EVALUATION OF THE POTENTIAL ROLE OF THYMOSIN ALPHA 1 IN BONE REMODELING

Thesis Submitted for the Award of the Degree of

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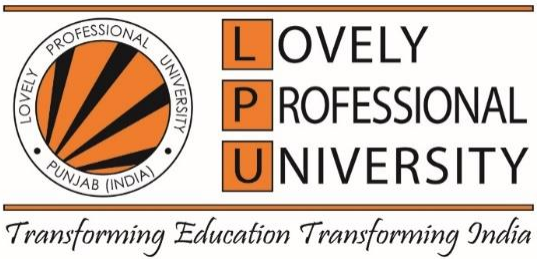

LOVELY PROFESSIONAL UNIVERSITY, PUNJAB

2024

DECLARATION

I hereby declare that the thesis entitled, **"Evaluation of the potential role of Thymosin alpha 1 in Bone remodeling"** submitted for Ph.D. in Clinical Biochemistry Degree to School of Allied Medical Sciences, Lovely Professional University is entirely original work and all ideas and references have been duly acknowledged. The research work has not formed the basis for the award of any other degree.

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CERTIFICATE

This is to certify that **Ms. Indu Bala (Registration No. 41700089)** has completed her Ph.D. Clinical Biochemistry titled, **"Evaluation of the potential role of thymosin alpha 1 in bone remodeling"** under my guidance and supervision. To the best of my knowledge, the present work is the result of her original investigation and study. No part of this thesis has ever been submitted for any other degree or diploma. The thesis is fit for the partial fulfillment of the condition for the award of the degree of Ph.D. in Clinical Biochemistry.

Signature of Supervisor
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ABSTRACT

The International Classification of Diseases recognizes around 150 diseases affecting the musculoskeletal system, encompassing muscles, bones, joints, tendons, and ligaments. These conditions vary in duration and impact, ranging from temporary issues like sprains to lifelong disabilities. Musculoskeletal problems, characterized by pain and limitations in mobility, can adversely affect an individual's work capacity, social engagement, mental health, and community well-being. Common conditions include osteoarthritis, back and neck pain, bone fragility-related fractures, traumas, and inflammatory disorders like rheumatoid arthritis. The Global Burden of Disease (GBD) study reveals the substantial disability caused by musculoskeletal disorders. Lower back pain has been a leading global cause of disability since 1990, and musculoskeletal issues ranked second in global disability in the 2016 GBD research. Approximately 20% to 33% of the world population experiences discomfort from musculoskeletal ailments. Bone is a complex structure composed of cells, blood vessels, and calcium compounds arranged in a crystalline pattern. Its primary functions are multifaceted. Firstly, bone plays a crucial role in maintaining "calcium homeostasis" and serves as a vital repository for storing essential elements such as phosphate, magnesium, potassium, and bicarbonate. Secondly, it provides crucial structural support to soft tissues and functions as a lever facilitating muscular movement. Lastly, bones serve as the primary site for the haematopoiesis process, contributing significantly to the formation of blood cells in our body. In essence, bone is a dynamic and integral part of our physiological framework, fulfilling key roles in mineral storage, structural support, and the generation of blood cells. To maintain optimal health, bones undergo a continuous cycle of growth, repair, and breakdown, a process known as remodeling. Osteoclasts and osteoblasts, specialized cells, play key roles in orchestrating these changes. Bone remodeling unfolds in a four-stage process, where old or damaged bone is broken down through resorption by osteoclasts, followed by the formation of new bone tissue by osteoblasts. This dynamic cycle ensures the constant adaptation and renewal of bone structure, contributing to overall skeletal health. Bone remodeling is a dynamic and continuous four-stage process essential for maintaining bone health. The activation phase initiates the cycle by recruiting osteoclasts, specialized cells responsible for bone breakdown. In the subsequent resorption phase, osteoclasts actively resorb the existing bone, paving the

