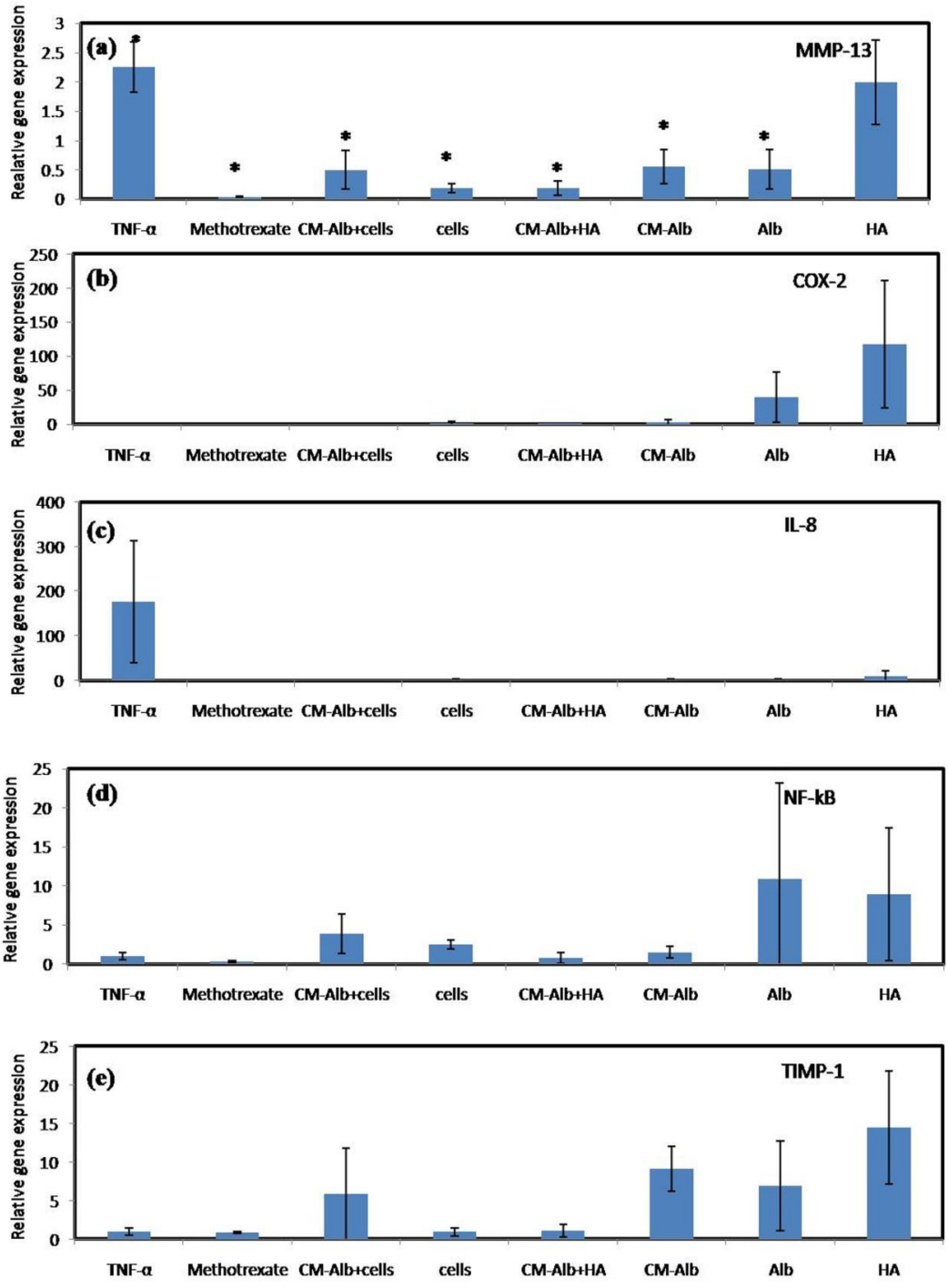


Figure 28: Relative gene expressions of disease model and treated tissues.*Graphical representation of qRTPCR data in terms of relative gene expressions on the 35th day demonstrating the inflammatory status of TNF-α (500ng) induced inflammation model and the treated tissue; (a) MMP-13; (b) COX-2;(c)IL-8; (d)NF-kB; and (e)TIMP-1. Values are represented as Mean±SD; n=3.P < 0.05 (*); P < 0.01 (**); P < 0.001 (***).Fold change is quantified relative to GAPDH expression using the $2^{-\Delta\Delta Ct}$ method, upon normalization with normal tissues.*

4.14.4. Histo-morphology of H&E Stained tissues

The H&E stained images of cartilage sections are represented in Fig 29 (a to i). It can be clearly observed from the images that in the normal tissue which is the NC (Fig 29a) cells are in proper alignment in their typical columnar arrangement. Whereas in TNF-α treated group (Fig 29b), the typical columnar arrangement of cells has been disrupted and is evident from the image that the inflammation has caused a scattered pattern. Inflammation induced tissues treated with CM-Alb (Fig 29g), CM-Alb+ChC (Fig 29d) and CM-Alb+HA (Fig 29f) showed normal columnar arrangement of cells similar to NC. The scattered and disrupted arrangement of cells can be observed in tissues treated with methotrexate (Fig 29c), ChC alone transplanted (Fig 29e) and Alb (Fig 29i) treated group. However, a moderate level of columnar arrangement was observed in HA (Fig 29h) treated group. Overall, the columnar alignment of cells can be graded as follows: (CM-Alb)>(CM-Alb+HA)>(CM-Alb+ChC)>HA>ChC> MET >Alb. The observation indicated that ChC or MET or Alb have not helped in reversing the deleterious effect of TNF-α on tissue, in terms of morphology of cell alignment.

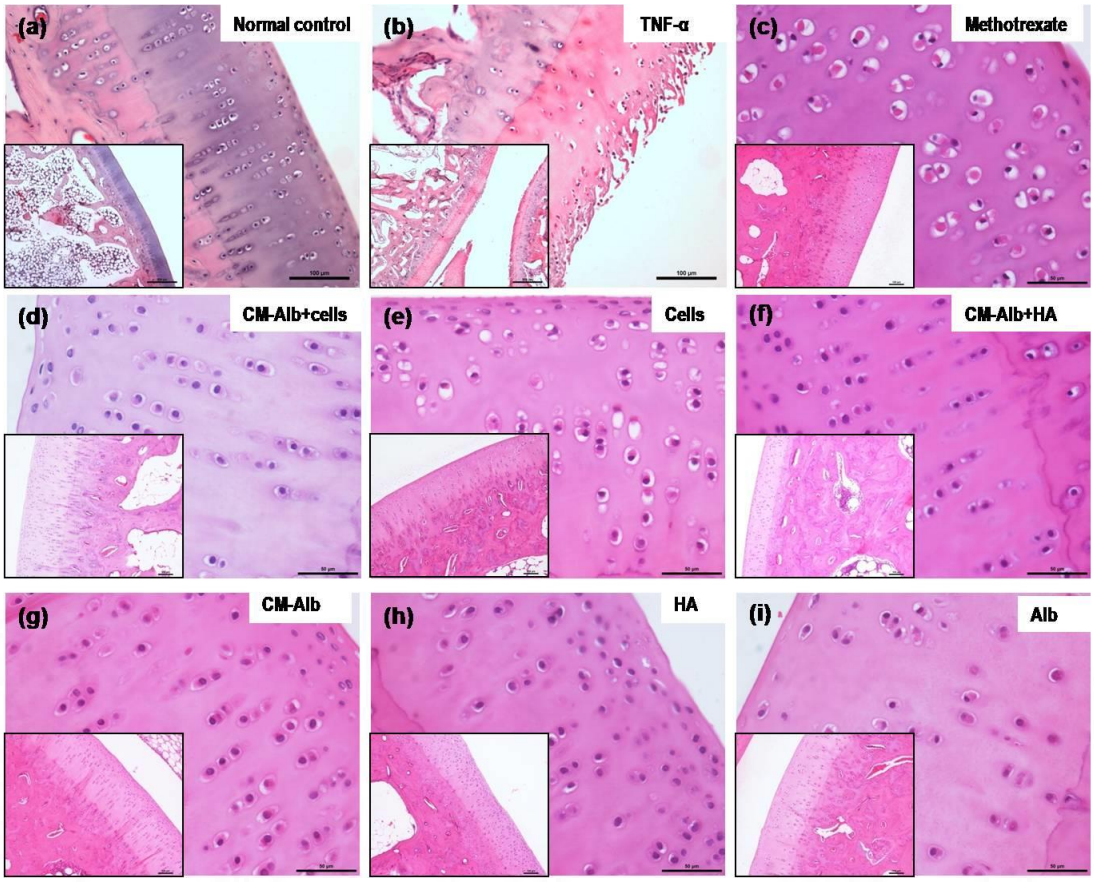


Figure 29: *Representative H&E stained images of cartilage sections of tissue explanted after inducing inflammation before and after different treaments (a) Normal control;(b) TNF-α-induced; (c)TNF-α-induced&Methotrexate-treated;(d)TNF-α-induced & (CM-Alb+rChC)-treated;(e)TNF-α-induced & rChC-treated;(f)TNF-α-induced & (CM-Alb+HA)- treated;(g)TNF-α-induced & (CM-Alb)-treated;(h)TNF-α-induced & HA–treated; and (i)TNF-α-induced & Alb-treated.*

4.14.5 Collagen staining

The picrosirius red staining of collagen in the extracellular matrix of cartilage sections are represented in Fig 30 (a to i). CM-Alb (Fig 30g) treated tissues showed maximum area of collagen deposition in addition to normal columnar arrangement of cells. The TNF-α induced tissues showed minimum collagen deposited area (Fig 30b). CM-Alb+ChC- treated (Fig 30d), ChC- treated (Fig 30e), HA-treated (Fig 30h), and Alb-treated (Fig 30g), tissues were also comparable to TNF-α induced tissues. Methotrexate (Fig 30c) and CM-Alb + HA-treated (Fig 30f) tissues were comparable with normal tissues. Collagen deposition can be visually graded as follows: (CM-Alb)>(CM-Alb+ChC)>(CM-Alb+HA)>(ChC)>(MET)>(HA)>Alb).

