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Title of PhD Thesis

***The chondropathies: study of antagonism between
growth factors and reactive oxygen species; clinical
applications in the early stages of chondral lesions***

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Contents

Abbreviations	3
Abstract	5
CHAPTER 1	
INTRODUCTION.....	6
1.1 Hyaline Cartilage	7
1.2 Chondropathy	9
1.21 Symptoms of chondropathies.....	10
1.22 Epidemiology	10
1.23 Diagnosis.....	10
1.3 Aetiology.....	11
1.4 Reactive oxygen species (ROS)	12
1.41 Oxidative stress in cartilage	13
1.5 Sources of ROS	14
1.6 ROS generation stimulated by cytokines	15
1.7 Role of ROS on chondrocyte functions.....	16
1.8 Role of ROS in cartilage damage.....	17
1.9 ROS and Matrix metalloproteinases	18
2.0 Mitochondria and ROS	19
2.1 Antioxidant system	20
2.2 Growth factors	22
CHAPTER 2	
AIM OF THE WORK	24
CHAPTER 3	
MATERIAL AND METHODS	28
3.1 Reagents.....	29
3.2 Study subjects	29
3.3 Cell culture and treatments	30
3.4 Measurement of intracellular ROS levels	30
3.5 Determination of cytoplasmatic and mitochondrial redox status	31
3.6 Measurement of cell proliferation.....	32

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

3.7 Measurement of superoxide dismutase (SOD) activity	33
3.8 Measurement of catalase activity	34
3.9 Measurement of Glutathione (GSH) activity	34
3.10 Statistical analysis	35
CHAPTER 4	
RESULTS.....	36
4.1 Effect of emartri and haematic sera on H ₂ O ₂ - elicited oxidative stress.....	37
4.2 Effect of emartri and haematic sera on H ₂ O ₂ - induced changes of the cytoplasmatic redox status.	38
4.3 Effect of emartri and haematic sera on H ₂ O ₂ - induced changes of the mitochondrial redox status.	40
4.4 Effect of emartri and haematic sera on H ₂ O ₂ - induced changes of cell proliferation.	42
4.5 Effect of emartri and haematic sera on H ₂ O ₂ - induced changes of SOD activity.	43
4.6 Effect of emartri and haematic sera on H ₂ O ₂ - induced changes of catalase activity.	45
4.7 Effect of emartri and haematic sera on H ₂ O ₂ - induced changes of GSSG/GSH ratio	46
CHAPTER 5	
DISCUSSION	48
CHAPTER 6	
BIBLIOGRAPHY	59
Acknowledgement.....	70

Abbreviations

ATP - adenosine triphosphate

AP-1 - activating protein1

BMP - bone morphogenetic protein

BrdU - 5-bromodeoxyuridine

COMP - cartilage oligomeric matrix protein

DCF - dichlorofluorescein

DMEM - Dulbecco's Modified Eagle's Medium

ECM - extracellular matrix

FCS - fetal calf serum

Fnf - fibronectin fragments

GAGs - glycosaminoglycans

GDF5 - growth differentiation factor 5

GSH - glutathione

GSSG - oxidised form of glutathione

GPX - glutathione peroxidase

HA - hyaluronic acid

H₂DCFDA - 2',7'-dichlorodihydrofluoresceindiacetate

H₂DCF - 2',7'-dichlorodihydrofluorescein

H₂O₂ - hydrogen peroxide

HCa - human articular chondrocytes

HBSS - Hank's balanced salt solution

HEPES- (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

ICRS - international cartilage repair society

IGF-1 - insulin like growth factor 1

IL-1 - interleukin 1

LIF - leukocyte inhibitory factor

ROS - reactive oxygen species

roGFP - redox sensing green fluorescent protein

MitMP - mitochondrial membrane potential

MMP - matrix metalloproteinases

Dr. Smitha Tulasigeri.Totiger

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

MRC - mitochondrial respiratory chain
MRI - magnetic resonance imaging
MTX roGFP - mitochondrial redox sensing green fluorescent protein
NO - nitric oxide
NOS - nitric oxide synthase
NADPH enzyme complex - nicotinamide adenine dinucleotide phosphate oxidase enzyme complex
OA - osteoarthritis
OPA - ortho-phthalaldehyde
OP-1 - osteogenic protein 1
PDGF - platelet derived growth factor
PG - proteoglycan
PBS - phosphate buffered saline
PCA - perchloric acid
SOD - superoxide dismutase
SLRPs - small leucine rich protein
TGF beta - transforming growth factor beta

Abstract

Oxidative stress is one of the risk factors for chondropathies. Deterioration of the cell functions due to oxidative stress has been found to be the initial stage changes involving disruption of the cartilage homeostasis. Eventually these changes progress to the final stage of osteoarthritis involving series of catabolic damage mediated by pro-inflammatory cytokines, MMPs, aggrecanases and cell death. Our study investigated the role of fluid, isolated from post operation knee drainage (emartri), of patients who underwent prosthesis replacement surgery, against hydrogen peroxide (H_2O_2) - induced oxidative stress on normal human articular chondrocytes (HCa). HCa were pre-treated with emartri and then exposed to H_2O_2 to mimic oxidative stress. Intracellular reactive oxygen species (ROS) levels were measured by using the molecular probe H_2DCFDA ; intracellular oxidative status in both the cytoplasm and mitochondrial compartment was measured by using HCa infected with lentiviral particle harbouring the redox sensing green fluorescent protein (roGFP); cell proliferation was determined by measuring the rate of DNA synthesis with BrdU incorporation. Moreover, superoxide dismutase (SOD), catalase and glutathione levels from the cell lysates were measured. Haematic sera from the same patient, obtained post-operation, were included in our study to compare and evaluate the response given by the two types of sera. Our study showed that emartri is able to overcome the H_2O_2 - elicited oxidative stress by decreasing the intracellular ROS levels, maintaining both cytoplasm and mitochondria in a reduced status, inhibiting the cell death due to oxidative stress and up-regulating the antioxidant defence system in chondrocytes. Emartri is assumed to be an important source of growth factors and anti-inflammatory substances that are attracted to the site of surgery. Our results support and propose an important effect of emartri-contained substances in maintaining HCa functions by mediating the up-regulation of the anti-oxidative system and protect cells from oxidative stress. We hypothesized that emartri could be used as an autologous treatment for patients in the early stage undergoing an operation of positioning of the prosthesis, with chondropathies and/or arthrosis or a developing osteoarthritis in the initial stage in the same or other locations in the body.

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

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CHAPTER 1

INTRODUCTION

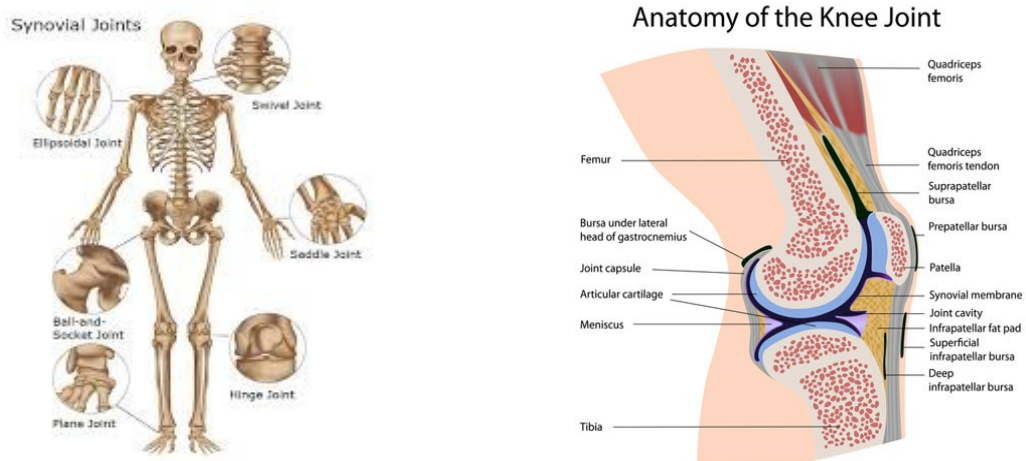
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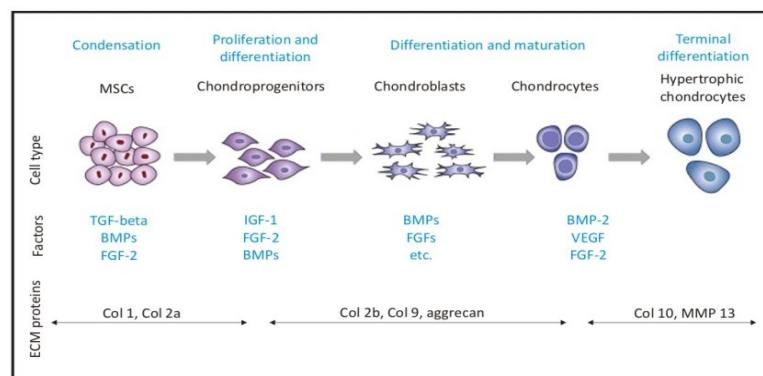
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1.1 Hyaline Cartilage

Hyaline cartilage is a smooth, white thick connective tissue found in the synovial joints of knee, hips, shoulder, wrist and elbow. It possess excellent load bearing functions. Cartilage accounts for the easy mobility of joints as the bones involved in the articulation are encapsulated by cartilage and can easily glide against each other during locomotion.



Cartilage is a heterogeneous dense connective tissue [1]. It consist the only type of differentiated cells, chondrocytes, which account for less than 5% of the total wet weight [2].



Chondrogenesis. (from lookfordiagnosis.com)

Following chondrogenesis, the mature chondrocytes remain as resting cells and secrete collagen II, proteoglycans and non-collagenous proteins which are the extracellular matrix (ECM) components. Chondrocytes get embedded in the abundant ECM secreted by them.

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

15-22% of total wet weight consists of collagen which provides the high tensile stiffness, strength and resiliency to the tissue [3], with collagen type II making about 95% and scarce amount of collagen IX and XI. 5-10% of the total wet weight is constituted by polyanionic proteoglycans which are a mix of large aggregating aggrecans associated with hyaluronic acid (HA) (50-85%) and large non-aggregating (10-40%) proteoglycans. They are responsible for pressure elasticity due to presence of glycosaminoglycans (GAGs) namely chondroitin sulphate and keratan sulphate which are negatively charged and their interactions with water provide osmotic properties to resist compressive loads and deformation [2].