way for the reversal phase. During this stage, osteoclasts undergo apoptosis, and osteoblasts are recruited to the site. Osteoblasts, crucial for bone formation, then usher in the final stage—the formation phase. Here, these cells diligently lay down a new organic bone matrix, which undergoes subsequent mineralization. This intricate process ensures the constant adaptation and renewal of the bone structure, contributing to the overall resilience and health of the skeletal system. Local regulation of bone remodeling is influenced by a range of hormones and cytokines. The delicate balance between new bone growth and degradation is crucial for skeletal health. Bone loss, symptomatic of various skeletal system illnesses, can manifest on a systemic or local scale. Conditions like osteoporosis and rheumatoid arthritis (RA) exemplify situations where bone loss outpaces formation. RA, a prominent systemic autoimmune disorder, is characterized by persistent synovial inflammation, resulting in joint and cartilage deterioration. Growing evidence suggests that immunological dysfunctions, particularly an abundance of pro-inflammatory responses, contribute to the chronic nature of arthritis. Both regional and systemic abnormalities in the body's natural defense appear to play a role in the initiation and progression of the disease. Recent advancements in RA pharmaceutical therapy have enabled many patients to achieve remission, enhancing their quality of life and minimizing long-term consequences. Early intervention and continuous care play pivotal roles in reducing damage. However, a notable challenge is the high cost associated with traditional RA treatments, primarily stemming from disease-modifying anti-rheumatic medications (DMARDs) and selective synthetic DMARDs. Despite the benefits, concerns arise, including an elevated risk of cardiovascular disease and disease flare-ups. A recent focus has been on the exploration and acceptance of biosimilars, generic substances resembling biological DMARDs. Some studies suggest their effectiveness, providing a significant alternative to cut costs, broaden treatment options, and reduce access disparities between affluent and developing nations. Even after exhausting therapeutic options, the common occurrence of therapy failure in RA patients emphasizes the need for novel treatments and understanding the mechanisms behind therapy toxicity and failure in non-remissive cases. Adverse effects, coupled with the high cost, pose significant barriers to patient adherence to medications. A novel substance Thymosin alpha 1, a synthetic peptide consisting of 28 amino acids and originally isolated from thymus tissue, is now produced synthetically. In healthy individuals, the naturally occurring levels of serum thymosin alpha-1 range from 0.1 to 1 ng/mL, indicating its

endogenous presence. T alpha 1 promotes the production of natural killer (NK), CD4, and CD8 cells, along with an increase in Th1 cytokines like IFN, IL2, IL3, and IL2 receptor expression. Additionally, it possesses anti-inflammatory properties. Its applications extend to the treatment of hepatitis B virus (HBV) and hepatitis C virus (HCV), cancer, severe sepsis, and its use as an adjuvant for vaccine enhancement. Studies suggest that T alpha 1 may influence immunological activity and have contrasting effects on joint complaints in breast cancer survivors. Notably, individuals with psoriatic arthritis and other chronic inflammatory autoimmune conditions exhibit lower T alpha 1 serum levels compared to healthy controls. Considering the immune-modulating and anti-inflammatory properties of Thymosin alpha-1, we designed a study to assess its potential role in bone remodeling using the Collagen-induced arthritis model in rats for rheumatoid arthritis (RA). After administering Thymosin alpha-1 treatment, noticeable changes were observed in various parameters, including body weight, paw swelling, arthritic score, and both biochemical and hematological parameters. Tissue histology of hind paw and Bone remodeling biomarkers were also improved after the administration of Thymosin alpha-1. Thus, it can be concluded that Thymosin alpha-1 can be used in the treatment of Rheumatoid arthritis and other bone related disorders.