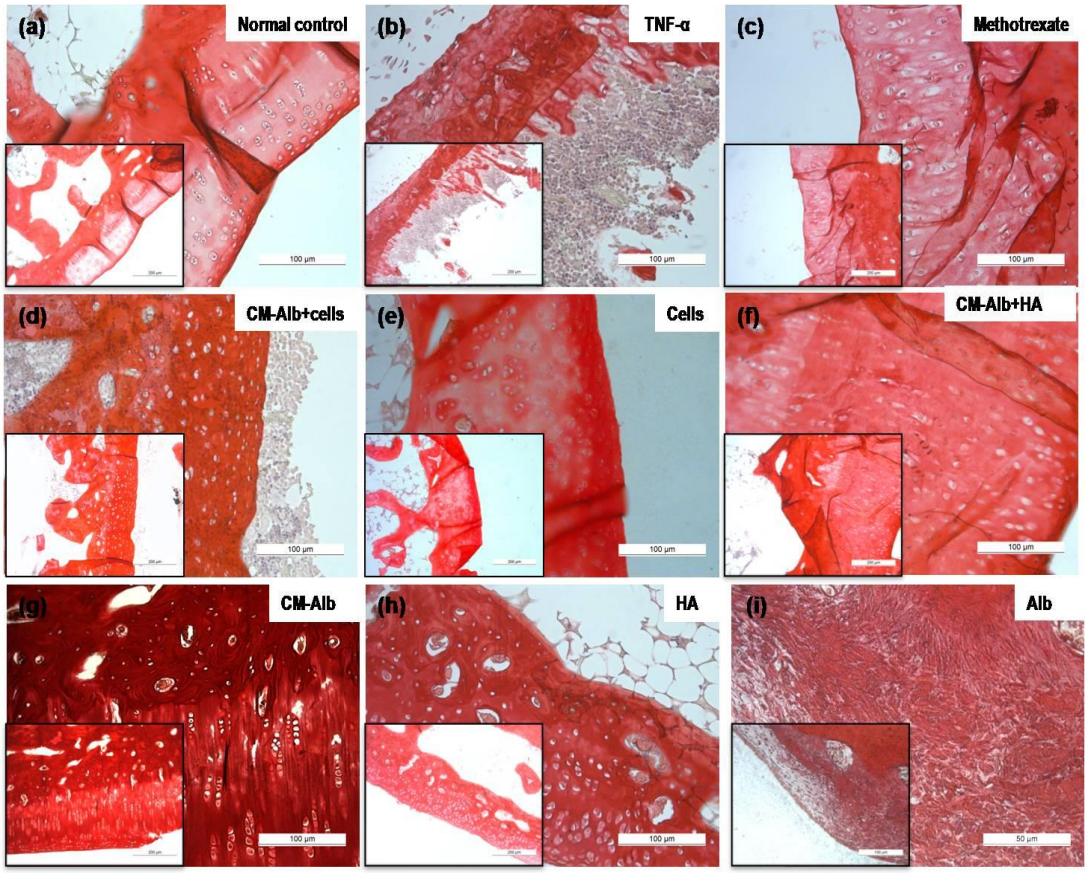


Figure 30: Representative images of cartilage tissue sections stained with picrosirius. *Red stained regions depicts the presence of collagen, processed from animals subjected to different treatment conditions (a) Normal control,(b) TNF-α-induced; (c)TNF-α-induced & Methotrexate-treated; (d) TNF-α-induced & (CM-Alb+ChC)-treated; (e) TNF-α-induced & ChC-treated; (f) TNF-α-induced & (CM-Alb+HA) treated; (g) TNF-α-induced-& (CM-Alb)-treated; (h) TNF-α-induced-& HA-treated; (i)TNF-α-induced & Alb-treated. Inserts in all images are of the magnification 10X and enlarged images are of the magnification 20X.*

4.14.6 Glycosaminoglycans (GAGs) staining

Deposition of GAGs in the ECM of cartilage sections stained by alcian blue is represented in the Fig 31 (a to i). The TNF-α- induced tissue treated with CM-Alb (fig 31g) was comparable with normal tissue (Fig 31a) in terms of GAG deposition. The TNF-α treated tissue (Fig31b), and the induced tissue treated with ChC (Fig 31e) or with Alb (Fig 31i) were comparable in terms of GAG deposition. The TNF-α treated tissue (Fig31b), treated with (CM-Alb+ChC) (Fig 31d) or (CM-Alb+HA) (Fig 31f) treated tissues were comparable and GAG deposition was closer to normal tissue. Lowest GAG deposition was found in Alb (Fig 31i) treated group. The TNF-α

treated tissue (Fig31b), treated with HA (Fig 31h) or methotrexate (Fig 31c) treated tissue also showed moderate GAG deposition. GAG deposition may be visually graded as:(CM-Alb)>(CM-Alb+ChC)>(CM-Alb+HA)>(MET)>(HA)>(ChC)>(Alb).

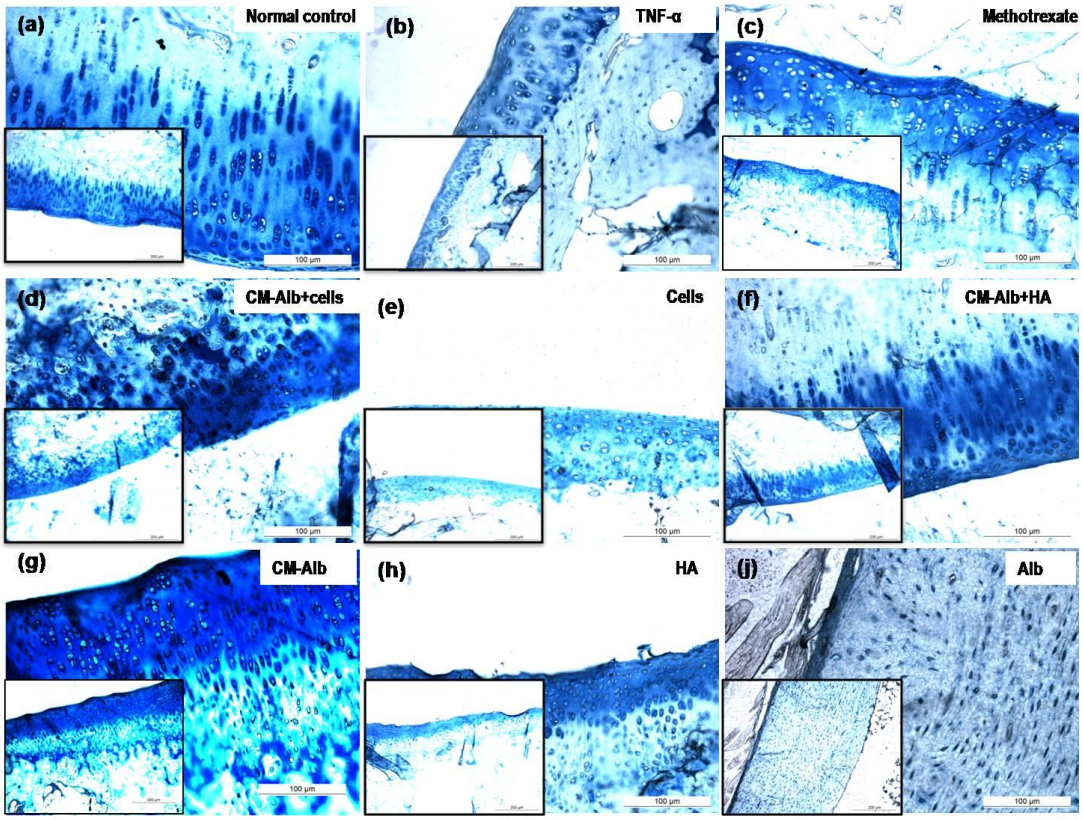


Figure 31: Representative images of cartilage tissue sections stained with alcian blue. *Blue stained regionsdepicts cartilage tissue sections stained with alcian blue depicting the presence of GAGs, processed from animals subjected to different treatment conditions:(a) Normal control;(b) TNF-α-induced; (c)TNF-α-induced & Methotrexate-treated; (d) TNF-α-induced & (CM-Alb+ChC)-treated; (e) TNF-α-induced-& ChC-treated; (f) TNF-α-induced & (CM-Alb+HA) treated; (g) TNF-α-induced & (CM-Alb)-treated; (h) TNF-α-induced & HA-treated; (i)TNF-α-induced & Alb-treated. Inserts in all images are of the magnification 10X and enlarged images are of the magnification 20X.*

4.14.5 MMP-13 Staining

Cartilage sections stained with anti-MMP-13 are represented in Fig (32a to i). CM-Alb (Fig 32g) treated group showed negligible level of MMP-13 expression. Following this, CM-Alb+cells (Fig 32d) treated tissue showed very faint expression of MMP-13. HA (Fig 32h) treated tissue also showed negative for MMP-13 expression. CM-Alb+HA (Fig 32f) treated tissue however showed mild level of

MMP-13 expression. It was observed that methotrexate (Fig 32c) treated tissues followed by Alb (Fig 32g) showed significant. Expression of MMP-13 was comparable in TNF-α induced and TNF-α induced and ChC treated tissues (Fig 32e). Expression of MMP-13 may be graded as: (CM-Alb)<(CM-Alb+cells)<(HA)<(CM-Alb+HA)<(Cells)<(Alb)<(MET).