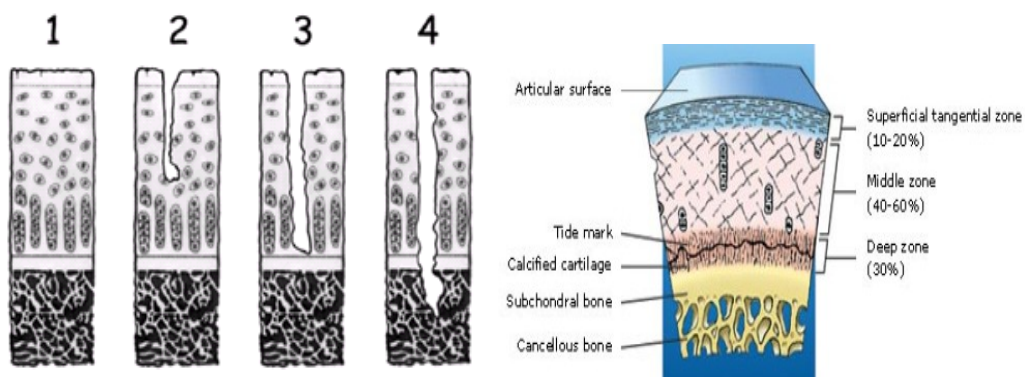
Lubrication, smooth articulation, load transmission and distribution are some of the functions of cartilage attributed by the proteoglycans [4]. The fibrous collagen network prevent the over swelling of hydrophilic proteoglycan content and accounts for tensile strength of the tissue. Thus matrix can deform increasing the joint contact areas and distribute the mechanical stress. Non collagenous proteins like small leucine rich proteins (SLRPs), cartilage oligomeric matrix protein (COMP), matrilin-1, elastins, integrins, decorin, fibromodulin, biglycan are responsible for the collagen fibril assembly, matrix organization and maintenance. Cartilage differs in its composition and distribution of matrix molecules and also chondrocyte morphology from superficial to deep zones [2]. Interaction of ECM components with chondrocytes determines chondrocyte phenotype and also homeostasis of the cartilage as they regulate binding, storage, release, presentation of and responsiveness to soluble mediators [5]. Mechanical and structural capacity of the cartilage highly depends on the composition and integrity of extracellular matrix

which allows it to achieve and maintain proper biomechanical function over long years [6, 7].

Chondrocytes normally maintain the matrix in a low turnover state by maintaining a balance between synthesis and degradation of ECM components by sensing the changes in matrix composition [5]. But have limited capacity to repair, remove and regenerate the cartilage damage as it lacks blood supply.

1.2 Chondropathy

Chondropathy refers to the diseases of the cartilage which involve cartilage destruction. International Cartilage Repair Society (ICRS) classifies the cartilage damage, in grades, based on the appearance of the articular cartilage when seen at arthroscopy.



Grade 1: A: Softening of cartilage

B: Minimum fissures

Grade 2: The lesions less than 50% of the cartilage thickness

Grade 3: A: The lesions affecting more than 50% of the cartilage thickness

B: Depth to the calcified cartilage

C: Depth to subchondral bone

D: Cartilage fibrillating flap

Grade 4: Exposure of subchondral bone

Dr. Smitha Tulasigeri.Totiger

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

1.21 Symptoms of chondropathies

Any kind of damage to the cartilage leads to disability of the joint movement. There is a poor correlation between the degree of anterior knee pain and cartilage damage [8]. Since cartilage is not an innervated tissue, damage to the cartilage goes asymptomatic unless the damage progress and involves the adjacent innervated tissue becoming symptomatic with pain [9]. Swelling, stiffness, athletic and functional disabilities are the common symptoms. Patello femoral pain can decrease on resting and physiotherapy [10] but, if persists with debilitating pain and accumulating inflammation in the articulation, over the years can transform to chronic degeneration leading to osteoarthritis (OA) involving cartilage damage [11].

1.22 Epidemiology

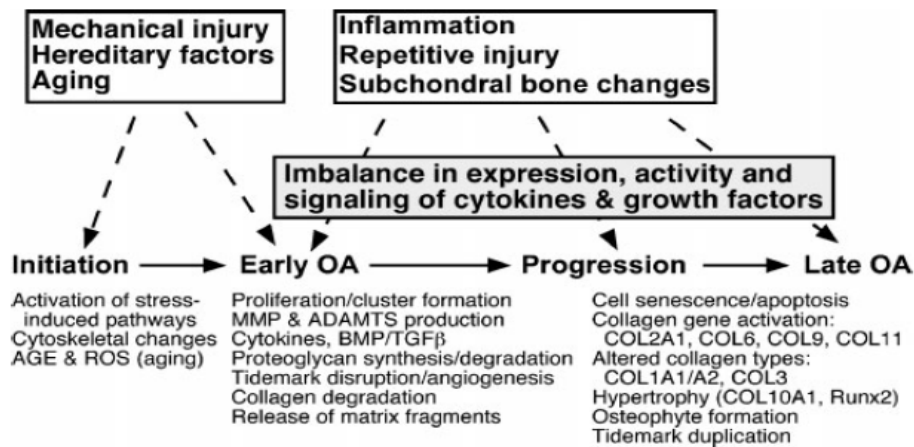
Joint pain can occur in young and aged [12]. But the chronic joint pain is the leading cause of disability in elderly people, aged above 50, worldwide and females are more affected compared to males [13]. The prevalence of OA increases with age [14].

1.23 Diagnosis

Clinical examination includes a comprehensive and systematic means of assessing the cartilage defects. Defining and characterising the symptoms, physical examination, evaluation of previous management and familial history, ultrasonography [15], radiological identification of joint space narrowing(JNS) together with immunoassay for synthesis and degradation markers in body fluids [2, 16, 17], magnetic resonance imaging (MRI) detection of cartilage damage may help in predicting the clinical outcome and disease progression for effective management and treatment [18].

1.3 Aetiology

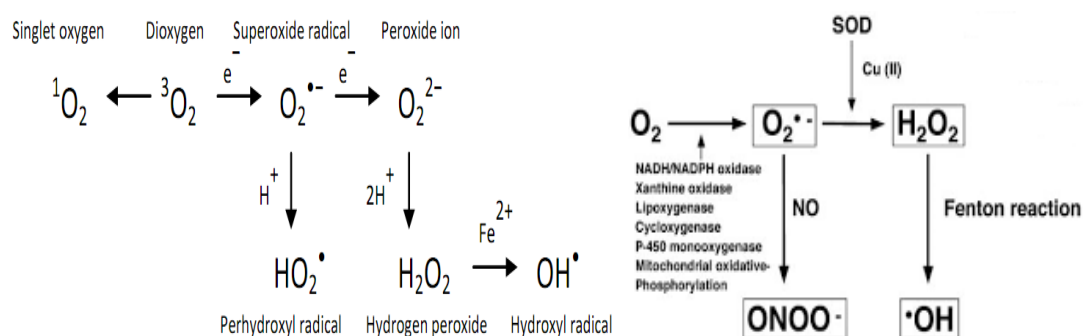
Chondropathies are multi-factorial diseases. The potential risk factors include biomechanical stress [19] [20] due to over load, overuse, injuries during sport [21, 22], age [23-25], instability and inadequate muscle strength or endurance, structure and mal-alignment of the joints [26, 27], obesity [28] and family history (genetic predisposition) [29, 30], polymorphism and mutations in extra cellular matrix genes [31-33], trauma with low systemic inflammation of surrounding tissue [34]. All these factors can disturb the homeostasis of the tissues in the joint ultimately leading to cartilage weakening and/or destruction of the joint [35]. How eventually the aforementioned risk factors lead to changes in the composition and mechanical properties of the articular cartilage? What are the underlying mechanisms mediating and / or perpetuating these changes, have not been fully understood. Yet the current hypothesis include, chondrocyte senescence [36], reduced response of chondrocyte to growth factors [37], increased chondrocyte catabolism mediated by pro-inflammatory molecules, cytokines and matrix metalloproteinases (MMPs) [38], change in structure and thus functioning of extracellular matrix proteins [4, 24, 39], changes in tissues besides cartilage [14, 40], excessive production of cytokines and growth factors by the inflamed synovium and activated chondrocytes [41], **oxidative stress** [42, 43].



Scheme of events in the initiation of Osteoarthritis (OA) and progression to late OA. Osteoarthritis, MARY B. GOLDRING AND STEVEN R. GOLDRING*

1.4 Reactive oxygen species (ROS)

Reactive oxygen species (ROS) are chemically reactive free radicals containing oxygen. Oxygen radicals formed from molecular oxygen by one-electron transfer reactions are collectively called as ROS. They are unstable hence react with other molecules to achieve stability and during this process generate a chain of free radical intermediates.



(from www.intechopen.com and www.chinaphar.com)

ROS are by-products of normal cellular metabolism and can produce either beneficial or even indispensable or harmful effects depending on their intracellular levels. The beneficial effects of reactive oxygen

species occur in host defence mechanism and in a number of cell signalling systems [44, 45]. Role of ROS in driving the age related catabolic imbalance is supported by many evidences [46-48]. ROS have been found to regulate the chondrocyte metabolism during normal and pathological conditions as they are relevant in chondrocytes, both as (physiological) intracellular signalling molecules [49] as well as molecules, which are very detrimental [50, 51]. In chondrocytes ROS are involved in modulating ionic homeostasis which is important for chondrocyte functions [52] and thus maintain the appropriate pH under altered O₂ tensions [53]. Excess ROS has harmful effects where they can damage macromolecules including DNA [54], lipids [55], protein [39] and inhibit their normal functioning.

1.41 Oxidative stress in cartilage

Two mechanisms majorly contribute to the oxidative imbalance in cartilage. They are 1) depletion of anti-oxidative capacity of chondrocytes, 2) nutrition of cartilage.

Cartilage is avascular. It depends on synovial and subchondral bone compartment from where the nutrients are diffused [56]. Oxygen and metabolic end products also have to diffuse over a relative long distance resulting in low O₂ tension in cartilage [57]. Oxidative metabolic turnover of cartilage produces ROS required for the maintenance of intracellular signalling mechanisms [58]. Superoxide anion [59, 60] and nitric oxide (NO) [61, 62] are the main ROS produced by chondrocytes and their respective derivative radicals are hydroxyl radical [63], hydrogen peroxide [64] and peroxynitrite [43, 65]. When the anti-oxidative capacity of chondrocytes is unable to combat the excess ROS generated, will result in oxidative stress condition.

1.5 Sources of ROS

ROS are generated endogenously and their sources in chondrocytes have been well identified. The potent sources include:

- NADPH oxidase
- Nitric oxygen synthases (NOS)
- Mitochondria
- Xanthine oxidase (XO), glucose oxidase and
- Cytochrome P-450

Superoxide anion radicals are produced by isolated human chondrocytes [66]. It is reported that chondrocytes possess NADPH enzyme complex located on the plasma membrane [59, 60] and produce superoxide in response to cytokine stimulation [67].

Nitric oxide is synthesized by nitric oxide synthases (NOS) enzymes in cytoplasm. Chondrocytes are the primary producers of NO compared to other cells in the articular joints and express the eNOS which are constitutively expressed and iNOS which are regulated by variety of growth factors, cytokines and endotoxins [61, 67, 68] is found to be expressed highly in osteoarthritic (OA) chondrocytes [69].

Mitochondria are the major sink for O₂ reduction hence one of the major sources of ROS found in chondrocytes [70]. 2-3% of oxygen consumed during electron transport chain is rather reduced to superoxide ion at complex I and III.