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INDU BALA

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LIST OF ABBREVIATIONS

Ta1	Thymosin alpha1
RA	Rheumatoid Arthritis
GBD	Global Burden of Disease
SAGE	Study of Global Ageing and Adult Health
TNF-α	tumor necrosis factor-alpha
IL	Interleukin
IFN γ	Interferon gamma
GM-CSF	granulocyte monocyte-colony stimulating factor
MMPs	matrix metalloproteinases
SF	Synovial Fluid
PBMCs	peripheral blood mononuclear cells
CIA	Collagen induced arthritis
RANKL	receptor activator of NF-kappa B ligand
VEGF	vascular endothelial growth factor
CRP	C-Reactive Proteins
NK cells	Natural Killer cells
CSIF	cytokine synthesis inhibitory factor
NKSF	natural killer cell stimulatory factor
CTL	cytotoxic T lymphocytes
ELISA	enzyme-linked immunosorbent assay
DNA	Deoxyribonucleic Acid
OA	osteoarthritis
ACPA	anti-citrullinated protein antibodies
TRAP	tartrate-resistant acid phosphatase
OPG	Osteoprotegerin
DMARDs	Disease-modifying anti-rheumatic medications
NSAIDs	Non-steroidal anti-inflammatory drugs
MTX	Methotrexate
HIV	human immunodeficiency virus
MHC	Major Histocompatibility Class

BRM	Biological response modifier
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HBV	Hepatitis B virus
SLE	Systemic Erythematosus Lupus
BSALP	Bone specific alkaline phosphatase
CTX-1	C-terminal telopeptide-1
OC	Osteocalcin
LPS	lipopolysaccharide
ALT	Alanine Transaminase
ALP	Alkaline Phosphatase
EDTA	Ethylene diamine tetra acetic acid
H&E	Haematoxylin & Eosin
PCR	polymerase chain reaction
NO	Nitric Oxide
iNOS	inducible nitric oxide synthase
RNA	Ribonucleic Acid
SD	Standard Deviation
SNP	Single Nucleotide Polymorphism
SPSS	Statistical Package for the Social Sciences
WHO	World Health Organization

INTRODUCTION

CHAPTER-1

INTRODUCTION

There are about 150 different diseases and disorders recognized by the International Classification of Diseases that can have an impact on the organ system responsible for body and body organ movement, which includes the “muscles, bones, joints, and related tissues like tendons and ligaments”. Some are acute and temporary, like sprains and strains, while others can last a lifetime and cause constant discomfort or even disability. A person's capacity to work and engage in social responsibilities, as well as their mental health and the prosperity of their communities, can be negatively impacted by musculoskeletal problems, which are characterized by pain and limits in mobility, dexterity, and functional ability. Osteoarthritis, back and neck discomfort, fractures related to bone fragility, traumas, and systemic inflammatory disorders like rheumatoid arthritis are among the most frequent and disabling musculoskeletal conditions. The information provided by the GBD study demonstrates the severity of the disability load caused by musculoskeletal disorders. Lower back pain has been the leading source of disability worldwide since the metric was first recorded in 1990, and musculoskeletal problems were the second biggest contributor to global disability in the 2016 GBD research. Between 20% and 33% of the global population experiences discomfort from a musculoskeletal ailment (1). This percentage varies widely depending on age and diagnosis. As per the recent from USA, the prevalence of musculoskeletal disorders among adults is equal to that of cardiovascular and chronic respiratory diseases (2). Based on an examination of data derived from the “World Health Organization's Study of Global Ageing and Adult Health (SAGE)”, arthritis exhibits higher prevalence in economically disadvantaged and middle-income nations, particularly among individuals with economically poor sections (3).

Bone is a cellular, vascular, and crystal structure of calcium compounds. The most important function of bone is:

a. plays a very important and significant role in “calcium homeostasis” and is also key player in the storage of “phosphate, magnesium, potassium, and bicarbonate”

b. offers structural assistance to the soft tissues and serves as a lever for muscular movement; and

c. is the prime location where haematopoiesis process takes place our body (4).