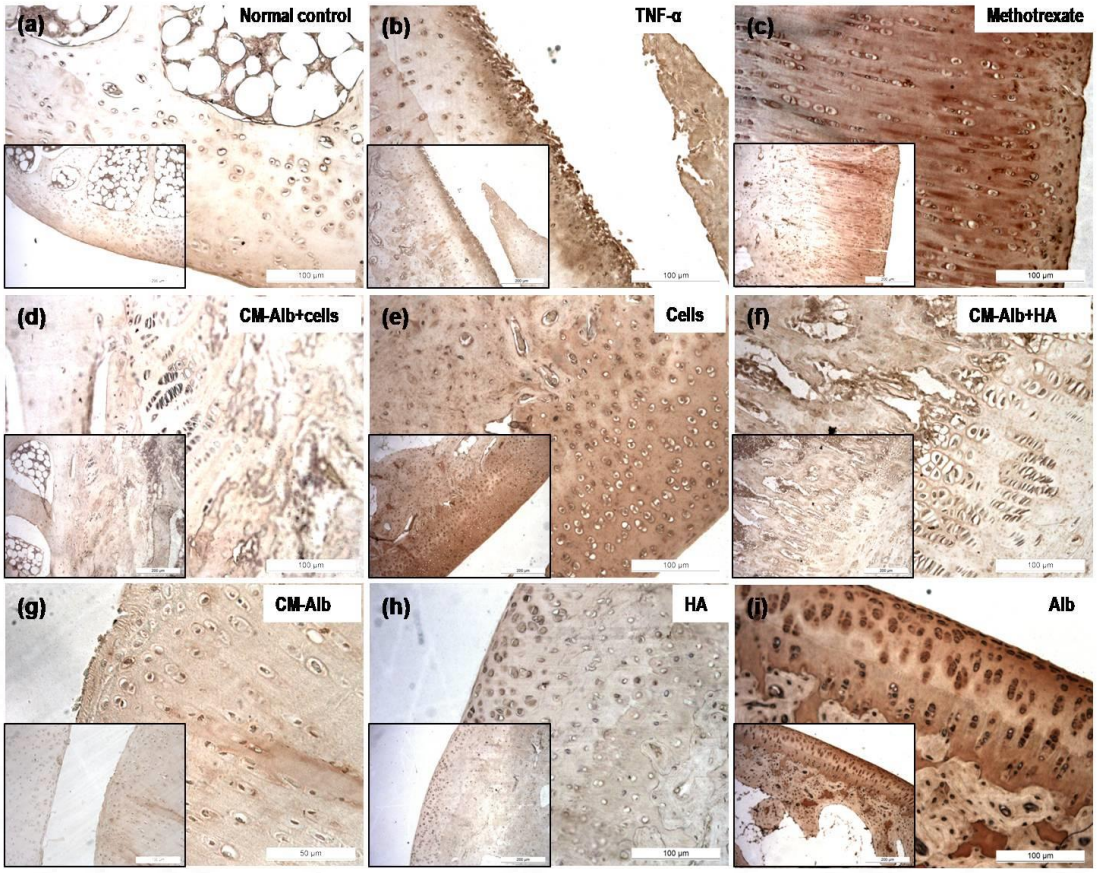


Figure 32: Representative images of cartilage tissue sections stained with anti-MMP-13. *Brown stained regions depicts cartilage tissue sections, processed from animals subjected to different treatment conditions showing presence of MMP-13: (a) Normal control,(b) TNF-α-induced; (c)TNF-α-induced & Methotrexate-treated; (d) TNF-α-induced & (CM-Alb+ChC)-treated; (e) TNF-α-induced & ChC-treated; (f) TNF-α-induced & (CM-Alb+HA) treated; (g) TNF-α-induced & (CM-Alb)-treated; (h) TNF-α-induced & HA-treated; (i)TNF-α-induced-& Alb-treated. Inserts in all images are of the magnification 10X and enlarged images are of the magnification 20X.*

4.14.6 TIMP-1 Staining

Immunohistostaining of TIMP-1 in cartilage sections are represented in the Fig 33 (a to i). Higher levels of TIMP-1 was expressed in CM-Alb+cells (Fig 33d) treated

tissue followed by CM-Alb+HA (Fig 33f), CM-Alb (Fig 33g) and HA (Fig 33h) treated tissues. However, cells alone (Fig 33e) treated showed very mild expression of TIMP-1 indicating that CM-Alb contributes to the over expression of TIMP-1 thereby proving its role as a chondroprotective molecule. Expression of TIMP-1 was graded as:(CM-Alb+cells)>(CM-Alb+HA)>(CM-Alb)>(HA)>(MET)>(Cells)>(Alb).