Cytochrome P450 monooxygenases, xanthine oxidase during oxidation of hypoxanthine and glucose oxidase are capable of one electron transfer to oxygen yielding superoxide anion.

1.6 ROS generation stimulated by cytokines

Signs and symptoms like joint swelling, effusion, redness and stiffness reflect the presence of synovial inflammation [34]. Clinical studies have found an association between inflammation and progression of structural changes in cartilage by the levels of cartilage oligomeric protein (COMP), C-reactive protein, hyaluronic acid in synovial fluid [17, 71]. Synovium and activated chondrocytes are the sources of pro-inflammatory cytokines majorly IL-1beta and TNF-alpha [34] which stimulate their own production, induce chondrocytes and synovial cells to produce other cytokines such as IL-6, IL-8, IL-4, IL-10, IL-11, IL-17, leukocyte inhibitory factor (LIF) and promote catabolic state in cartilage [72]. They are also involved in increasing the ROS generation.

IL-1 beta and IL-17 increase the production of nitric oxide (NO) [67, 73]. Elevated levels of NO induced by IL-1, decrease the IL-1 receptor antagonist synthesis, further increasing the IL-1 level and its associated damage to cartilage [74]. IL-1 and to lesser extent IL-6 lead to transient accumulation of H₂O₂ in mitochondria as they are involved in spatial and temporal stimulation of Mn-SOD associated with delayed increase in glutathione peroxidase levels and decreased catalase levels leading to oxidative stress [75]. IL-1, Interferon gamma and tumor necrosis factor (TNF) prime the chondrocytes to produce significant amounts of H₂O₂ [64, 76]. In normal human chondrocytes IL-1, TNF alpha stimulation decreased the MRC complex I, ATP production and mitochondrial membrane potential and also have been found to reduce the proteoglycan (PG) content in extracellular matrix of normal cartilage [77]. ROS act as signalling intermediates [67, 78], mediate the signalling pathways induced by inflammatory cytokines [79]. Superoxide radicals have been

found to play a pivotal role in inflammation especially in inflammatory joint disease like Osteoarthritis (OA) thus ROS promote cartilage matrix protein destruction and are also involved in inflammatory transformation promoting the transition of clinically silent cartilage to osteoarthritis (OA) [51]. Of the cytokines studied IL-4, IL-10 and IL-13 are the anti-inflammatory cytokines found to decrease the production of IL-1 β , TNF- α and MMPs; up-regulate IL-1 receptor antagonist and tissue inhibitor of matrix metalloproteinases [34].

1.7 Role of ROS on chondrocyte functions

High doses and /or inadequate removal of ROS resulting in oxidative stress damage biological macromolecules and cause severe metabolic malfunctions. Presence of oxidative damage in aging and degenerating cartilage has been indicated in cartilage explants of elder and osteoarthritis donors compared to young donors by the over expression of an oxidative stress marker, nitrotyrosine [43]. Oxidative stress has been found to induce cell senescence *in vitro* [80]. Cell growth was observed to decrease with increase in oxygen derived radicals produced in rabbit articular chondrocyte cultures [81]. Induced oxidative stress on cultured human articular chondrocytes has been found to decrease the replication potential, induce telomere instability as observed with short telomere length. And antioxidant treatment was reported to rescue the chondrocytes by increasing the replication potential and elongating the telomere length indicating the role of oxidative stress in cell senescence by inhibiting the rate of chondrocyte proliferation [82]. The same study which investigated the catabolic changes in cartilage found that under oxidative stress condition, glycosaminoglycan (GAG) content was decreased compared to control and antioxidant treated, which had

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

maintained the GAG content better than control. Thus oxidative stress may be closely involved in chondrocyte senescence that may contribute to the risk for cartilage matrix degeneration [83, 84].

1.8 Role of ROS in cartilage damage

Studies have shown that ROS generated due to mechanical strain [20], overload, impact injury [85], local intra-articular lesion and consecutive synovial inflammation is involved in cartilage damage. An experimental mouse model injected with intra-articular glucose oxidase, studied the *in vivo* H₂O₂ mediated cartilage damage and inflammation of nearby tissues in the knee joint which was inhibited upon catalase administration to the site [86]. ROS imbalance the anabolic and catabolic activities involved in the extra cellular matrix formation in chondrocytes. Oxidative stress has shown to decrease the proteoglycan (PG) production by chondrocytes [87], induce telomere instability and down regulate the chondrocyte function [82]. Collagen fibril architecture is impaired and susceptible for degradation by matrix metalloproteinases (MMPs) when small leucine rich repeat proteins (SLRPs) are deficient, whose function depend on core protein and GAG [4]. Thus ROS mediated GAG loss could induce structural and functional alterations in cartilage matrix [82]. H₂O₂ produced by xanthine oxidase and glucose oxidase systems in cultures of bovine articular cartilage has shown a decrease in hyaluronic acid [88], polymerization of HA [20]and PG synthesis [88, 89]. Superoxide radicals, NO can initiate lipid peroxidation reactions which is shown to be involved in oxidation of cartilage collagen in isolated rabbit chondrocytes [55]. NO has shown anti-anabolic role by inhibiting the PG synthesis and anti-catabolic role with respect to PG degradation in different zones of articular cartilage [90]. Proteolytic cleaved

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

fragments of fibronectin in the degenerating cartilage, release and degrade the collagen binding molecules [91], and along with fragmented collagen II and hyaluronan, stimulate the MMP, aggrecanases production [92, 93] mediated by ROS but not NO as obligatory secondary messengers [94]. Extracellular matrix is a key site and regulator of cytokine, growth factor binding and release. Modification of its composition and integrity by oxidative damage is expected to alter its role [95].

1.9 ROS and Matrix metalloproteinases

Matrix metalloproteinases (MMP) are the large group of enzymes that have a role in tissue remodelling as well as cartilage and bone destruction in arthritic joint. They are involved in degrading collagen II which is the major component of cartilage matrix [96, 97]. Of the 27 MMPs four classic collagenases are MMP-1, MMP-8, MMP-13 and MMP-14. MMP-1 is involved in degrading newly synthesised collagen and MMP-13 degrades collagen resident in superficial and middle zone of cartilage [98]. Cleavage of collagen II by MMP-13 is found to initiate chondrocyte differentiation and matrix mineralization [99]. ROS play as key regulators of MMP production by directly regulating the activity of redox sensitive transcription factor activating protein (AP-1) required for MMP production, in many disease pathologies including degenerative disorders [67, 78, 100]. It has been found that exogenous and endogenous NO in chondrocytes is able to induce metalloproteinase activity [101] and MMP-3, MMP-13 expression [102]. H₂O₂ and IL-1 have found to increase MMP-1, MMP-3 expression [103]. Aggrecan is cleaved by both different MMPs and aggrecanases.

2.0 Mitochondria and ROS

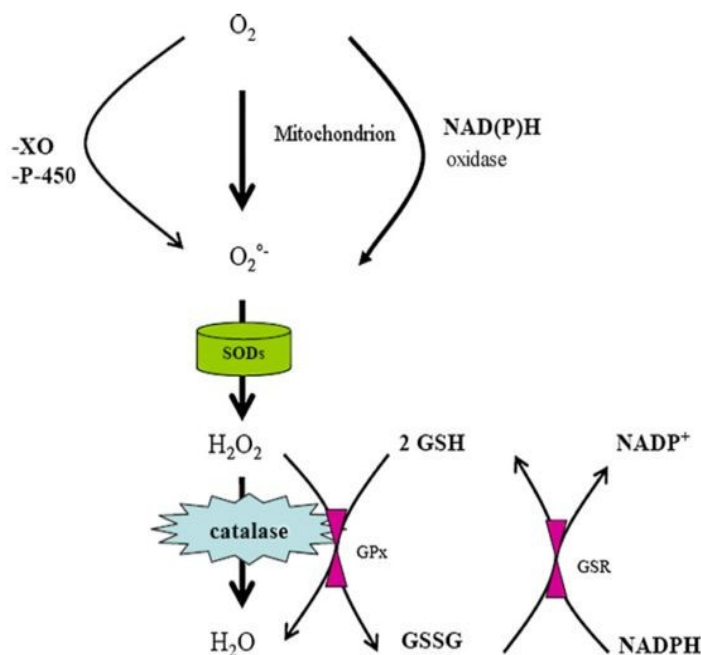
Although cartilage has anaerobic metabolism with glycolysis being the primary ATP source, mitochondria serve as ATP reserve in stressed chondrocytes. Also chondrocyte mitochondrial impairment has been investigated to be one of the mediators in cartilage functional compromise, onset and progression of OA [104]. It has been reported that mitochondrial respiratory chain (MRC) activity and mitochondrial membrane potential (MitMP) are reduced in cultured human OA chondrocytes compared to normal donors [105]. In normal human articular chondrocytes, NO was found to reduce the MRC complex IV activity, MitMP and decrease the chondrocyte survival [106]. A low concentration of ROS in presence of NO might promote apoptosis, while a high concentration of ROS might promote necrosis [107, 108]. From the study of Grishko et al., [54] it can be found that oxidative stress in chondrocyte mitochondria lowers the mtDNA repair capacity accumulating the mtDNA damage in OA chondrocytes to a great extent than normal, correlating with decreased viability and increase in apoptosis induction [109]. Loss of mitochondrial dysfunction resulting in excess ROS stimulate the activation of caspases and leads to cell death [110-112]. ROS produced by the mitochondria are the key regulators of MMP production [100]. Inhibition of mitochondrial respiratory chain complex III and V with antimycin-A and oligomycin has shown to induce ROS synthesis in human articular chondrocytes [113] and increase in MMP-1 and MMP-3 expression.

2.1 Antioxidant system

Cells possess anti-oxidative defence system that delays or prevents the oxidation of cellular molecules from ROS [114]. When oxidative stress arises as a result of pathologic event, the defence system promotes the regulation and expression of anti-oxidative enzymes. Chondrocytes constitutively synthesize a well co-ordinated enzymatic antioxidant system to protect the cells against the harmful effects exhibited by ROS on cell functions [115, 116]. These include superoxide dismutases (SOD), which are the first line of antioxidant defence and catalyse the dismutation of the reactive superoxide into hydrogen peroxide (H_2O_2). Three isoforms of SOD has been found in different compartment in the cell. SOD1 (Cu/Zn-SOD) is found in cytoplasm, SOD2 (Mn-SOD) in mitochondria and SOD3 in association with GAGs such as heparin and heparan sulphate in extracellular space [48, 117]. Reduction of H_2O_2 by the Fenton reaction to the highly toxic hydroxyl radical can be harmful to the cell and chondrocytes do synthesize H_2O_2 detoxifying enzymes majorly catalase and glutathione peroxidase (GPX) which converts it into molecular oxygen and water. Catalase is one of the efficient enzymes against severe oxidant stress as it cannot be saturated by H_2O_2 at any concentration. It is widely expressed in peroxisomes and also found in mitochondria and cytosol. Cytosolic and mitochondrial glutathione peroxidase catalyzes the reduction of variety of hydroperoxides (ROOH and H_2O_2) at the expense of glutathione (GSH). Glutathione redox cycle is major source of protection against low oxidative stress in the cell.