In order to be healthy, bone is constantly expanding, repairing, and breaking down (resorption). The term "remodeling" is used to describe all of these changes to bones. The activities of osteoclasts and osteoblasts lead to remodeling. Bone remodeling is a four-stage process (5):

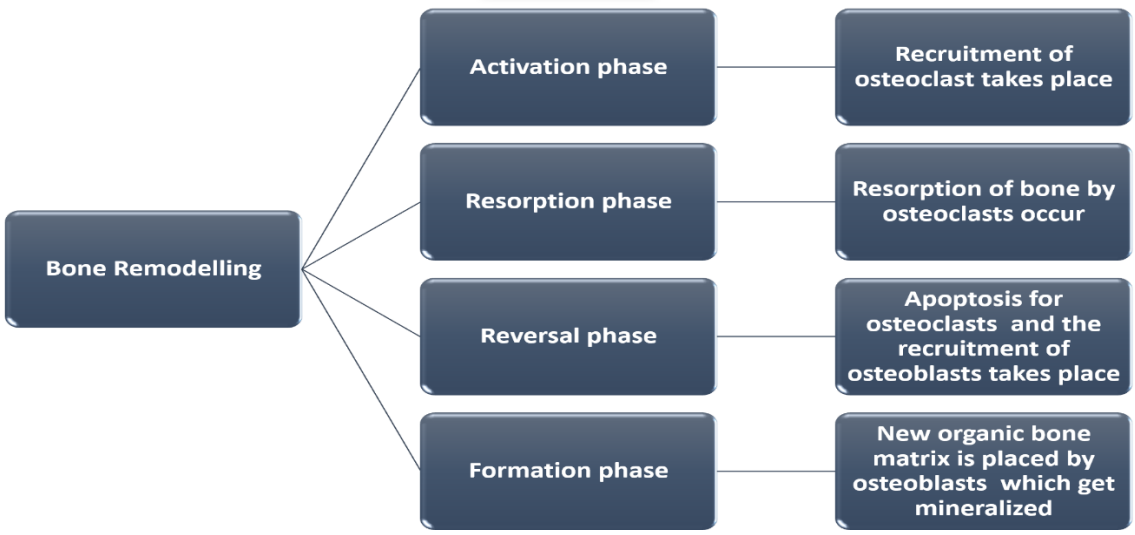

Fig-1: Phases of Bone Remodeling

Hormones and a variety of cytokines are examples of the local regulation that influence bone remodeling. Bone loss occurs when new bone growth is not equal to bone degradation. Bone loss can occur on a systemic or local scale, and it is a symptom of many skeletal system illnesses. Conditions like osteoporosis and RA are examples of situations where bone is lost at a faster rate than it is formed (6). Among the several systemic autoimmune disorders, RA ranks high in prevalence. Synovial inflammation persists over time, leading to the wear and tear of joints and cartilage. There is mounting evidence that immunological dysfunctions linked to a preponderance of pro-inflammatory responses underlie the chronicity of arthritis. It appears that both regional and systemic abnormalities in the natural defences of our body may have a function in the onset and progression of the disease. Initiation and maintenance of the chronic inflammation in RA are mostly dependent on helper T cells (Th cells), according to a recent study (7). T cells equipped with CD4 marker on their surface are critical to the pathophysiology of rheumatoid arthritis (8), and they are able to differentiate into pro- and anti-inflammatory subpopulations depending on the cytokine microenvironment.

Thymosin alpha-1 (Ta-1) is a pure synthetic amino-terminal acylated peptide of 28 amino acids that was first isolated from thymus tissue. Serum Ta-1 levels in healthy persons have been measured to be between 0.1 to 1 ng/mL (9), indicating its presence endogenously. T alpha 1 encourages the body to make more natural killer (NK), CD4, and CD8 cells (10). Production of Th1 cytokines such as IFN, IL2, IL3, and IL2 receptor expression is also elevated (11–13). In addition, it reduces inflammation. Treatment for Hepatitis B (HBV) and Hepatitis C (HCV) viruses, along with therapies for cancer and severe sepsis, as well as the use of Ta-1 as an adjuvant to boost vaccine efficacy (14). Researchers found that Ta-1 might modify immunological activity and had opposing effects on joint complaints in breast cancer survivors (15). Patients with psoriatic arthritis and other chronic inflammatory autoimmune illnesses have been shown to have reduced Ta-1 serum levels compared to healthy controls (16). Keeping in mind the immune modulating and anti-inflammatory properties of Ta-1, we have designed this study to evaluate the potential role of Ta-1 in bone remodeling through the Collagen-induced arthritis model in Rats for RA. The results of the current study will help in the management or improvement of different bone diseases as well as in joint disorders.