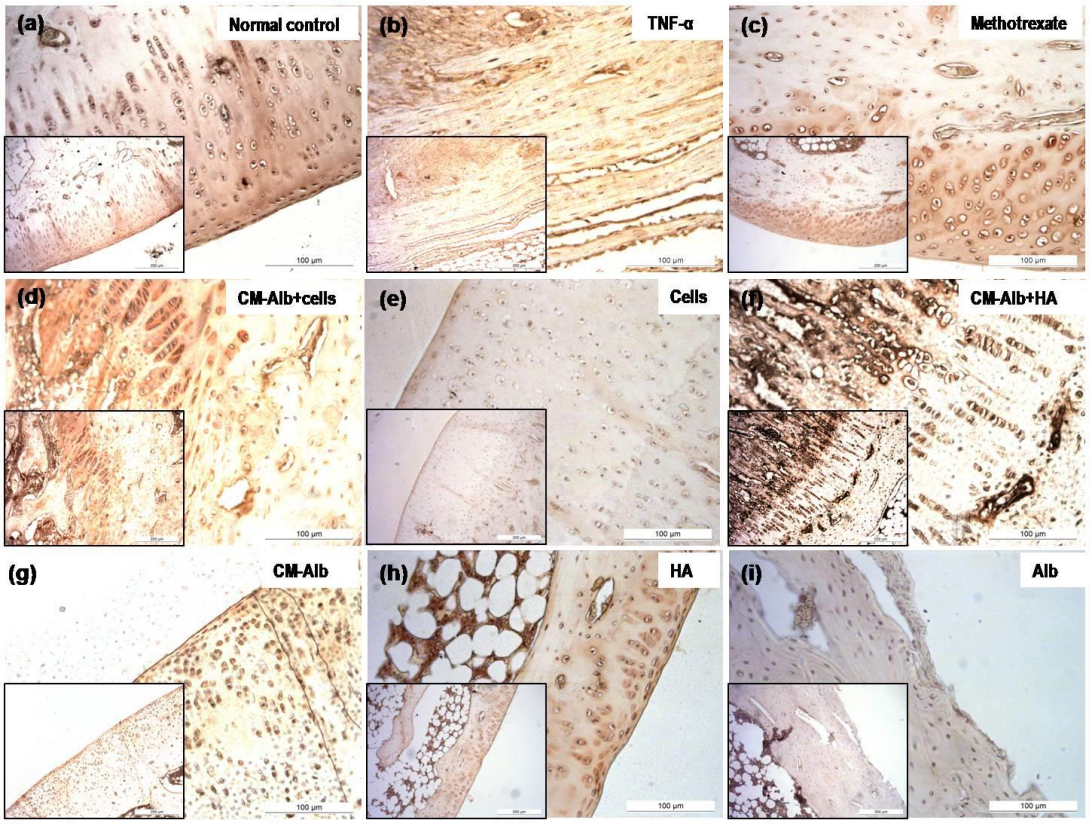


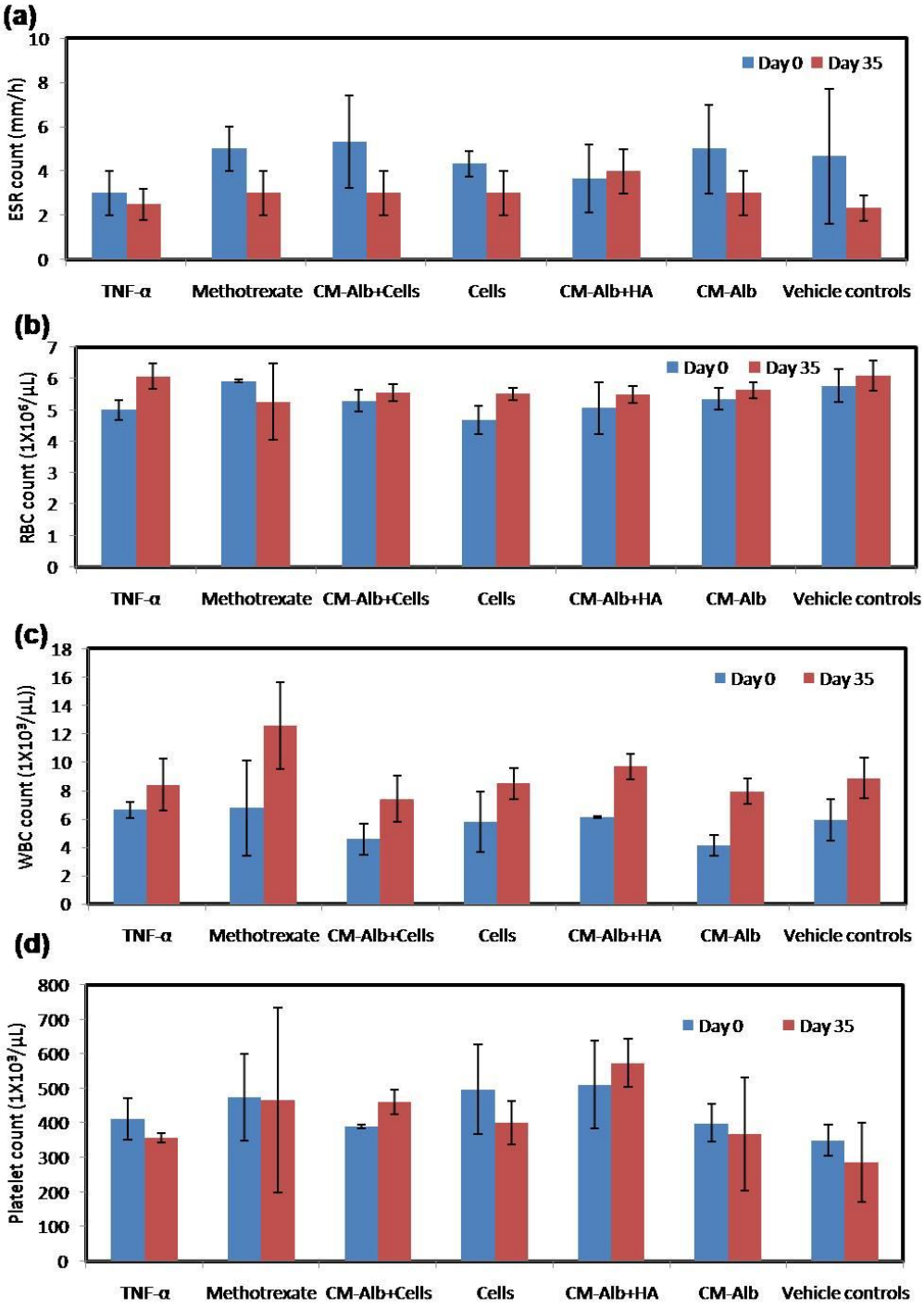
Figure 33: Representative images of cartilage tissue sections stained with anti-TIMP-1. *Brown stained regions depicts cartilage tissue sections, processed from animals subjected to different treatment conditions showing presence of TIMP-1: (a) Normal control; (b) TNF-α-induced; (c)TNF-α-induced-& Methotrexate-treated; (d) TNF-α-induced-& (CM-Alb+ChC)-treated; (e) TNF-α-induced-& ChC-treated; (f) TNF-α-induced-& (CM-Alb+HA) treated; (g) TNF-α-induced-& (CM-Alb)-treated; (h) TNF-α-induced-& HA-treated; (i)TNF-α-induced-& Alb-treated. Inserts in all images are of the magnification 10X and enlarged images are of the magnification 20X.*

Over all, considering all tested parameters, the recovery of TNF-1α induced tissues were completely disrupted even after 35 days. The best recovery from the disrupted stage was achieved when tissues were treated with CM-Alb and CM-Alb+HA in terms of columnar cell arrangement and expressions of markers. ChC

treatment was no effective to recover the daage done by TNF- α and even in the presence of CM-Alb, ChC treatment did not result in complete recovery. Neither Alb or HA alone did not result in any significant benefit in terms of columnar alignment of cells or other marker expressions. On the other hand CM-Alb and HA together seems to have the best regulation in collagen & GAG synthesis and and the resultant regenerated tissue was much smilar to the normal tissue.

4.14.7 Analysis of blood parameters and body weight

Upon measuring, various blood parameters (Fig 34 a to f) such as ESR, RBC count, WBC count, Platelet count, Hemoglobin and Hematocrit , at 0^{th} and 35^{th} day, it was observed that there was no significant difference in any of the parameters among the treated groups and that the values fall under the normal range. In terms of body weight (Fig 34g) also, there was no significant changes observed.


(a)
ESR count (mm/h)
Day 0
Day 35
TNF-α
Methotrexate
CM-Alb+Cells
Cells
CM-Alb+HA
CM-Alb
Vehicle controls
(b)
RBC count (1X10⁶/µL)
(c)
WBC count (1X10³/µL))
(d)
Platelet count (1X10³/µL)

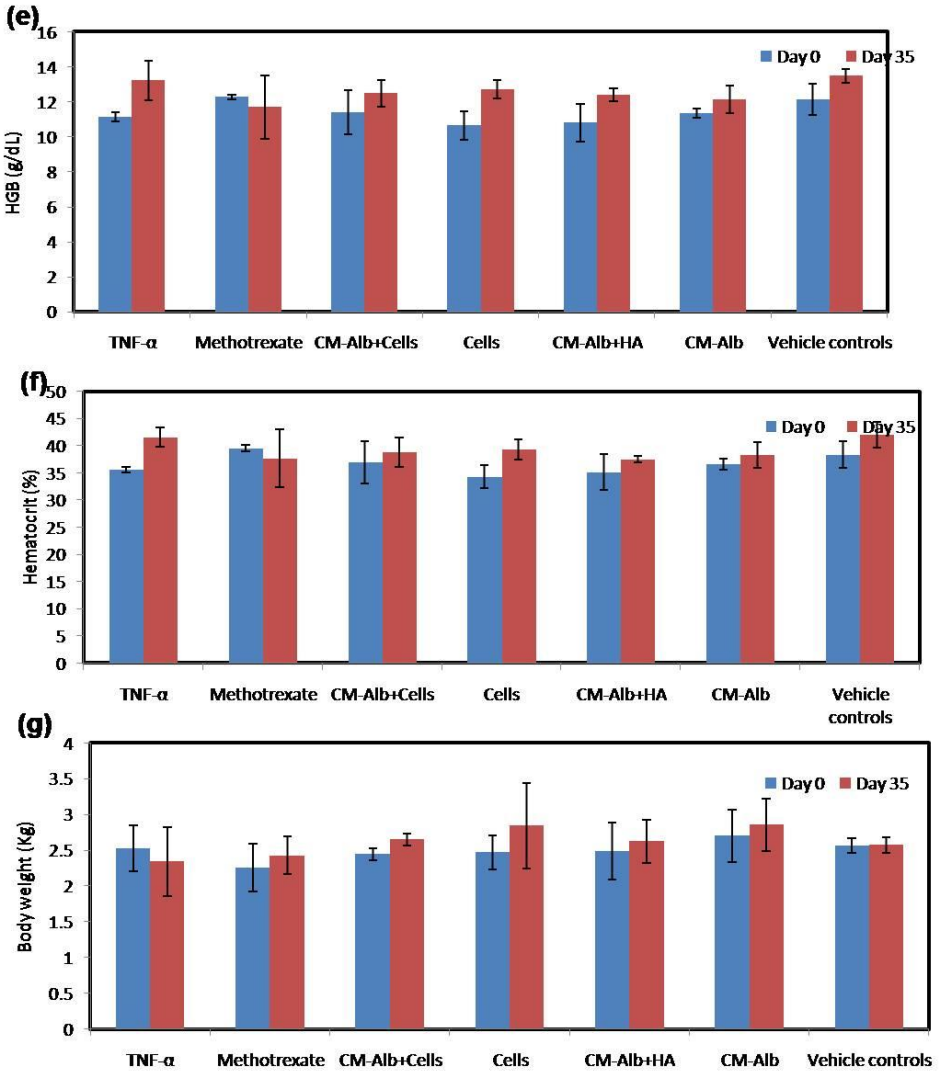


Figure 34: *Graphical representations of blood parameters (a)ESR,(b) RBC count, (c) WBC count, (d) platelet count, (e)Hemoglobin, (f) Hematocrit at 0^{th} and 35^{th} day;(g) body weight at 0^{th} and 35^{th} day.*

CHAPTER 5

5. DISCUSSION

This chapter provides detailed discussion on results presented in chapter 4 relating to the current knowledge in the field and literature published. Also the study hypothesis is considered and results are discussed to the light of the defined objectives to evaluate the study outcome.

The major goal of the study was to establish the effect of aqueous soluble CM in controlling the inflammatory response responsible for progression of diseases such as atherosclerosis and arthritis. CM is normally a hydrophobic and insoluble molecule which is difficult to be applied for its drug action. Therefore, obtaining CM conjugated to human serum albumin (HSA) to obtain high concentration of soluble CM was evaluated for its stability when stored in liquid and solid form. Once highly soluble and stable formulation is available, its effect in controlling inflammation was explored. ECs and ChCs are the major affected cells in atherosclerosis and arthritis, respectively. Therefore, the strategy adopted to test the anti-inflammatory effect was to first obtain both EC and ChC into an inflammatory phenotype. Once the level of gene expressions of inflammatory marker proteins are established in both cell types, the effect of soluble CM conjugate on the marker proteins could be studied. Though preliminary *in vitro* studies are valuable for screening the drug actions, *in vivo* studies are essential to confirm the effect. Therefore, an inflammatory model in the rabbit knee joint was proposed and created to analyze the effect of water soluble CM. The *in vivo* experiments and tissue level analysis is valuable in establishing the role of the drug for reversing the inflammatory response. Promising study results were obtained establishing the effect of CM acting at the molecular and cellular level to correct the inflammatory changes at the tissue level. The results are discussed systematically in the following sections.