Glutathione (GSH) a major tri-peptide (Cys-Glu-Gly) cellular antioxidant molecule detoxifies the H_2O_2 and is also involved in

scavenging peroxynitrite [118]. Cysteine possesses a sulfhydryl group (thiol moiety) that serves as a proton donor and is responsible for the biological activity of GSH. Glutathione reductase regenerates reduced GSH from oxidised GSSG at the expense of NADPH. Inhibition of peroxide metabolizing systems like catalase and GSH has shown cartilage becoming vulnerable to proteoglycan synthesis inhibition by H_2O_2 , projecting the role of these defence systems in maintaining the normal biosynthetic rates during oxidative stress [115]. Apart from these enzymes peroxiredoxin5 (PRDX5) a newly discovered thioredoxin peroxidase is able to detoxify H_2O_2 [119]. Water soluble ascorbate and lipid soluble vitamin E are able to terminate peroxidative chain reactions preceding the oxidation of cartilage collagen [55] and maintaining its stability [120].



ROS detoxification by antioxidants

(From: [Joint Bone Spine](#). 2007 Jul;74(4):324-9. Epub 2007 May 24. Reactive oxygen species and superoxide dismutases: role in joint diseases. [Afonso V¹](#), [Champy R](#), [Mitrovic D](#), [Collin P](#), [Lomri A](#).)

Dr. Smitha Tulasigeri.Totiger

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

2.2 Growth factors

Growth factors that regulate the metabolism of articular cartilage are produced from chondrocytes and are also derived from synovial fluid and surrounding tissues. Insulin like growth factor-1(IGF-1) is a major serum mitogen found to increase chondrocyte proliferation, matrix production increasing collagen II, IX, XI but not collagen X and proteoglycan secretion in chick embryo sternum isolated chondrocytes [121]. Growth factors apart from their true anabolic activity, have displayed potential anti-catabolic effects in cartilage repair and injury. The review by Schmidt et al., [122] summarizes the effect of IGF-1 and PDGF on cartilage repair, where IGF-1 *in vitro*, stimulates the proteoglycan (PG) synthesis in cultured chondrocytes and meniscal explants, slows the PG catabolism in cartilage. *In vivo* cartilage repair was seen, on IGF supplementation, with defects been filled with hyaline like repair tissue well integrated. IGF has shown to decrease the synovial inflammation. PDGF increases the cell proliferation, proteoglycan synthesis although it does not seem to have any effect in increasing collagen production, maintaining the chondrocyte phenotype in cultured monolayer chondrocytes. GDF5 and BMP5 are essential in the maintenance and repair of synovial joints as well as chondrogenesis and chondrocyte proliferation.

Many studies on growth factor effect on chondrocytes, highlight the role of growth factor OP-1, TGF-beta1, BMP-2 in maintaining articular cartilage by synthesising proteoglycans, collagen and balancing the anabolic and catabolic functions of chondrocyte [123-127]. Of the growth factors studied, basic fibroblast growth factor (bFGF) stimulation in bovine articular chondrocytes has shown to produce ROS by NADPH

oxidase [78] which could explain one of the way growth factor mediated ROS play a role in maintaining physiological signalling pathways. bFGF has been found to have anabolic as well as catabolic effects in maintaining cartilage homeostasis [128].

Dr. Smitha Tulasigeri.Totiger

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

CHAPTER 2

AIM OF THE WORK

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

Chondrocytes the only cell type present in articular cartilage is the primary mediator of anabolic and catabolic process. Deterioration of the cell functions due to oxidative stress has been found to be the initial stage changes involving disruption of the cartilage homeostasis. Eventually these changes progress to the final stage of osteoarthritis involving series of catabolic damage mediated by pro-inflammatory cytokines, MMPs, aggrecanases and cell death [58, 129]. Though cartilage damage is the principal feature of OA, it is a whole joint disease involving surrounding tissues with synovial inflammation and changes in the subchondral bone with aging [130]. The very first action of increasing and accumulating ROS is to disturb the homeostasis in chondrocyte and mediate catabolic changes in both chondrocyte and extra cellular matrix. ROS mediated cell senescence with decreased proliferation and increased cell death is the major cell function damage [51]. In addition to it one of the fact that the depletion of, and/ or inability of cells to keep in pace the synthesis of anti-oxidative enzymes to protect the cells against ROS induced cellular and molecular damage determines the progression of the disease pathology.

We know that cartilage is an avascular tissue with limited ability for auto-regeneration after the damage. It has less density of chondrocytes, lacks migration of mesenchymal cells. Turnover of ECM molecules by the chondrocytes is very slow in normal adult cartilage. Many treatments including subchondral penetration of bone, microfracture technique [131], articular lavage and debridement of damaged cartilage, abrasion arthroplasty [132] aimed at mesenchymal stem cell stimulation showed initial improvement but deteriorating effects overtime. *In vivo* the subchondral bone mesenchymal cells may form connective tissue which is not a true hyaline cartilage as produced by chondrocytes but is

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fibrocartilaginous in nature with type I collagen [133]. It has poor mechanical stability, with diminished resilience and wear characteristics. Cartilage defects replacement with fresh allograft, autograft have the limitation posed by the potential risk of disease transmission and abnormal stress- strain distribution in cartilage respectively [134]. Autologous cartilage implantation is promising and also an expensive treatment. Research in biomaterial engineering is yet to achieve satisfactory results on human models. All these therapies may diminish the symptoms by reducing the pain and inflammation but possess a major challenge to restore original hyaline cartilage making the integration of the repair tissue into surrounding tissue problematic.

Growth factor stimulation of cartilage repair and regeneration is one of the promising effective treatments. Much of the studies carried out have evaluated the effect of a single growth factor. Given the fact that growth factors work in concert to regulate and maintain the homeostasis in cartilage, it is unlikely that a single growth factor can affect the damaged cartilage. *In vitro* studies have shown the importance of autologous human serum in chondrocyte monolayer expansion, in maintaining proliferation and minimising apoptosis, and 3D cartilage construct development [135-137]. Platelet rich plasma therapy for arthritis is gaining prominence.

Since oxidative stress is the mediator of pathogenic changes seen at the initial stages of osteoarthritis, we wanted to demonstrate the ability of growth factors to antagonize the death and/or cell damage due to oxidative stress in cultures of healthy chondrocytes. Thus our work aimed to study the effect of combination of growth factors against H₂O₂ induced oxidative stress on cultured chondrocytes.

With this background for our study we concentrated on the fluid that is released as a natural inflammatory response in order to heal the major wound occurred during the knee operation done for a prosthesis replacement. This kind of fluid rich with growth factors, anti-inflammatory substances secreted by adjacent tissue in the articulation, is collected in wound drainage reservoir but is usually discarded. The post-operation drainage was obtained and isolated sera from it (emartri). Our study aimed at investigating if emartri has any protective role against the H₂O₂ - elicited oxidative stress in chondrocytes. Haematic serum obtained post-operation was included in our study to compare and evaluate the difference in response given by these two kinds of sera. Following which our study involved the three objectives.

1. Investigation of changes in intra-cellular ROS levels and redox status in cellular compartments in chondrocyte culture pre-treated with emartri and haematic sera with subsequent exposure to H₂O₂ induced oxidative stress.
2. Investigation of changes in proliferative ability of chondrocytes in culture under the same condition of objective 1.
3. Investigation of changes in anti-oxidative status in the cell extract of chondrocyte culture under the same conditions of objective 1.

CHAPTER 3

MATERIAL AND METHODS

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

3.1 Reagents

- HC-a cell line and chondrocyte medium along with supplements was purchased from Innoprot.
- SOD, catalase, GSH assay kits were purchased from Bio vision.
- BrdU cell proliferation elisa kit was from Roche.
- H₂DCFDA was from Molecular Probe, Eugene.
- FCS was from Gibco.
- L-Glutamine and trypsin were from Lonza.
- Cytobuster protein extraction buffer was from Novagen.
- DMEM, penicillin-streptomycin antibiotic, HBSS, Protease inhibitor, phosphatase inhibitor cocktail 2 and 3 were purchased from Sigma Aldrich.
- HEPES was purchased from Invitrogen.
- Phosphate buffered saline was prepared.

3.2 Study subjects

Patients who were undergoing prosthesis replacement at knee were selected for collecting the post operation fluid from the operated site, in a wound drainage reservoir. Patients were in the age group between 50 to 65years. The sera isolated from the fluid were called as emartri. Post-operation, the haematic sera from the peripheral blood of the same patients were isolated.

3.3 Cell culture and treatments

Human primary articular chondrocytes (HC-a, Innoprot) were cultured in chondrocyte medium supplemented with growth factors (Innoprot) and antibiotic (Innoprot) at 37°C in a humidified atmosphere with 5% CO₂. On reaching confluence the cells were split using Trypsin-EDTA and sub-cultured in DMEM complete medium (10% FCS, 0.68mM L-Glutamine, 100Upenicillin, 100ug streptomycin) and used within five passages.

Unless specified the cells were grown in 96wells black plates (Costar) until 70 to 80% sub-confluence. In all the experiments carried out, the cells underwent a serum free adaptation for 8hours before treating with emartri and haematic sera at 5% final concentration for 12hours respectively. Except for the ROS evaluation and redox status evaluation in cytoplasmatic and mitochondrial compartment, due to the limitation of the probe used in evaluation, the cells were treated with H₂O₂ at 300uM final concentration for 3 hours without removing the serum treatment.

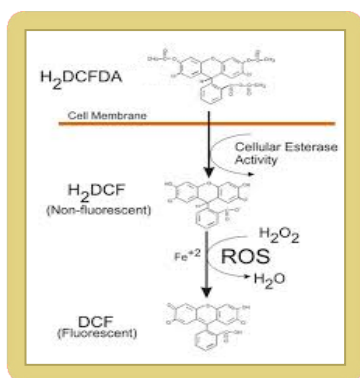
3.4 Measurement of intracellular ROS levels

Intracellular ROS levels were determined by using the ROS molecular probe 2',7'-dichlorodihydrofluoresceindiacetate (H₂DCF-DA) (Molecular Probe, Eugene, OR) as previously described with minor modification [138]. Upon entering the cells H₂DCF-DA are cleaved at the diacetate groups by esterase and the reduced form of the probe, H₂DCF, thus gets trapped inside the cell. Intracellular ROS oxidize H₂DCF, yielding the fluorescent product, dichlorofluorescein (DCF).

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari



After 12hrs of incubation of chondrocytes with the sera, the treatment is removed and the cells are incubated with Hanks balanced salt solution containing probe H₂DCF-DA at 5uM concentration for 15minutes. Later which the probe is removed, cells are washed with PBS. Oxidative stress is induced by incubating with Hanks balanced salt solution containing H₂O₂ at a sub lethal dose of 300uM. Maintained control for each sera treatment without adding H₂O₂ in HBSS respectively.