REVIEW OF LITERATURE

CHAPTER-2
REVIEW OF LITERATURE

[A] BONE: a dense connective tissue

Bone is a rigid, dense connective tissue that makes up the skeletal system in vertebrates. It provides structural support to the body, protects internal organs, facilitates movement through its interaction with muscles, and serves as a reservoir for minerals, such as calcium and phosphorus. Bones are dynamic tissues that undergo constant remodeling, involving processes like bone formation (ossification) and resorption. The cellular structures, blood arteries, and calcium crystals that make up bone are what make it a porous and mineralized structure. The proportion varies depending on the different types of bones and where they are located. The intricate mechanisms underlying cellular differentiation, which ultimately culminate in the development of the skeletal system, are orchestrated by a series of genes. These genes play a pivotal role in establishing the initial blueprint of the skeletal structure, primarily manifested as cartilage and mesenchyme. Subsequently, the process unfolds as these cartilaginous and mesenchymal components are gradually supplanted by bone, a transformation facilitated by the differentiation of specialized cells known as osteoblasts. Genes are responsible for the development of the skeleton. Extracellular matrix, collagen, and cells are the constituents that make up the structural components of bone. Cortical bone and trabecular bone are the two forms of bone that are typically seen in an adult human skeleton (17). Both the macroscopically and microscopically distinct forms have the same chemical composition. The only difference between them is in their scale. The outer layer of every skeletal structure consists of cortical bone, comprising 80% of the total skeletal mass. It is complicated, compacted, has a slow turnover rate, and provides significant resistance to bending and twisting. The cortical bone, comprising the predominant portion of the skeletal structure, is primarily characterized by its calcified nature. Its principal function revolves around providing mechanical fortitude and safeguarding vital anatomical components. Nevertheless, it is worth noting that cortical bone possesses the capability to engage in metabolic processes, particularly in instances where a notable or enduring insufficiency of minerals is present. Nevertheless, it is crucial to note that a substantial proportion, specifically 80%, of the bone external area is situated within the confines of the

elongated bones dispersed throughout the various anatomical regions of the vertebral column. Additionally, it is pertinent to acknowledge that these elongated bones are not the sole repositories of bone surface area, as the interior regions of the pelvis and other prominent flat bones also contribute significantly to this metric. It is noteworthy that trabecular bone, also known as spongy or cancellous bone, comprises a mere 20% of the overall skeletal mass. In contrast to cortical bone, which assumes the primary responsibility for a plethora of metabolic functions, trabecular bone exhibits a considerably diminished density, heightened elasticity, and an augmented rate of turnover. Trabecular bone, also known as spongy or cancellous bone, serves a dual purpose in the human body. Firstly, it acts as a reservoir of mineral supply during episodes of acute mineral insufficiency. This crucial function ensures that the body's mineral requirements are met, thereby maintaining optimal physiological functioning. Secondly, trabecular bone plays a significant role in providing mechanical support to the body, particularly in weight-bearing bones like the vertebrae. By contributing to the structural integrity of these bones, trabecular bone aids in maintaining the overall stability and functionality of the skeletal system.

[B] BONE CELLS: PLAYERS OF BONE REMODELLING

Bone cells take on specialized forms to carry out the various functions of bone, such as production, resorption, mineral homeostasis, and repair. These forms can be differentiated based on their morphology, function, and typical position in the bone. “Mesenchymal stem cells” are responsible for developing preosteoblasts, osteoblasts, bone lining cells, and osteocytes. Hematopoietic stem cells are responsible for the development of circulating or marrow monocytes, preosteoclasts, and osteoclasts.