5.1. Solubilizing and stabilizing of CM

Inflammation serves as a checkpoint in progression of many diseases. So controlling/regulating the inflammatory processes has become a mandatory requirement and criteria of anti-inflammatory drugs. The commonly available anti-inflammatory drugs (NSAIDs) have shown to posses harmful side-effects upon long

term administration (Kahlenberg and Fox, 2011). As an alternative to this, CM has been known to posses immense therapeutic actions and has been employed to achieve recovery from various ailments. It has been widely used in treating wounds, scars, cancer, gastrointestinal discomforts, and inflammatory disorders. However, its poor aqueous solubility and bioavailability has been two major concerns that restrict its therapeutic action (Prasad et al., 2014b). So selection of a suitable drug carrier that can improve the solubility is a major research interest. It must also be responsible for maintaining appropriateness in target delivery, least affecting the normal tissues, not altering the drug action and also carrier as such being harmless once administered into the body (Dusad, 2013). Alb has been used in combination with several anti-inflammatory drugs to improve their bioavailability and it has been found to be overproduced at the region of inflammation thereby adding additional benefits of targeting the drugs to their site of action (Dusad, 2013). Also, reports suggest that, the presence of specific ligand binding sites on Alb, improves the stability of the drug by enhancing the half life of the drug (Kratz, 2008). In this study, a formulation of CM and Alb was successfully obtained to enhance the biological functioning of CM. A study depicting the importance of CM-BSA conjugate with enhanced bioavailability along with effective anti-cancer activity has been reported (Thomas, Pillai and Krishnan, 2014) (Aravind and Krishnan, 2016). It showed that the conjugation does not affect the biological activity of the drug molecule in terms of cytotoxic action. In this study, Alb conjugation with CM has been confirmed by the FTIR spectra. Furthermore, the UV-Visible spectra and HPLC data confirms stability of conjugate in aqueous medium and lyophilised state, thereby proving that Alb is responsible for maintaining the intact nature of CM protecting it from degradation. Free CM has shown a trend of rapid degradation in aqueous medium which again confirms the important role played by Alb in protecting CM from degradation. Different cell types with respect to their sources has been shown to behave differently upon exposure to CM. Studies have reported that exposing cells in culture to different doses of CM has resulted in reduced cell viability. In a study, wherein, giant cell tumor cells were treated with CM, showed a gradual decrease in cell viability at 24 and 48h of exposure almost between 80 to 60% (Cao et al., 2015). However this reduced viability is of low potential when we consider with what was

observed in another study in which the cancer cells were exposed with CM-Alb for a period of 24 and 48h, it showed cell viability of below 50% (Aravind and Krishnan, 2016b). Therefore from the latter study it can be said that Alb conjugation has enhanced the interaction of CM with cells thereby proving the critical role played by Alb in improving the efficacy of the drug molecule without altering the functional aspects. In addition to this, studies have reported that tumor cells express a special type of receptor known as secreted protein acidic and rich in cysteine (SPARC) which binds specifically to Alb (Merlot et al., 2014). So the presence of this receptor adds up on the advantage of using the technology of Alb in combination with CM in anti-cancer therapies. In this study, the endocytosis of CM-Alb has been clearly depicted by both cell types ECs and ChCs. In ECs, one of the most important category of Alb specific receptors come in the group known as Albondin/gp60 that helps in transporting Alb into the cell cytoplasm (Merlot et al., 2014). Such receptors are responsible for disrupting the barrier function of endothelial layer. Accumulation of Alb has been observed in osteoarthritic conditions also at the inflamed joints. This process has been exploited by many pharmaceutical companies in targeting anti-inflammatory drugs to treat the inflamed joints (Wunder et al., 2003). This might be the reason for uptake of Alb conjugated drug by the ChCs. This way, Alb performs explicitly the dual functions of solubilizing the drug molecule thereby ensuring its availability at the abnormal/diseased tissue regions as well as easing up/facilitating the uptake of drug molecule by cells.

5.2. Optimization/identification of non-toxic/safe dose of CM-Alb

Studies have shown that higher doses of CM cause cell death in tumor cells and normal cells by necrosis. At the same time, lower doses results in cell death in tumor cells by apoptosis whereas normal cells did not show any remarkable cell death (Syng-ai et al., 2004). This study observed a minimal cytotoxic effect at lower concentrations in ECs while maintaining viability above 80% at higher concentrations upon 24h exposure. The effect was in correlation with dose and time of exposure of cells to CM-Alb conjugate. Free CM however has shown reduced cell viability in a time and dose dependent manner which can be correlated with another study which showed a decreased cell viability in retinal endothelial cells upon exposure to free CM even at very low concentrations (Premanand et al., 2006). This

in turn shows that free CM lacks proper control over its action whereas, Alb conjugated CM shows a regulated action which might be accounted due to the critical role played by Alb. The maintenance of cell viability in ECs can in turn be correlated with the data obtained by establishing the potential of EC to proliferate in the presence of conjugated CM. There was no reduction in proliferation observed upon treatment with conjugate at lower concentrations. This might be because the ECs used for *in vitro* experiments were primary cells and as studies have reported that CM has no cytotoxic effect on normal primary cells which can be correlated with the cell proliferation observed (Syng-ai et al., 2004). In case of ChCs, the lower proliferation rates observed upon exposure to conjugate can be correlated to the fact that ChCs are terminally differentiated cells, upon reaching a differentiated stage most of the differentiated mature cell types usually will not undergo proliferation. The lower cell viability observed in ChCs upon 24h exposure compared to ECs exposed for the same time period can be correlated with the lack of proliferation potential of differentiated ChC cells (Adams and Shapiro, 2002). However a reversal in cell viability observed upon treatment with conjugate for a period of 48h might be attributed to the contribution of the proliferating progenitor cells present in the culture. As these ChCs were obtained by differentiation of hADMSCs, presence of progenitor cells cannot be ruled out. The identification of a safe and optimum dose of CM-Alb based on cytotoxicity assay is very critical for carrying out anti-inflammatory studies. The major concern for selecting a dose of CM-Alb for anti-inflammatory action puts forward the mandatory requirement that the dose selected must exhibit its therapeutic role at the same time must ensure remarkable cell viability. ECs and ChCs being non-tumorous cells, they are less prone to cytotoxic effects of CM-Alb at lower doses. This suggests that lower concentration of CM is ideal for ensuring cell viability in normal cells.

5.3. Anti-inflammatory role of CM-Alb *in vitro*

The anti-inflammatory role of CM is well known in the traditional Indian medicine. But the scientific proof signifying the dose and time dependent effect of CM on inflammatory conditions has not been well studied. For demonstrating the anti-inflammatory action of any drug molecule, selection of ideal candidates for *in vitro* studies is very critical. ECs and ChCs serve as suitable *in vitro* models for

studying anti-inflammatory properties of drug molecule as both cell types are critical in the clinical scenario of inflammatory diseases atherosclerosis and arthritis, respectively. In this study, inflammatory models were created *in vitro* by treating the cells in culture with the help of pro-inflammatory inducers/cytokines. During inflammation, macrophages are one of the major types of cells that are recruited to the site of action and these cells serve as an important source for secretion of various cytokines that can be either pro or anti-inflammatory in action depending on the need of the situation. Studies have reported that the pro-inflammatory cytokines such as TNF-α, IL-6, IL-1β etc are secreted by macrophages and these molecules play an indispensable role in initiating the various cellular responses (Zhang and An, 2007). As a result of these inflammatory reactions, several downstream molecules in the inflammatory pathways get activated and determining the expression of these molecules can make them to be recognizable as ideal markers of inflammation. Few of such inflammatory markers expressed by these cell types include NF-kB, ET-1, COX-2, MCP-1, VCAM-1, MMP-13, IL-8, TIMP-1 etc. In this study, it was found that, in ECs except VCAM-1, all other inflammatory markers: NF-kB, ET-1, COX-2 and MCP-1 were found to be downregulated upon treatement with CM-Alb at lower concenrations whereas at higher concentrations it was found to be upregulated. In case of ChCs the following inflammatory markers MMP-13, COX-2 and IL-8 showed downregulation at lower concentrations wheras at higher concentrations upregulation was observed. The expression of TIMP-1 molecule was greatly enhanced at lower doses of CM-Alb suggesting that the latter does play a critical role as a chondroprotective moleculewhereas higher doses had no effect in its expression. This is in correlaion with a study that proved the chondroprotective role of CM in ChCs (Csaki et al., 2009). So from thisit can be inferred that CM-Alb exhibits a biphasic effect based on dose. This can be correlated with a recent study published that highlights the biphasic/hormetic effect of CM (Moghaddam et al., 2019). The transcription factor NF-kB is involved in the regulation of transcriptional stimulation of various genes during inflammation and the relationship between TNF-α and NF-kB is such that both the molecules are involved in the mutual activation of each other during unfavourable conditions (Hewlings and Kalman, 2017). Various studies have