3hours kinetic measurement of the DCF fluorescence is measured using GENios plus microplate reader (Tecan, Männedorf, CH) at excitation and emission wavelengths 485nm and 535nm respectively. All fluorescence measurements were corrected for background fluorescence. Intracellular ROS levels were evaluated for each sera treatment maintaining triplicate wells.

3.5 Determination of cytoplasmatic and mitochondrial redox status

Intracellular oxidative status in both the cytoplasm and mitochondrial compartment was measured by using HCa infected with lentiviral particle harbouring the redox sensing green fluorescent protein (roGFP) [139]. roGFP has a significant dynamic range and responds linearly to increasing doses of a well known oxidant. It can be targeted to various

Dr. Smitha Tulasigeri.Totiger

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

cellular compartments [140]. Stable transfectants constitutively expressed roGFP in cytoplasmatic and mitochondrial compartment respectively as observed under fluorescence microscope.

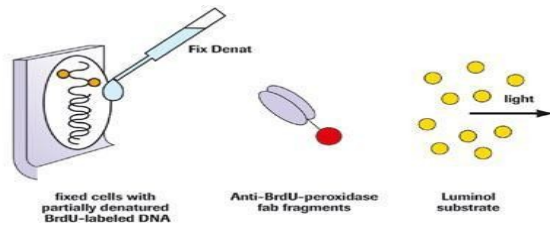
After the treatment the transduced chondrocytes with roGFP or mitochondrial roGFP were washed with PBS and incubated with HBSS containing 300uM H₂O₂ and HBSS without H₂O₂ to serve as control in each treatment group. Measured the fluorescence by GENios plus microplate reader (Tecan, Männedorf, CH) where the oxidised form of roGFP has fluorescence excitation maxima at 400nm and the reduced form of roGFP has fluorescence excitation maxima at 485nm. The ratio of fluorescence at 400nm and 485nm gives the information about the extent of oxidative status in cytoplasmatic and mitochondrial compartment respectively. Oxidative status was evaluated for each sera treatment maintaining triplicate wells.

3.6 Measurement of cell proliferation

Cell proliferation is measured by an Elisa assay based on the detection of 5-bromodeoxyuridine (BrdU, a pyrimidine analogue of thymidine) incorporated into the genomic DNA of proliferating cells.

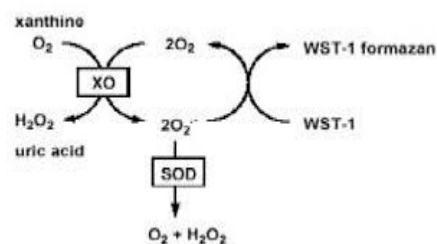
After the treatments cells are incubated with DMEM with 2.5% FCS containing BrdU probe at 10uM concentration for 10hours. During this labelling period, BrdU is incorporated in place of thymidine into the DNA of proliferating cells. With the removal of the labelling medium cells are fixed and the DNA is denatured in one step by incubating with FixDenat for 30' at ambient temperature. Following with the removal FixDenat, cells are incubated with anti-BrdU-POD antibody for 90' at ambient temperature; anti-BrdU-POD is bound to the BrdU incorporated into the newly synthesized cellular DNA. The immune complexes are

detected by the subsequent substrate reaction. The reaction product is quantified by measuring the luminescence in GENios plus microplate reader (Tecan, Männedorf, CH). Cell proliferation was evaluated for each sera treatment maintaining triplicate wells.



3.7 Measurement of superoxide dismutase (SOD) activity

Superoxide dismutase (SOD) activity was determined using Bio vision kit reagents. The kit utilizes WST-1 that produces water-soluble formazan dye upon reduction with superoxide anion, liberated due addition of enzyme working solution in the kit. The rate of reduction with superoxide is linearly related to the xanthine oxidase activity, and is inhibited by SOD. The inhibition activity of SOD can be determined colorimetrically.



To extract the proteins the cells were plated in 6well plate (BD Falcon). After the treatments the cells were washed with chill PBS and incubated with cytobuster reagent, containing protease and phosphatase inhibitors at 1X concentration, on ice for 5minutes to lyse the cells. Using cell

scrapers the cells were scrapped from the wells, collected in a vial and spun at 16000xg, 4°C for 10 minutes. The supernatant was collected and determined the total protein content. The volume of supernatant of each samples containing equal protein amount was used to measure the SOD activity.

3.8 Measurement of catalase activity

Catalase activity was determined using Bio vision fluorometric assay kit protocol and reagents. In each extract maintained a high control sample by adding the stop solution that inhibits the activity of catalase. 1mM H₂O₂, prepared from the kit stock, is added in each wells and incubated for 30 minutes at ambient temperature according to the protocol. The reaction in sample wells is stopped by adding stop solution. Upon incubation with developer mix for 10 minutes at ambient temperature read the fluorescence at excitation 535nm and emission 590nm in GENios plus microplate reader (Tecan, Männedorf, CH).

The unconverted H₂O₂ reacts with OxiRed™ probe present in the developer to give the fluorescence. Thus difference measured between fluorescence of high control and sample is used to determine the H₂O₂ converted by the catalase present in the sample with respect to the H₂O₂ standard curve generated. The catalase activity in each sample was corrected to the total protein present in the extract.

3.9 Measurement of Glutathione (GSH) activity

Glutathione (GSH) activity was determined using Bio vision fluorometric assay kit protocol and reagents. ortho-phthalaldehyde (OPA) reacts with GSH (not GSSG), generating fluorescence, thus

specifically quantifying GSH. Addition of a reducing agent converts GSSG to GSH, so (GSH + GSSG) can be determined.

To extract the proteins the cells were plated in 6well plate (BD Falcon). After the treatments cells were washed with chill PBS and incubated with assay buffer provided in the kit for 5minutes on ice. Scrapped the cells and transferred the cell suspension into the vial containing perchloric acid provided in the kit at the ratio 3:1 to concentrate the GSH. Vortex to form an emulsion and then incubated on ice for 5minutes. Centrifuged the emulsion at 13000xg , 4°C for 2minutes to collect the supernatant.

Some volume of cell suspension after the scrapping was centrifuged at 16000xg, 4°C for 10mintutes. From the supernatant collected determined the total protein by Bradford assay.

The GSH before and after adding reducing was quantified using the GSH standard curve generated simultaneously in the 96black plate. The fluorescence values were corrected for background fluorescence after addition of reducing agent.

The difference between GSH and GSH+GSSG gives the oxidised form of GSSG in the extracts. Ratio of GSSG to GSH will give the information about the redox status maintained by the GSH in the cell.

3.10 Statistical analysis

Results are presented as the mean \pm SD. One way ANOVA followed by post comparison Tukey test was used to assess statistical significance among treatments. Unpaired t-test was used to compare two data means. P values less than or equal to 0.05 were considered as statistical significance.

CHAPTER 4

RESULTS

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4.1 Effect of emartri and haematic sera on H₂O₂ - elicited oxidative stress.

Studies have shown that ROS are involved in the initiation of changes in cartilage metabolism involved in the early phase of OA. In our study we mimicked the oxidative stress condition by treating the chondrocytes with H₂O₂ at 300uM concentration. We wanted to test if the emartri and haematic sera have any effect on the intracellular ROS levels due to H₂O₂ - elicited oxidative stress. Thus, the chondrocytes were pre-treated with emartri and haematic sera for 12hours before inducing the oxidative stress. The controls for each group were not stimulated with H₂O₂ stress. Our results (fig.1) shows that in emartri pre-treated group upon induction of oxidative stress by H₂O₂ the increase in intracellular ROS levels was non-significant compared to its control. Whereas, in haematic pre-treated group we found a significant increase in intracellular ROS levels compared to its control.

Effect of emartri and haematic sera on intracellular ROS levels

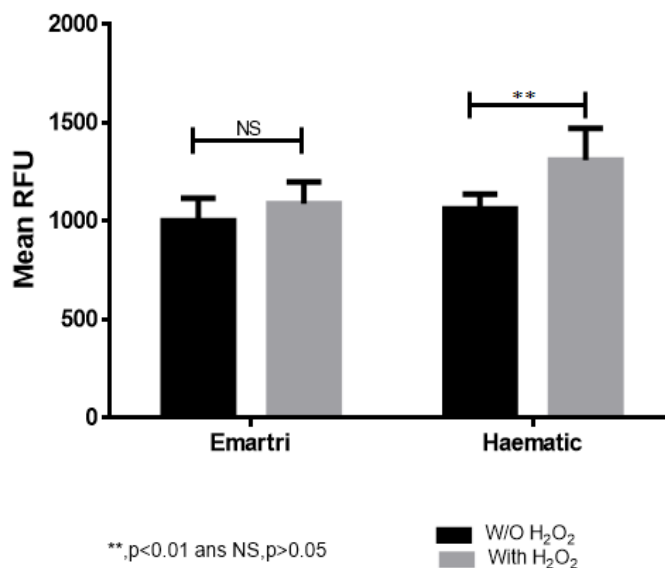


Figure 1

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

The increase in intracellular ROS levels due to induced oxidative stress was minimal in emartri than the haematic sera treated group.

We see a protective effect of emartri against the induced oxidative stress compared to the haematic sera which can be better understood in the second graph (fig.2), where we assessed the difference in relative fluorescence unit (RFU) between H₂O₂ treated and control in both sera treated group.

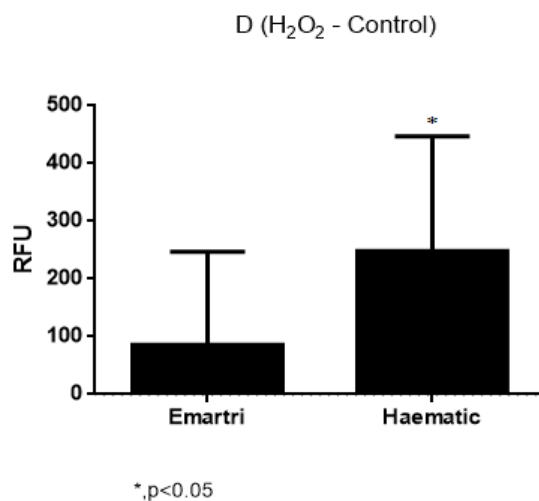


Figure 2

Emartri was able to minimise and/or counteract the effect of H₂O₂ as shown by a minimal increase in intracellular ROS levels due to induced oxidative stress. Whereas the haematic sera was not able to effectively counteract the oxidative stress as the increase in intracellular ROS levels was significantly higher as observed in the graph.

4.2 Effect of emartri and haematic sera on H₂O₂-induced changes of the cytoplasmic redox status.

Cytosol is the venue for many ROS synthesis. Redox dependant signalling pathways are channelized through cytoplasm. Hence we were interested to know the effect of emartri and haematic sera pre-treatment on the redox status of cytoplasm against H₂O₂ - elicited oxidative stress.