(a) Osteoblasts:

The osteoblast is the cell that is in charge of producing the components that make up the bone matrix. Osteoblasts do not act independently but rather are found in clusters along the bone surface. These clusters line the layer of bone matrix that osteoblasts are responsible for creating. They are derived from multipotent mesenchymal stem cells, which have the ability to develop into osteoblasts, adipocytes, chondrocytes, myoblasts, or fibroblasts (18). They are also known as multipotent mesenchymal stem cells. The findings of recent investigations pertaining to gene deletion have brought to light the significance of the absence of osterix, a downstream

factor, or runt-related transcription factor 2 in the process of osteoblast differentiation (19). The synthesis of osteoid matrix, the maturation of that matrix, and finally the mineralization of the matrix constitutes the three stages that make up the process of bone formation. These activities take place at the same rate in healthy adult bone, which ensures that the ratio of matrix creation to mineralization is maintained at a constant level. At first, osteoblasts make osteoid by rapidly depositing collagen. This is the first step in the process. After this, there is an increase in the rate of mineralization until it reaches the same level as the rate of collagen production. The final stage sees a reduction in the rate of collagen production, although mineralization proceeds up until the point where the osteoid is completely mineralized. In response to a wide variety of stimuli, osteoblasts produce several different types of growth factors. These include insulin-like growth factors, platelet-derived growth factor, basic fibroblast growth factor, transforming growth factor-beta, and bone morphogenetic proteins (20).

(b) Osteocytes:

Osteocytes are entrapped osteoblasts that have found a home in the osteoid. Osteoblasts continue to create matrix proteins even though their metabolic activity declines once they are completely encased in bone matrix. In order to organize the matrix prior to calcification, osteocytes have several complicated cell processes that are packed with microfilaments. The bone matrix is permeated by a network of these tiny canaliculi. The age of an osteoclast determines its functional activity and shape. Although similar in structure to an osteoblast, a juvenile osteocyte is smaller and produces less protein. An osteocyte that is older and more deeply embedded in the calcified bone has a smaller cell volume and more glycogen in its cytoplasm. Osteoclastic bone resorption culminates in the osteocytes being phagocytosed and digested (21). Although the osteocytic network is highly organized, its precise function is yet unknown. Osteocytes, in response to stress on bone tissue, likely boost bone remodeling activity by attracting osteoclasts to damaged areas (22). Although osteocytes may send signals to surface cells in response to bone tension or microdamage, no such evidence exists as of yet.

(c) Osteoclasts:

The osteoclast is the bone lining cell that is responsible for bone resorption. It is a huge multinucleated cell that may reach a diameter of up to 100 millimetres and originates from hematopoietic cells that are of the mononuclear lineage (23). Because

of its resorptive properties, it frequently interacts with calcified bone surfaces and resides within lacunae. Osteoclasts are characterized by the presence of many Golgi complexes, mitochondria, and transport vesicles that are packed with lysosomal enzymes. They have profound foldings of the plasma membrane in the area that faces the bone matrix as well as the surrounding zone of attachment, which is referred to as the sealing zone. Osteoclasts are responsible for the active synthesis of lysosomal enzymes, such as tartrate-resistant acid phosphatase and cathepsin K, which are then released into the bone-resorbing compartment via the ruffled border (24).

[C] FROSTIAN BONE MODELING AND REMODELING:

(a) Bone Remodelling:

The processes of development, reinforcement, and resorption occur continuously in living bone. The term "remodeling" is used to describe all of these changes. Living bone undergoes remodeling processes that allow it to adjust its histological structure in response to long-term loading changes. Defects such as microfractures are mended through the cooperation of osteoblasts and osteoclasts, which leads to remodeling. Bone remodeling, also known as bone resorption and creation, is the process by which bone responds to mechanical load and strain by constantly replacing old tissue with new (25).