reported that CM has an inhibitory effect on the functional activation of NF-kB. Usually NF-kB is found in association with inhibitor of kB (IkB) in the cell cytoplasm. Inflammatory stimuli disrupts this association by activating IkB kinases leading to the migration of the transcription factor into the cell nucleus and activate other genes (Tak and Firestein, 2001). COX-2 is an important enzyme that takes part in regulating inflammatory pathways. Normal healthy cells express COX-1 constitutively which is required for the smooth functioning of cell activities. However, upon stimulation by variety of inflammatory cytokines, cells begin to express another isoform COX-2. Both these forms convert arachidonic acid into prostaglandins during inflammatory pathways. And with the increase in intensity of inflammatory stimuli, studies have shown that the expression of COX-2 also increases meanwhile the expression of COX-1 remains normal (Caughey et al., 2001). So cyclo-oxygenase being an important and critical enzyme in inflammatory pathways, COX inhibitors have been developed by the healthcare industry. And in our study, CM-Alb has been effective in downregulating its expression in ECs and ChCs. Recruiting the monocytes and macrophages to the inflamed tissue is through MCP-1 (Yang et al., 2009). In the current study it is seen that in ECs the expression of MCP-1 increase significantly when treated with TNF-α for a period of 24h. When the inflammation induced ECs are treated with CM-Alb conjugate, 5 to 20µM concentrations have down-regulated MCP-1 expression significantly, compared to the TNF-α treated cells. During inflammation there is an enhanced expression of adhesion molecules on vascular walls which is involved in worsening of vascular diseases. This is accompanied by EC activation leading to the release of MCP-1 (Yang et al., 2009). At the time of inflammation, the recruited monocytes differentiate into classical M1 macrophages and non-classical M2 macrophages resulting in cholesterol ingested plaques. These plaques serve as a reservoir for the release of several inflammatory cytokines that deteriorates the plaque and promotes inflammation in the affected region of the vascular wall (Chistiakov et al., 2017).It has also been reported that MCP-1 increases the expression of VCAM-1 on ECs (Yang et al., 2009). Another major cytokine ET-1 released by ECs acts by promoting vasoconstriction of blood vessels during CVDs (Masaki and Sawamura, 2006). In the current study, ET-1 has been found to increase upon treatment with TNF-α; which is

significantly reduced upon treating with CM-alb conjugate at 5, 10, 20µM concentrations. TNF-α and IL-6 are known to induce the expression of VCAM-1, ICAM-1 and other cell adhesion molecules on their surface as a result of their response to pro-inflammatory mediators (Liao, 2013). In this study also, upon incubation with TNF-α for a period of 24h there is significant increase in the expression of VCAM-1 by ECs. Further, the treatment of inflammation induced ECs with CM-Alb conjugate at 5, 10, 20µM concentrations down regulated the expression of VCAM-1 significantly compared to the TNF-α treated cells. Another important aspect of an anti-inflammatory drug is that it has to be used for treating arthritis must ensure that the balance between MMP-13 and TIMP-1 is maintained. This in turn helps in maintaining the integrity of the cartilage which facilitates its biological functional aspects (Li et al., 2017). MMP-13 is the major culprit in destroying the ECM of cartilage which gets inhibited by TIMP-1 (Young et al., 2019). So, an ideal anti-inflammatory drug molecule which is intended to be used for arthritic treatment has to perform the dual roles of suppressing the expression of MMP-13 and at the same time promoting the expression of TIMP-1.

5.4. Anti-inflammatory role of CM-Alb *in vivo*

Once a drug candidate has proven its effectiveness in *in vitro* system, next hierarchal level is to test its action *in vivo* using a suitable animal model that can mimic the disease condition. Development of atherosclerotic animal model is a difficult task. Many studies have highlighted the possibilities of developing animal models with CVDs but failed to have a controlled disease condition as the process was accompanied with uninvited complications involving other vital organs along with delay in the onset of disease (Leong et al., 2015) (Zaragoza et al., 2011). So, as an alternative to this, developing a cartilage disintegrated model can serve the purpose for demonstrating the anti-inflammatory action of a drug molecule. Even though many small and large animal models have been discussed in published literature, based on some specific advantages rabbit was selected for creating an appropriate inflammatory model. Rabbit can serve as an ideal *in vivo* model as it satisfies all the conditions for developing cartilage disruption. Adequately sized joints along with sufficient thickness of cartilage are few of the conditions that are to be considered while selecting the animal model (Moran et al., 2016). Attainment of

skeletal maturity and suitable for long term studies in case of inflammatory aspects are also some other factors that are to be taken into account while choosing the animal model. In this study, inflammation was induced in articular cartilage of rabbit knee joints using TNF-α. Studies have reported the use of various chemicals to induce arthritis by application of invasive methods (Kuyinu et al., 2016).During the standardization of inflammatory model in knee joint using TNF-α, the cartilage disintegration was assessed histopathologically using H & E staining. As per published literature, type II collagenase has been proposed for inducing inflammation in knee joints of rabbit by intra-articular injection(Kikuchi et al., 1998). Initially as part of standardizing the inflammatory model, type II collagenase was administered intra-articular but remarkable changes were not observed in comparison with normal control. The *in vitro* studies showed significant upregulation of inflammatory markers and few other studies claim to have used TNF-α for inducing inflammation *in vivo*, this study also explored the use of this cytokine and successfully achieved inflammatory changes. Normal and healthy cartilage is characterized by the presence of ChCs arranged in columnar alignment especially in the deep zone of cartilage (Sophia Fox et al., 2009). ChCs are the only cell types present in cartilage that performs the function of supporting the ECM by contributing towards the formation and degradation of matrix. The number, size and arrangement of these cells do matter for the proper functioning of cartilage which in turn is marked by the maintenance of adequate consistency of the matrix and it is this matrix that is responsible for protecting ChCs from stressful stimuli (Sophia Fox et al., 2009). Along with disruption in columnar arrangement of ChCs, fibrillation of the cartilage surface is another characteristic feature of damaged cartilage (Akkiraju and Nohe, 2015). This is in correlation with the results obtained in this study. Upon treatment with TNF-α, the disrupted and sparse distribution of ChCs observed along with surface fibrillations proves that the cartilage has been damaged by the intra-articular intervention. Whereas, the normal cartilage depicted a smooth and intact surface layer. The implanted rChCs were tracked by tagging the cells with PKH26 dye. There are reports that emphasizes on the use of PKH26 owing to its non-toxic effect and tracking of implanted cells *in vivo* (Duek et al., 2017). The presence of PKH26

tagged cells detected via *in vivo* imaging might be an indicator of the retention of cells at the site of administration. As observed in *in vitro* experiments, CM-Alb was able to enhance the expression of TIMP-1*in vivo* also. The downregulation of MMP-13, COX-2, NF-kB and IL-8 upon treatment with CM-Alb can be correlated with the *in vitro* data obtained in inflammatory studies carried out using ChCs. The expression pattern of MMP-13 can be correlated with the immunohistochemical staining of the cartilage tissue sections, wherein lowest expression was observed in CM-Alb treated tissues and highest expression was however observed for methotrexate treated tissues followed by Alb treated ones. The expression of TIMP-1 can also be correlated with immunohistochemical staining of cartilage tissue sections wherein tissues that were treated with formulations comprising CM-Alb showed higher expression and lowest was observed in Alb treated ones. The H&E staining showed normal columnar arrangement of cells in CM-Alb treated group, (CM-Alb+ChC)- treated tissues, (CM-Alb+HA)- treated tissues and HA treated tissues show the effectiveness of the drug molecule to preserve the structural integrity of cartilage which is a critical requirement for functioning of healthy cartilage. At the same time, poor alignment of cells in methotrexate treated tissues, cells treated tissues and lower density of cell distribution in Alb treated tissues projects their inefficiency in restoring the damaged cartilage. Methotrexate was used for the *in vivo* study for comparison of tissue response because it is a clinically used anti-inflammatory drug. But it is known to be accompanied with side effects especially in patients with malfunctioned kidneys (Nedelcu et al., 2019) (CUTOLO et al., 2001). HA has been recommended by many clinicians as an alternative to reduce the pain at joints by providing a cushioning effect for the joints (Medvedeva et al., 2018). These are available commercially as modified forms in order to achieve effectiveness in the therapeutic applications. It is reported that HA contributes to anti-inflammatory effect by serving as an inhibitor molecule to many pain sensitive receptors (Nicholls et al., 2017). But as contradictory to this, upon clinical trials, no much significant changes are observed towards recovery of patients (Medvedeva et al., 2018). Lower levels of inflammatory reactions were observed at the joints upon treatment with HA, but whether the molecule as such is responsible for such reactions or the procedure of injection is causing it has not been clearly distinguished so far (Wen, 2000). Both

advantageous as well as no effects at all have been reported through clinical trials. The proposed reason for such huge variations might be because of the different amounts of HA degrading enzymes present in different individuals (Bowman et al., 2018).Collagen is one of the most abundant constituent of articular cartilage and offers a major contribution to the mechanistic properties of cartilage (Eyre, 2002). So detecting the presence and distribution of collagen serves as an important criterion for assessing the integrity of cartilage. Collagen present in the ECM of cartilage is also responsible for ensuring the proper structural features for executing its involvement in activities that puts forwards the demand for mechanical strength (Sophia Fox et al., 2009). In this study, CM-Alb treated tissues showed maximum collagen deposition and Alb effected a disorganised/poor deposition of collagen compared to all other tissues. This can be correlated with the result obtained in terms relative gene expression and immunohistochemical staining wherein MMP-13 expression was greatly reduced thereby preventing the cleaving of collagen. One of the primary function of MMP-13 is disrupting the ECM by cleaving collagen present in the matrix (Howes et al., 2014). As the TNF-α caused upregulation of MMP-13 expression, it results in the cleaving of collagen thereby reducing its deposition and distribution throughout the ECM. From the histochemical staining it can be seen that the collagen deposition is reduced in TNF-α treated tissue which can be correlated with higher expression of MMP-13 in immunohistochemical staining. So from this it can be inferred that the collagen deposition in cartilage is dependent on the absence of MMP-13. As a result of this, anti-inflammatory drug that are to be designed for cartilage regeneration must specifically target the suppression of MMP-13 and must serve as MMP-13 inhibitors. Another important criterion to assess the functionality of cartilage is by detecting the presence of GAGs. These molecules play an important role in providing hydration to the cells and its surrounding environment (Casale and Crane, 2020). The hydration of cartilage is necessary to provide flexibility during moving activities. In this study, the maximum deposition of GAGs was found to be in tissues treated with formulations comprising CM-Alb. Another important function of GAGs is to control the activity of MMPs by binding to specific sites on the latter and inhibiting or activating it based on the stimuli (Tocchi and Parks, 2013). In this

study, GAGs is seen to be present in CM-Alb treated tissues which can be correlated to the fact that this tissue showed least expression of MMP-13.