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

Lentiviral vector harbouring redox sensing green fluorescent protein based detection of oxidative status is more sensitive and accurate since the protein can be channelized to particular compartment in the cell.

We investigated if there is any protection accounted by the emartri against induced oxidative stress on cytoplasmatic redox potential.

Effect of emartri and haematic sera on H₂O₂ - induced changes of cytoplasmatic redox status

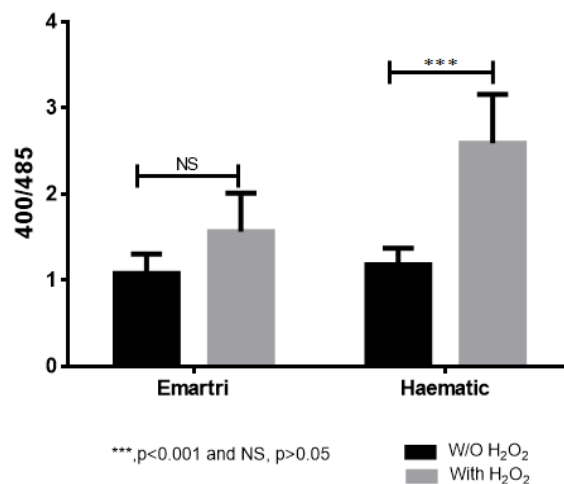
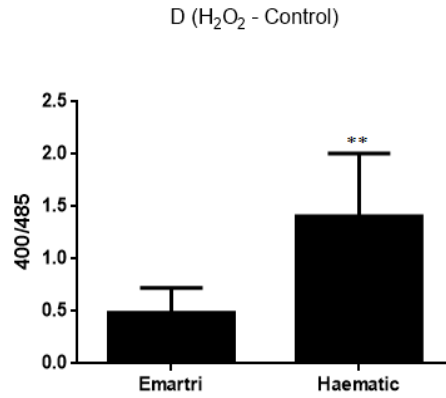


Figure 3

We see that in haematic sera treated group, upon induction of oxidative stress by H₂O₂ the extent of oxidation in cytoplasm was significantly higher than the respective control (fig.3). Whereas in emartri treated group, upon induction of oxidative stress the extent of oxidation in cytoplasmatic compartment was not significantly higher than respective control. The increase in oxidative condition was minimal in emartri treated group compared to haematic sera treated group.

We see a protective effect of emartri under induced oxidative stress condition compared to the haematic sera which can be better understood by assessing the difference in ratio of the fluorescence between H₂O₂ treated and respective control in each group.



**₂, p<0.01

Figure 4

From the graph (fig.4) we see that there is a minimal increase in oxidative status due to induced oxidative stress under emartri pre-treatment and thus emartri is able to minimise and /or counteract the effect of H₂O₂ in cytoplasm. Whereas the haematic sera is unable to effectively counteract the oxidative stress as the increase in extent of oxidation was significantly higher.

4.3 Effect of emartri and haematic sera on H₂O₂ - induced changes of the mitochondrial redox status.

Since mitochondria are one of the major source and also target of ROS, mediating the functional damage to chondrocytes [111], we therefore investigated if there is any protection accounted by the emartri against H₂O₂ - elicited changes of the mitochondrial redox potential.

The following graph in fig.5 shows the extent of oxidation determined in the mitochondrial compartment in each treatment group. We obtained the similar protective response exhibited by emartri against the induced oxidative stress on mitochondrial redox status as that of cytoplasmic compartment.

Effect of emartri and haematic sera on H₂O₂ - induced changes of the mitochondrial redox status

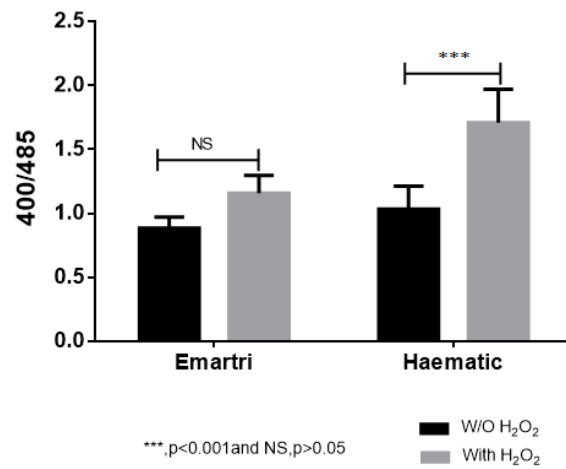


Figure 5

We see a protective effect of emartri evident compared to haematic sera under induced oxidative stress condition which can be better understood in the second graph.

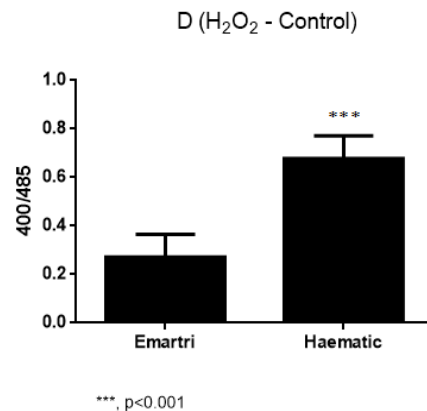


Figure 6

In this graph (fig.6), the difference in ratio of the fluorescence between H₂O₂ treated and respective control in both of the sera treated group is assessed. Emartri was able to minimise and /or counteract the effect of H₂O₂ as shown by a minimal increase oxidative status due to induced oxidative stress. Whereas the haematic sera was not able to effectively counteract the oxidative stress as the increase in extent of oxidation in mitochondria was significantly higher due to induced oxidative stress.

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

4.4 Effect of emartri and haematic sera on H₂O₂ - induced changes of cell proliferation.

The following graph in fig.7 shows the chondrocyte proliferation levels measured by luminol luminescence corresponding to the BrdU incorporated in newly synthesized DNA.

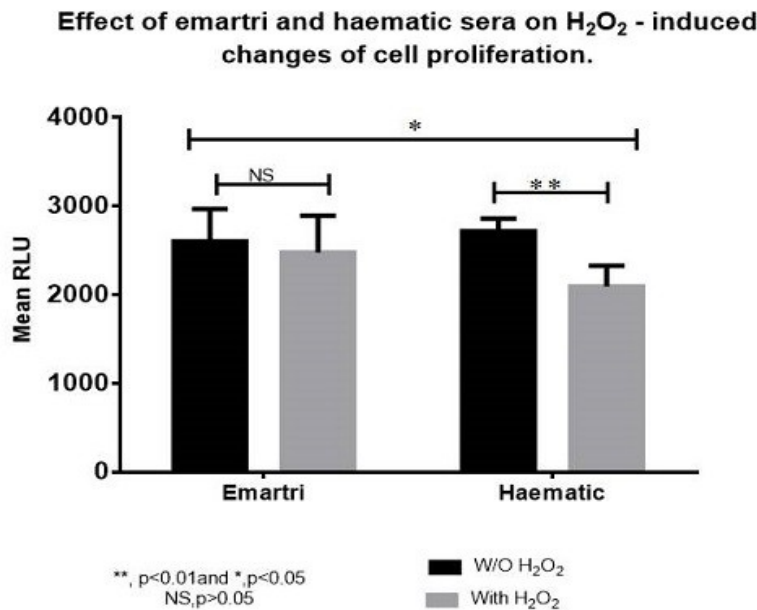


Figure 7

We see that in haematic sera pre-treated group, on inducing the oxidative stress by H₂O₂ the cell proliferation level was significantly lowered than the respective control. Whereas in emartri pre-treated, upon induction of oxidative stress by H₂O₂ the cell proliferation levels did not decrease significantly than respective control. The decrease in cell proliferation due to induced oxidative stress level was minimal in emartri treated group.

We see a protective effect of emartri under induced oxidative stress condition compared to the haematic sera which can be better understood from the second graph (fig.8).

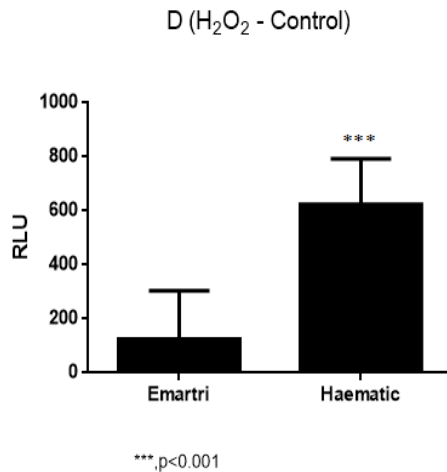


Figure 8

We assessed the difference in the luminescence values between H₂O₂ treated and respective control in both of the sera treated group. Delta here indicates the extent of cell death due to induced oxidative stress.

Emartri was able to minimise and/or counteract the effect of H₂O₂ as shown by a minimal increase in cell death due to H₂O₂ elicited oxidative stress. Whereas the haematic sera were not able to effectively counteract the H₂O₂ elicited oxidative stress as the increase in cell death was significantly higher.

4.5 Effect of emartri and haematic sera on H₂O₂ - induced changes of SOD activity.

The graph in fig.9 shows the SOD activity represented in % inhibition activity of SOD. We see that in both emartri and haematic sera pre-treated group, upon induction of oxidative stress by H₂O₂ the SOD level were lowered than the respective control but not significantly. It can be observed that in emartri treated group SOD activity remains higher than haematic treated group.

Effect of emartri and haematic sera on H₂O₂ - induced changes of SOD activity

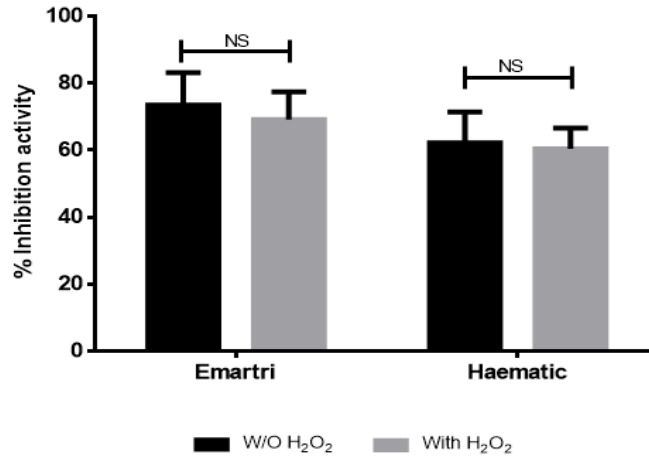


Figure 9

On assessing the difference in SOD activity between H₂O₂ treated and respective control in each treatment group. The delta obtained is the indicative of xanthine oxidase activity liberating superoxide radicals that are left un-neutralized because of decrease in SOD activity although the decrease in SOD activity is not significant between emartri and haematic sera treated group due to H₂O₂ induced oxidative stress (fig.10).

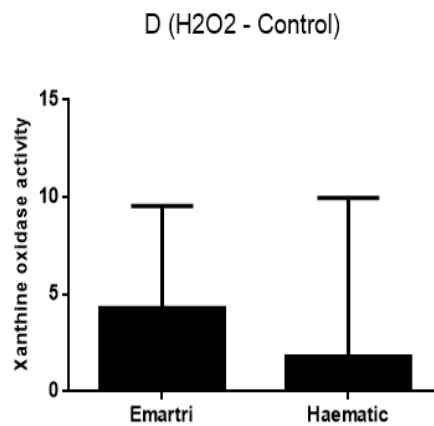


Figure 10

4.6 Effect of emartri and haematic sera on H₂O₂ - induced changes of catalase activity.

Next we determined the catalase activity represented in mU/mg protein.

Effect of emartri and haematic sera on H₂O₂ - induced changes of catalase activity

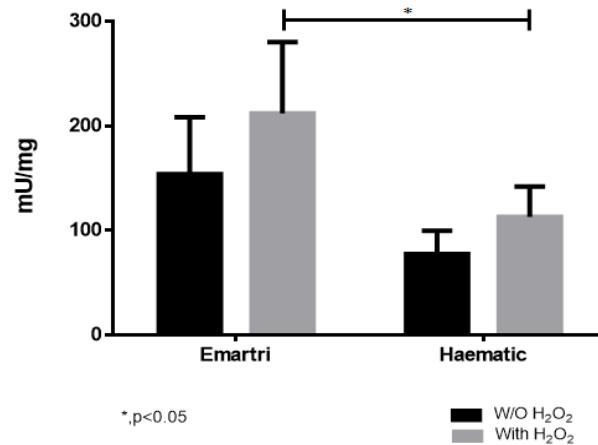


Figure 11

We see that in both emartri and haematic sera pre-treatment groups upon elicitation of oxidative stress by H₂O₂, the catalase activity increased but not significantly with respective controls (fig.11). We see a significant increase in catalase activity in emartri treated under oxidative stress compared to haematic sera treated under oxidative stress. If we assessed the difference in values of catalase activity between H₂O₂ and respective control in each sera treated group the increase in catalase activity due to H₂O₂ induced oxidative stress was higher in emartri than haematic sera pre-treated, although not significantly (fig.12). We see a protective role of emartri against oxidative stress by triggering the catalase synthesis.

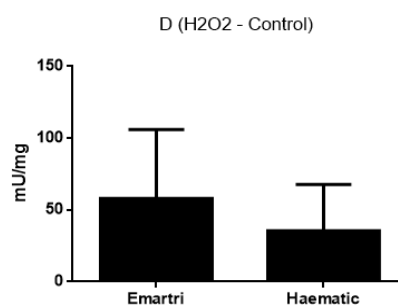


Figure 12

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

4.7 Effect of emartri and haematic sera on H₂O₂ - induced changes of GSSG/GSH ratio

Lastly we determined the GSH levels, both reduced and oxidised form in the cell lysate. We assessed the ratio between oxidised to reduced form of GSH to determine the oxidative status maintained due to GSH in the chondrocytes. In the fig.13 we see that both haematic sera and emartri pre-treated group upon induction of oxidative stress by H₂O₂ minimised the effect of oxidative stress by maintaining GSSG/GSH level to that of respective controls. But emartri pre-treated group was able to decrease the oxidative status significantly than the haematic sera pre-treated group as seen with lower GSSG/GSH ratio. It indicates that emartri pre-treatment was able to trigger the GSH synthesis to minimise the oxidative stress significantly than the haematic pre-treated group.

Effect of emartri and haematic sera on H₂O₂ - induced changes in GSSG/GSH ratio

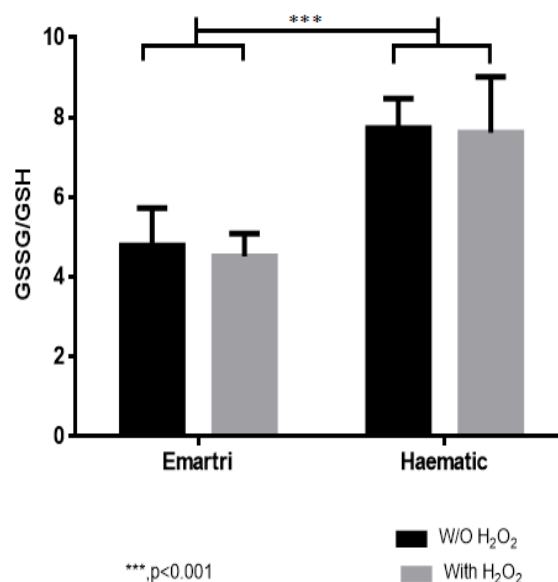


Figure 13

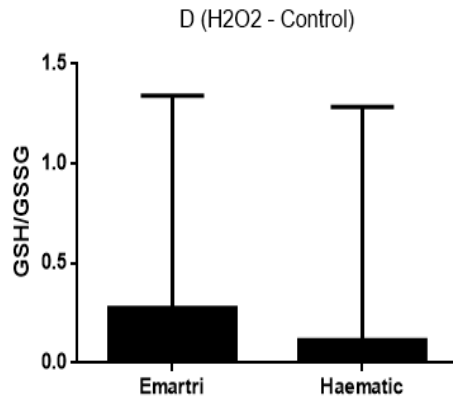


Figure 14

When we assess the difference in GSSG/GSH ratio between H₂O₂ treated and respective control in both sera treated group, we obtain the GSH/GSSG ratio, represented in the graph above (fig.14), which indicates that emartri is able to increase the intracellular redox status maintained by GSH compared to the haematic sera, although the increase was not significant.

CHAPTER 5

DISCUSSION

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

Reactive oxygen species (ROS) are not just involved in initiation of catabolic changes in cartilage but also in consistently mediating the redox sensitive signalling pathways regulated by pro-inflammatory cytokines and growth factors during progression of the disease pathology to the osteoarthritic (OA) stage. Even at advanced stages of OA when normal matrix contacts have been lost and autocrine survival signalling is reduced, endogenous ROS mediate cell death and administration of antioxidants have rescued the cell death in chondrocyte culture models [141].

Osteoarthritis patients who underwent an operation for knee replacement with prosthesis were the important subjects for our study. Emartri is isolated from the post operation knee drainage fluid, obtained at the site of operated knee, accumulated as a result of natural inflammatory response of the surrounding tissue, in order to heal the major injury that occurred during knee operation. This fluid has to be removed post operation, if not accumulates causing swelling. Thus normal post operation practise is to collect this fluid in a wound drainage reservoir to discard. Since this fluid is rich in anti-inflammatory substances and growth factors that are attracted to the site of injury, our study aimed at isolating the sera from it, called as emartri, and studying the effects of emartri on chondrocytes exposed to H₂O₂ - elicited oxidative stress. In OA condition, the soluble factors mediating the cartilage damage may be found in synovial fluid [142] as they get diffused into it in the normal process of exchange. Synovial membrane has the blood supply and some soluble factors like inflammatory and anti-inflammatory cytokines can be present in patient blood [142, 143]. Thus we involved in our study the haematic sera isolated from peripheral blood, of the same subjects, collected after the operation. We were interested to investigate and

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

compare the role exhibited by emartri and haematic sera against H₂O₂ - elicited oxidative stress on the chondrocytes.

H₂O₂ is involved in cell signalling, at a physiological level that is linked to reductive oxidative based mechanism, by oxidative activation or inactivation of enzymes and signalling molecules [144]. H₂O₂ can traverse the lipid bi layer by aquaporins to access the cytoplasm where most of its target protein exists [145]. In chondrocytes H₂O₂ has been found to suppress proteoglycan synthesis *in situ* and *in vitro* [89, 146] and reduce the proliferation [81]. H₂O₂ mediated oxidative damage was found on glycolytic enzymes decreasing the ATP levels which affected the chondrocyte functions including DNA and protein synthesis [147]. Thus H₂O₂ could induce substantial oxidative stress and associated damaging effects on chondrocyte functions *in vitro*. Hence, in our study we used H₂O₂ to induce oxidative stress in chondrocytes. 300uM concentration was found to be sub lethal on chondrocytes as observed from the preliminary study of H₂O₂ dose response on chondrocyte vitality. In all the experiments carried, chondrocytes were pre-treated with each type of sera at 5% concentration and then exposed to H₂O₂ induced oxidative stress.

Firstly, we investigated the effect or role of emartri and haematic sera against H₂O₂ - induced oxidative stress on intracellular ROS levels. In fig.1, controls in both the emartri and haematic sera pre-treatments showed almost same extent of intracellular ROS levels indicating that emartri, which is rich with pro-inflammatory, anti-inflammatory substances and growth factors, is able to maintain the ROS levels to that of physiological levels. When the chondrocytes pre-treated with emartri and haematic sera were exposed to H₂O₂ - induced oxidative stress, the intracellular ROS levels rose significantly compared to control in

Dr. Smitha Tulasigeri.Totiger

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Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

haematic pre-treated cells but not in emartri pre-treated. This confirms that soluble factors in emartri are able to protect the cells from H₂O₂ - induced oxidative stress but not by haematic sera which could be due to low, although physiological, levels of soluble factors, not sufficient enough to decrease the ROS levels. The difference in relative fluorescence values (RFU) between control and H₂O₂ exposed in each sera pre-treated group give the increase in ROS levels due to H₂O₂ - induced oxidative stress. From fig.2, our results on assessing the role of emartri and haematic sera against induced oxidative stress on the levels of intracellular ROS showed that chondrocytes pre-treated with emartri were able to sustain the oxidative stress, as evident by the low levels of rise in intracellular ROS where as in haematic sera pre-treated a significant rise in the intracellular ROS levels.

Cytoplasm has many enzyme systems that generate ROS as a by product during the reactions including NADPH oxidase, nitric oxide synthases (NOS), xanthine oxidase. Mitochondria are the source of ROS and in turn are vulnerable to ROS mediated damage. Study undertaken to investigate the role of mitochondria in degenerative disease like OA revealed higher mitochondrial dysfunction [105], mtDNA damage [54] in isolated OA chondrocytes compared to normal chondrocytes as shown by decreased activities of respiratory chain complexes II and III as well as reduction in mitochondrial membrane potential. Antimycin-A and oligomycin (specific MRC inhibitors) inhibited the MRC complex III and V activity respectively and induced ROS synthesis. Human chondrocytes have limited capacity to repair oxidative damage [54] as observed by a decrease in repair of mtDNA after an oxidative damage. This mtDNA damage can lead to mtDNA mutations that accumulate to produce aberrant MRC subunits ending with augmented ROS production

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

as observed in aging and degenerative diseases [148]. Also it has been found that inhibition of MRC in human articular chondrocytes induced inflammatory responses [113, 149] and cell death [106].