5.5. Effect of combination therapy with CM-Alb

The different parameters evaluated correlated well and established the effectiveness of (CM-Alb) in reversing the inflammatory response of tissue to TNF-α. However, an increased collagen deposition in (CM-Alb)-treated tissue as compared to normal tissue is a matter of concern (Fuerst et al., 2009). Other 2 combinations of (CM-Alb) used for treatment were (CM-Alb+HA) and (CM-Alb+rChC). Most of the results in (CM-Alb+HA)-treated tissues were very close to the (CM-Alb)-treated tissues. On the other hand, the collagen deposition in (CM-Alb+HA)-treated tissues showed a pattern more close to the normal tissue. The result suggest that in the combination treatment, HA regulated collagen deposition much better than that in (CM-Alb)-treated tissues. In line with the observation the regulated collagen deposition, in (CM-Alb+HA)-treated tissue, the MMP-13 expression is found to be higher than that in (CM-Alb)-treated tissues. Also, the TIMP-1 expression is high in the (CM-Alb+HA)- treated tissues as compared to (CM-Alb)- treated tissues. Therefore, it may be expected that the excessive breakdown of the deposited collagen by MMP-13 may be regulated by the TIMP-1. This interplay between MMP-13 and TIMP-1 in (CM-Alb+HA)-treated tissue seems to have regulated the collagen content. Therefore, in terms of collagen required for regulated regeneration of the inflamed cartilage tissue, combination of (CM-Alb) and HA seems to be preferable.

Also, the GAG deposited in (CM-Alb)-treated tissues are high as compared to normal tissue. On the other hand in (CM-Alb+HA)-treated tissues, the GAG content is comparable to the normal tissues. Therefore, it appears that HA in the combination has regulated the GAG deposition. The columnar cell arrangement and tissue morphology is comparable in (CM-Alb) –treated and (CM-Alb+HA)-treated tissues. On the contrary, combination of (ChC +CM-Alb) was not found to help. In spite of the retention of transplanted ChC, there was no benefit for preserving the tissue morphology assessed as columnar cell arrangement. Regenerative approaches using ChC transplantation has been a major research focus aiming to reduce the morbidity caused by chronic arthritis (Minas et al., 2010). However, poor preservation of tissue

morphology and associated with low collagen organization is indicated by bright red stained tissue sections upon ChC- transplantation. MMP-13 expression in ChC-transplanted tissue was high which may be responsible for reduced collagen organization. In the ChC-transplanted tissue GAG deposition was also low. Expression of TIMP-1 was low in ChC- treated tissues. Combination of ChC and (CM-Alb) has improved collagen and GAG depositions, reduced MMP-13 expression and increased TIMP-1 expressions. Even then, the preservation of normal tissue morphology was not achieved by combining (CM-Alb) with ChC for treating disease tissues. Therefore, the deleterious effect of ChC was not overcome by including (CM-Alb). Based on the well-correlated multi-parameter results of this study, scope for cell transplantation therapy, with or without (CM-Alb), may not be advocated for regeneration of inflamed cartilage.

It is evident in all stained sections, such as in H&E, Collagen, GAG, MMP-13, and TIMP-1, that the tissue morphology was well-preserved with cells arranged in the columnar alignment and well-formed meniscus when the combination of CM-Alb and HA were used for treating the tissues disrupted by TNF-α. In addition, optimal deposition of collagen and GAG, regulated by the HA added with (CM-Alb) indicates that the combination treatment may be considered for further evaluation in animal models. Even though, HA alone helped preservation of TNF-induced inflammation, poor collagen and GAG deposition, high MMP-13 expression, low TIMP-1 expression etc. confirms that injection of HA alone may not be sufficient for regeneration of disease tissue. The CM-Alb alone is able to preserve the tissue morphology; however, for regulation of collagen and GAG depositions, combining HA with the conjugate seems to be beneficial and may be further evaluated in detail.

5.5. Limitations of the study

Four doses of TNF-α seems to have resulted in significant disruption of the cartilage tissue on 35th day. However, the effect of each cytokine administration and the kinetics of the changes were not evaluated. Also, the treatment was given along with the 3rd dose of induction. If the induced tissues were not explanted on 35th day and allowed to remain for prolonged period such as 90 days, what could be the effect on tissue morphology? If the treatment is started on the 90th day, instead of starting

on 14th day, what could have been the effect was not studied. Thus upon analyzing the results of this study, several limitations have been identified.

CHAPTER 6

6. SUMMARY AND CONCLUSION

Major objective of this study was to explore the possibilities of solubilizing CM in aqueous medium and demonstrating anti-inflammatory property of aqueous soluble CM-Alb conjugate using *in vitro*/*in vivo* models of inflammation. The study projected that conjugating CM with human serum Alb can improve solubility of the former and primary cells relevant in clinical scenario could become suitable in *vitro* models for assessing the anti-inflammatory action of CM. *In vitro* studies using ECs and ChCs further proved that this conjugation did not affect the functional properties of CM. The uptake of conjugate by both cell types was proven quantitatively and qualitatively by endocytosis assay. Optimum and safe dose of conjugate critical for maintaining cell viability in ECs and ChCs was determined by MTT assay. With the use of TNF-α, *in vitro* models of inflammation was standardized in ECs and ChCs. Following which, anti-inflammatory effect of soluble CM was demonstrated in inflammation induced ECs and ChCs. Further a suitable *in vivo* demonstration of anti-inflammatory effect of CM in inflammation induced with TNF-α in rabbit knee joint has substantiated the *in vitro* experiments. The levels of inflammation and anti-inflammation were assessed by determining inflammatory marker expression at transcriptional and translational levels. Histopathological assessment was carried out to determine the ECM changes in cartilage. Thus, switching on from NSAIDs to alternative sources from natural origin like CM associated with lesser side effects at lower doses will greatly improve the health index worldwide.

6.1 Summary

Preparation of soluble form of CM has been successfully achieved by simple conjugation with human serum Alb and its purification via gel filtration chromatography resulting with an approximate recovery of 85% for CM. CM remains unaltered by this conjugation has been proven by HPLC. Major problems of low aqueous solubility and poor stability of CM has been successfully overcome by Alb conjugation. This simple conjugation of CM with Alb has shown to improve the solubility of the drug molecule along with ensuring the stability of the drug molecule

in soluble as well as lyophilized state (short term storage in aqueous form up to 24h at 4ºC and long term storage in lyophilized form up to 6 months). Solubility has been demonstrated by UV-Visible spectroscopy. Stability has been demonstrated by UV-Visible spectroscopy, HPLC and FTIR.ECs and ChCs being the major cell types affected during major inflammatory disease conditions, atherosclerosis and arthritis respectively, was considered for *in vitro* inflammation/anti-inflammation studies. ECs isolated from human umbilical cord vein and ChCs obtained by differentiation of hADMSCs proved to be good sources for establishing *in vitro* models. ECs are critical in normal functioning of blood vessels and being first elements of encounter during progression of atherosclerosis serves as good responders to inflammatory stimuli. ChCs being the only cell type present in cartilage serves as another good option as an *in vitro* model of inflammation to mimic the inflammatory changes occurring during cartilage disintegration at the time of arthritic progression. Cellular uptake of the conjugate was established by both cell types *in vitro*. As CM is known to possess cytotoxic effect, identification of safe dose for cells that does not affect the cell viability is crucial which was determined in this study. The study showed that lower doses of conjugate are responsible for maintaining cell viability, exposure to higher doses results in reduced cell viability in both cell types. Free CM has shown to be more cytotoxic compared to CM-Alb proving that the conjugation is playing an important role in exhibiting controlled activity of the drug molecule. And also as the time of exposure to conjugate increased, cell viability showed a reduction pattern. So, the cell viability assay proved that there is a time and dose dependent effect on cells upon exposure to CM-Alb. Lower concentrations of CM-Alb showed no significant deleterious effect on cell proliferation in ECs. At the same time in ChCs, the cell proliferation was observed to be less due to terminal differentiation of the cells. TNF-α was proven to be a good inflammatory inducer as upon its treatment with cells, expression of inflammatory markers were found to be upregulated in both cell types. Eventhough, IL-6 and IL-1β were also used as inflammation inducers, there was no significant upregulation observed and hence were avoided from further experiments. The following were the inflammatory markers assessed: NF-kB, VCAM-1, ET-1, MCP-1, COX-2 in ECs and NF-kB, COX-2, IL-8, MMP-13, TIMP-

1 in ChCs and *in vivo*. Upon treatment with CM-Alb, most of the inflammatory markers showed significant downregulation. In addition to this, it was found that CM-Alb exhibited a biphasic effect on these marker expressions. Upon treating cells at lower concentrations of CM-Alb, markers were downregulated but at the same time higher concentrations of CM-Alb resulted in upregulation of the same markers. The results indicate that at high cytotoxic concentrations, it cannot be used as anti-inflammatory drug. The upregulated expressions of inflammatory markers in dying cells may not be reversed by CM-Alb conjugate. Upon inducing inflammation *in vivo*, different treatment strategies were applied. They are as follows: groups treated with methotrexate, CM-Alb, CM-Alb+rChCs, rChCs, CM-Alb+HA, HA, Alb. Further, histological assessment showed the variations in different treatment groups in terms of collagen deposition (Picro-sirius red staining), GAGs deposition (Alcian blue staining) and expression of MMP-13 and TIMP-1. Based on this assessment, CM-Alb treated group showed promising effect for reversing the inflamed cartilage. There were no changes observed in different treatment groups in terms of body weight and blood parameters.