Thus we were next interested to find overall red-ox status in cytoplasm and mitochondrial region in chondrocytes by using HCa infected with lentiviral particle harbouring roGFP reporter system targeted to cytoplasm and mitochondria respectively. In fig. 3, we can observe that controls in both emartri and haematic pre-treatment showed near to same extent of red-ox status in cytoplasm. Upon induction of oxidative stress by H₂O₂ the emartri pre-treatment did not allow significant increase in the extent of oxidation in cytoplasm, showing a protective effect against H₂O₂ - induced oxidative stress. Whereas, haematic pre-treatment was unable to exhibit any protective role as there is a significant increase in the extent of oxidation in cytoplasm compared to respective control when exposed to H₂O₂ - induced oxidative stress. A similar pattern of response was observed in mitochondrial compartment (fig. 5) except for a finding although non-significant, the control in haematic pre-treatment, showed higher extent of oxidation than the control in emartri pre-treatment. Thus to know how far, effectively, the emartri is able to exert a protective role against the H₂O₂ - elicited oxidative stress, we measured the increase in extent of oxidation in cytoplasm as well as in mitochondrial compartment, by taking the difference in the ratios of fluorescence at 400 and 485nm between controls and H₂O₂ treated in each sera treated group (fig 4 and 6). Indeed the chondrocytes pre-treated with emartri was able to stabilize the redox status significantly in cytoplasm and in mitochondria under H₂O₂ - induced oxidative stress condition than the haematic pre-treated as observed with significantly lower levels of increase in extent of oxidation.

Studies have shown that age related increase in the prevalence of osteoarthritis is contributed by the *in vivo* chondrocyte senescence and decrease in the cartilage repair efficacy [83, 84]. And it is found that oxidative stress may be closely involved in chondrocyte senescence decreasing the rate of proliferation [82]. Cha et al., have demonstrated that oxidative stress induced by H₂O₂ increased beta-galactosidase activity and collagen X expression, hallmark of senescence, in human chondrocytes [150]. Although age is a risk factor for OA, OA is not an inevitable consequence of aging hence, senescence is not an inevitable fate a cell especially in case of chondrocytes which are highly differentiated and quiescent cells. But due to its sole contribution in maintaining the homeostasis of the cartilage and absence of vascular mediated supply of other cellular and soluble factors in damage conditions, it is vulnerable for damage progression. Oxidative damage to DNA contributing for stress induced senescence could explain this fate of chondrocytes. Thus to prevent the development and progression of osteoarthritis and improve cartilage repair for middle-aged and older patients one of the strategies is to slow the progression of chondrocyte senescence or to replace senescent cells.

Thus we aimed at investigating if the emartri which decreased the intracellular ROS levels, stabilizing the extent of oxidation in cytoplasm and mitochondria under H₂O₂ - induced oxidative stress condition was able to maintain or increase the proliferative ability of chondrocytes under the same condition. From the results obtained we found that chondrocytes pre-treated with emartri, under H₂O₂ - induced oxidative stress condition, were able to maintain the proliferative ability, although not increased, compared to its respective control (fig.7). Under induced oxidative stress condition in chondrocytes pre-treated with haematic

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

sera, we found a significant decrease in proliferative ability (fig.7) in concordance with a significant increase in intracellular ROS levels (fig.1) which indicates the absence of protection by haematic sera against the H₂O₂ - elicited oxidative stress on cell proliferative ability. Our results corroborate with the results of Cha et al., [150] where they were able to demonstrate that decreasing the ROS levels by a ROS scavenger beta-mercaptoethanol (BME) and subsequent oxidative stress effectively delayed the onset of senescence and dedifferentiation of chondrocytes during *in vitro* expansion. On assessing the cell death due to oxidative stress by taking the delta between the H₂O₂ treated and control in both the sera treated group (fig.8) we found that cell death was minimal under H₂O₂ - induced oxidative stress condition, in emartri pre-treated than haematic sera pre-treated indicating the role of emartri-contained growth factors in allowing the cells to proliferate sustaining the oxidative stress.

It should be noted that response of chondrocytes to cytokines and growth factors are also dependent on cellular redox status and intracellular antioxidant systems defending the ROS which play a major role in this regard. Studies have shown that oxidative stress decrease the growth factor mediated proteoglycan synthesis and responsiveness of chondrocytes to growth factors [37, 151]. But these studies concentrated on single growth factor response. Combination of growth factors like IGF and OP-1 have shown to increase the survival of and matrix synthesis by normal and osteoarthritic chondrocytes [152] suggesting that study of cumulative effects of such or more growth factors could give us solidifying evidence in support of using growth factor therapy in improving the cartilage health during the damage.

Involvement of ROS in mediating the chondrocyte and ECM components damage has invoked many studies on role of endogenous and exogenous antioxidants during the disease progression and as a therapy in alleviating ROS respectively. Importance of antioxidants against oxidative stress mediated damage to chondrocyte functions and cartilage has been concluded in the recent studies [82, 87, 153, 154] and many are concentrating on the antioxidant properties of medicinal plants [155], resveratrol [156], NSAIDs [157] and SOD mimetics [48] with an aim at not just relieve the inflammation pain but also locally protect cartilage against deleterious effects of ROS. Increasing the antioxidant content through diet [120], oral supplements [158] and / or intra articular administration [102] have been suggested to fight the ROS and decrease their damaging effects on chondrocyte functions [159] as chondrocytes through the dysregulation of its antioxidant systems become more susceptible to oxidant-mediated cell death [160]. To determine the long term effects of antioxidant diet, supplements and drugs require large clinical studies. However, it should be noted that chondrocytes have potential to increase their antioxidant status. It has been observed that in response to heat shock, shear stress, heavy metals, and oxidative stress SOD1 transcripts increase indicating that this enzyme is involved in cell responses to various sources of stress [48]. Studies have shown SOD being enhanced to detoxify the ROS produced in chondrocytes subjected to mechanical strain [20], increasing catalase activity in H₂O₂ stimulated chondrocytes as a cellular response to the intracellular flux of H₂O₂. Although chondrocytes respond to the stress by increasing their antioxidant defence sometimes cannot pace up to overwhelming ROS. It has been shown from the studies done by Taschan T et.al, [161] that the active molecules with low molecular mass present in the conditioned

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

medium from high density cultures provided to chick chondrocytes in low density cultures promoted survival and that could be replaced by antioxidants, cysteine and dithioerythritol suggesting a possible role of anti-oxidants in decreasing the cell loss under culture conditions. Potential anti-oxidant role of growth hormones has not been much explored yet, except in one study [162], showed that IGF-1 stimulation was able to decrease the ROS levels, ROS mediated cell death and increase the glutathione peroxidase (GPX) activity in rat articular cartilage. From our first two objective results, since we observed a protective role of emartri in decreasing the intracellular ROS levels, maintaining reduced status in cytoplasm and mitochondrial compartment and increasing the proliferative ability of chondrocytes against H₂O₂ - induced oxidative stress, we therefore were interested to look if emartri has any positive effects in increasing the anti-oxidative level in the cells under the same experimental condition. In chondrocyte antioxidant mechanism, SOD and catalase have been found to be constitutively expressed [116]. We therefore, investigated the levels of relevant antioxidant enzymes SOD, catalase and glutathione (GSH) ROS scavenger activity in cell lysates. Chondrocytes pre-treated with emartri showed higher SOD activity compared to haematic sera pre-treated (fig.9). The SOD activity analysed was in cell lysate extracted following protocol for total SOD. Thus our result may determine the activity of Cu/Zn SOD and Mn-SOD. Study on OA chondrocyte mitochondrial dysfunction has detected decreased levels of Mn-SOD [163]. In our study when oxidative stress was induced by H₂O₂, SOD levels were decreased in both emartri and haematic pre-treated but not significantly. It shows that both sera were unable to increase the SOD levels under oxidative stress condition. One reason for limited role of SOD could be

Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

H₂O₂. Although H₂O₂ could elicit other ROS generation in the cells, H₂O₂ is itself a product of SOD activity.

Catalase is an efficient H₂O₂ detoxifying enzyme. It is evident from the fig.11 that emartri pre-treatment shows increased catalase levels compared to haematic pre-treated group. When exposed to H₂O₂ - induced oxidative stress we observed an increase in catalase levels with respect to the controls, but the emartri pre-treated group is able to increase the catalase synthesis significantly compared to haematic pre-treated under H₂O₂ - induced oxidative stress condition. Delta assessment (fig.12) indicated the effect of emartri compared to haematic pre-treatment on chondrocytes in triggering the synthesis of catalase in response to oxidative stress as shown with higher catalase levels. Presence of 3-nitrotyrosine in aged and OA cartilage suggests oxidative damage from peroxynitrite [43] with a decrease in intracellular GSH concentration, as GSH are effective peroxynitrite scavenger [118]. In our study results (fig.13), compared to haematic pre-treated the emartri pre-treated group showed lower levels of GSSG/GSH ratio significantly. When both treatment groups were exposed to H₂O₂ - induced oxidative stress, we could observe a decrease in GSSG/GSH ratio with respect to controls although not significantly. But the delta assessment (fig.14) indicated that emartri pre-treatment was able to increase the GSH/GSSG ratio compared to haematic pre-treated, though this increase was not significant. Our results demonstrate that emartri pre-treatment is able increase the GSH concentration or able to maintain the reduced form of GSH may be by up-regulating the enzymes GSH reductase and thus effectively maintain a reduced cytoplasmic status as evident by low GSSG/GSH ratio under oxidative stress condition. Reduced

cytoplasmatic redox status is important in keeping proteins, enzymes of signalling pathway in functional form which are redox sensitive.

There is an extensive literature about the oxidative associated cartilage changes during the aging process [129]. Age related increase in GSSG/GSH ratio in chondrocytes implies a higher basal level of oxidative stress association with cartilage aging [153]. Therefore we could predict a higher oxidative stress to exist in OA cartilage. Our study investigated the effect of emartri on normal human chondrocytes exposed to oxidative stress. Thus it would be an interesting goal further to investigate the effect of emartri on isolated chondrocytes from aged and OA cartilage.

Our study showed that emartri which is supposed to contain growth factors and anti-inflammatory molecules is able to overcome the oxidative stress and its effects on cell death, by decreasing intracellular ROS levels, maintaining the cell proliferative ability and reduced status in cytoplasm and mitochondria and by triggering the antioxidant system in chondrocytes. Our result of emartri treatment support and propose the emartri-contained substance mediated up-regulation of anti-oxidative enzymes playing a protective role against the H₂O₂ - induced oxidative stress on intracellular ROS and cell proliferation levels. We hypothesize that since emartri is an autologous serum, it would be practically clinically safe to administer by intra-articular injection as a therapy to the patients in early stage who undergo an operation of positioning of the prosthesis, with chondropathies and/or in any case of an arthrosis in the initial stage in the other locations. These sera may also be stored at -80°C and used to necessity in case the patient had chondropathies same or a developing osteoarthritis in other locations.

CHAPTER 6

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Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

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Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

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Dr. Smitha Tulasigeri.Totiger

The chondropathies: study of antagonism between growth factors and reactive oxygen species; clinical applications in the early stages of chondral lesions

Ph.D thesis in Biochemistry, Physiology and Biomolecular Sciences, International Ph.D school in Biomolecular and Biotechnological Sciences, University of Sassari

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Dr. Smitha Tulasigeri.Totiger

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