The major achievements of the study are as follows:

1. An ideal anti-inflammatory drug formulation was achieved by simple conjugation technique and purification by gel filtration chromatography.
2. FTIR spectra showed specific peaks for specific groups present in CM and Alb confirming the interaction between both CM and Alb.
3. Approximately 85.5% recovery was obtained for CM upon quantification of pooled fractions obtained via gel filtration chromatography.
4. The solubility of formulated conjugate CM-Alb was proven by UV-Visible spectroscopy.
5. The short term storage (24h) stability of conjugate in aqueous medium was proven by UV-Visible spectroscopy.
6. The long term storage (6months) stability in lyophilized state was proven by HPLC and FTIR.
7. The cellular uptake of FITC tagged CM-Alb by ECs and ChCs was effectively demonstrated qualitatively by microscopic imaging and quantitatively by flow cytometry analysis.

8. Minimally toxic concentration of conjugate was identified upon exposure to cells.
9. MTT assay showed that conjugate exerts time/dose dependent effect on cell viability with lower doses ensuring maximum viability upon short term exposures in ECs.
10. MTT assay showed reversal effect in ChCs upon 48h exposure due to the presence of chondrocytes progenitor cells in the culture.
11. The cell proliferation in ECs remained unaffected upon treatment with conjugate thereby proving the safe nature of drug formulation.
12. There was no much cell proliferation observed in ChCs confirming terminal differentiation of cells.
13. Significant upregulation of inflammatory marker expressions was observed in ECs and ChC upon treatment with pro-inflammatory molecule TNF-α.
14. Relative gene expression analysis showed effective and significant down regulation of inflammatory marker (MCP-1, ET-1, VCAM-1, NF-kB) expression in TNF-α stimulated ECs.
15. Relative gene expression analysis showed effective and significant downregulation of inflammatory marker (MMP-13 & IL-8) expression in TNF-α stimulated ChC upon treatment with conjugate as compared to free CM.
16. The anti-inflammatory effect was more prominent at lower concentrations of conjugate showing a biphasic nature at higher concentrations.
17. NF-kB at higher concentrations of conjugate is highly expressed which is responsible for upregulation of other pro-inflammatory genes.
18. In ChC, the expression of TIMP-1 molecule was significantly enhanced upon treatment with conjugate and free CM indicating chondroprotective role.
19. *In vivo* model of inflammation was developed in rabbit knee joint, by injecting specific concentration of TNF-α (500ng/ml).
20. The retention of PKH 26 tagged ChC in knee joint was observed denoting the retention of injected cells.
21. In TNF-α induced tissues, there was upregulation in MMP-13 and IL-8 expression was observed significant in the case of MMP-13.

22. CM-Alb treated group showed upregulation for TIMP-1 and downregulation for MMP-13 and IL-8 compared to TNF-α induced tissues.
23. Except HA treated tissues, all other tissues showed significant downregulation in MMP-13 expression.
24. Columnar alignment of cells were graded as follows: (CM-Alb)>(CM-Alb+HA)>(CM-Alb+cells)>(HA>Cells)>(MET)>(Alb).
25. Collagen deposition was visually graded as follows: (CM-Alb)>(CM-Alb+cells)>(CM-Alb+HA)>(Cells)>(HA)>(MET)>(Alb).
26. GAG deposition was visually graded as follows: (CM-Alb)>(CM-Alb+cells)>(CM-Alb+HA)>(MET)>(HA)>(Cells)>(Alb).
27. Expression of MMP-13 was quantified as follows:(CM-Alb)<(CM-Alb+cells)<(HA)<(CM-Alb+HA)<(Cells)<(Alb)<(MET).
28. Expression of TIMP-1 was quantified as follows:(CM-Alb+cells)>(CM-Alb+HA)>(CM-Alb)>(HA)>(MET)>(Cells)>(Alb).
29. The regeneration of inflamed cartilage tissue was found optimum, with normal tissue morphology cellular and extracellular matrix organizations, when combination of HA and CM-Alb was introduced as treatment.
30. The regeneration of inflamed tissue was not satisfactory when ChC was transplanted with or without CM-Alb, Alb alone or HA alone.

6.2. Conclusions of the study

1. Demonstrated high concentrations of stable albumin-conjugated CM in aqueous medium and the long shelf-life of lyophilized conjugate.
2. Established endocytosis of CM-Alb into EC and ChC.
3. Identified non cytotoxic dose response to EC and ChC for safe testing of anti-inflammatory activity.
4. Demonstrated anti-inflammatory effect of CM-Alb in inflammation induced ECs and ChCs *in vitro*.
5. Developed inflammation model *in vivo* in rabbit knee joint, by injecting specific concentration of TNF-α.

6. Achieved regeneration of the inflammation induced cartilage, at molecular and cellular level leading to preservation of the tissue morphology, upon treating with CM-Alb.
7. Established the benefit of HA, for regulating the tissue regeneration, upon combining with CM-Alb for treating the disrupted cartilage.

6.3. Future prospects of the study

1. Combination of HA and CM-Alb may be evaluated extensively to understand the regeneration potential in osteoarthritis using animal models
2. Clinical translation may be aimed after completing the above stated study.

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LIST OF PUBLICATIONS

Patents

- Indian patent: Lissy Kalliyana Krishnan, Renjith Parameswaran Nair, Mejo Chiratteparambil Korah and Deepa Sathee; A highly soluble intravenous formulation of dimethoxycurcumin-human serum albumin retaining proven biological activities and a process thereof. Application number: 202041020471 dated 15.05.2020.
- Indian Patent: Lissy K Krishnan, Sachin Shenoy, Sabareeswaran A. and Deepa S; Hyaluronic acid- and human serum albumin conjugated curcumin-composition for restoring inflammation induced damage of knee joint". Application No: 202041047225 dated 29.10.2020.

Original Papers

- Deepa Sathee, Lissy Kalliyana Krishnan. Aqueous Solubility and Bioavailability of Albumin Conjugated Curcumin in Endothelial Cells Allege Potential of the Formulation for Anti-Inflammation Therapy. Cardiology and Cardiovascular Medicine 5 (2021): 17-35. IF: 3.5
- Deepa Sathee, Sachin J Shenoy, Sabareeswaran A, Arya Anil, Manesh Senan, Lissy Kalliyana Krishnan. Curcumin protecting TNFα-activated inflammatory response in chondrocyte culture and rabbit knee cartilage. (Communicated)
- Deepa Sathee, Sachin J Shenoy, Sabareeswaran A, Arya Anil, Lissy Kalliyana Krishnan. Combinatorial application of Hyaluronic acid and Curcumin-Albumin conjugate for cartilage repair in TNF-α induced inflammation in Rabbit knee joint. (Under preparation)

Presentations and Conference Proceedings

- Deepa S and Lissy K. Krishnan. Anti-inflammatory property of Curcumin-Albumin conjugate established using primary cell cultures. 2018. 5th TERMIS-2018 World Congress, Kyoto, Japan. (Poster)

- Deepa S and Lissy K. Krishnan. Exploring anti-inflammatory property of curcumin-albumin conjugate using endothelial cell culture system. 2017. National seminar on Frontiers in Biotechnology held at Inter-University centre for Genomics and Gene Technology, Department of Biotechnology, University of Kerala, Kariavattom Campus, Trivandrum, India. (Poster)

APPENDIX

PBS (1000ml) pH 7.4

NaCl	-8g
KCl	-0.2g
Na_2HPO_4	-1.44g
KH_2PO_4	-0.24g

HBSS (1000ml) pH 7.4

KCl	-0.4g
KH_2PO_4	-0.06g
NaCl	-8g
Na_2HPO_4	-0.0482g

Resolving gel buffer (1.5M) pH 8.8

Tris base	-18.15g
Distilled water	-50ml

Stacking gel buffer (0.5M) pH 6.8

Tris base	-6g
Distilled water	-60ml

Acrylamide-bisacrylamide (100ml)

Acrylamide	-29.9g
Bisacrylamide	-0.8g
Distilled water	-73ml

10% SDS

SDS	-1g
Distilled water	-9ml

Sample buffer

Distilled water	-3.55ml
0.5M Tris HCL (pH 6.8)	-1.25ml
Glycerol	-2.5ml
10% SDS	-2ml
0.5% Bromophenol blue	-0.2ml

5X electrode running buffer	-1.5g
Tris base	-7.2g
Glycine	-0.5g
SDS	-95ml
Distilled water	

Transfer buffer	
Tris base	-0.58g
Glycine	-0.29g
SDS	-0.037g
Methanol	-20ml
Distilled water	-80ml
Developer solution (20ml)	
Nickel chloride	-0.1g
DAB	-0.01g
Hydrogen peroxide	-40µl
PBS	-20ml

Reagent A for Lowry (100ml)	
2% Sodium carbonate	-2g
0.1N NaOH	-0.4g
Distilled water	-100ml

Reagent B for Lowry (100ml)	
0.5% copper sulphate	-0.5g
1% Sodium potassium tartarate	-1g
Distilled water	-100ml

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Submitted	7/16/2020 10:04:00 AM
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