There are typically four stages to each given remodel. There are four distinct stages in bone formation: (a) the activation stage, during which osteoclasts are recruited; (b) the resorption stage, during which osteoclasts resorb bone; (c) the reversal stage, during which osteoclasts undergo apoptosis and the osteoblasts are recruited; and (d) the formation stage, during which the osteoblasts lay down new organic bone matrix that subsequently mineralizes(5). Migration of partially developed mononuclear preosteoclasts to the bone surface, where they differentiate into multinucleated osteoclasts, is the first step in the resorption process. When osteoclastic resorption is complete, a reversal phase begins, and mononuclear cells begin to populate the bone surface. These cells offer signals for osteoblast differentiation and migration, preparing the way for new osteoblasts to initiate bone production. After the resorption phase, the creation phase begins and osteoblasts begin laying down bone to replace the old. After this process concludes, the surface is coated with flattened lining cells and a long resting period begins until the next remodeling cycle is launched. The duration of time

spent in each phase of the remodeling cycle varies. Until the new bone structure unit is fully generated, resorption likely continues for another 2 weeks, the reversal phase may last up to 4 or 5 weeks, and formation may continue for another 4 months (26).

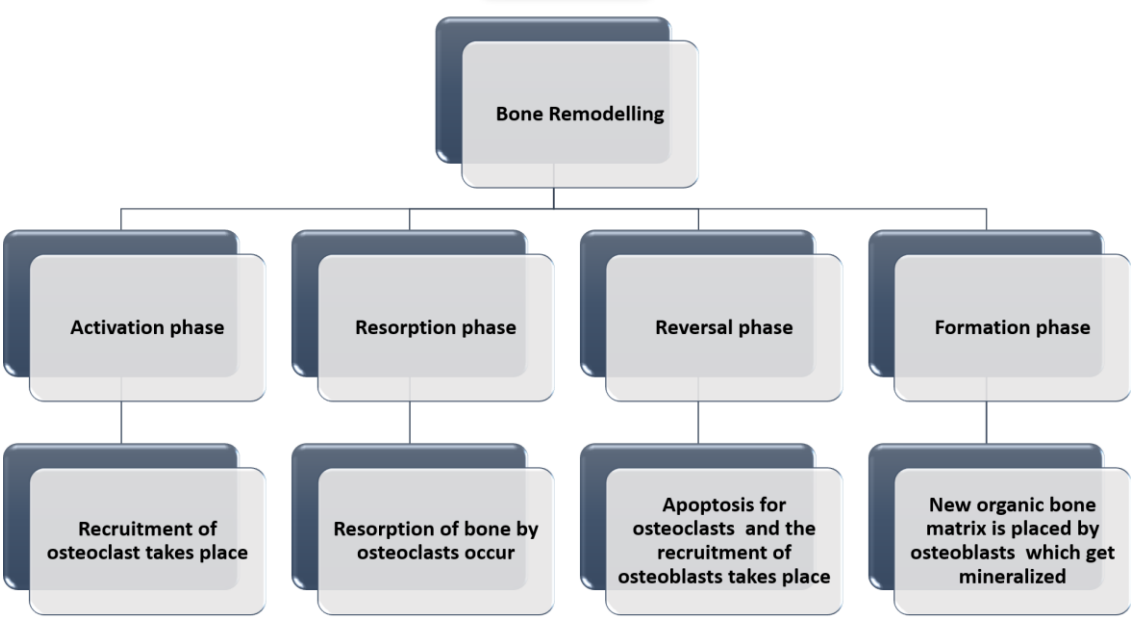

Fig. 2: Stages of Bone remodelling

However, the specific cells within the osteoblast lineage that are responsible remain elusive. These cells change their shape, release enzymes that break down proteins on the surface of the bone, and produce a 317-amino-acid peptide known as "receptor activator of NF-kappa B ligand (RANKL)", belonging to the "tumor necrosis factor (TNF)" superfamily. The progenitors of osteoclasts express a receptor termed RANK, which is activated by RANKL. Hematopoietic cells of the osteoclast lineage are activated, differentiated, and fused as a result of the RANKL/RANK interaction, allowing them to initiate the resorption process. Additionally, it inhibits apoptosis in osteoclasts, allowing them to live longer (27). Among other things, this demonstrates that RANKL links bone resorption and bone production. Osteoprotegerin (OPG) is a 120-kDa secretory dimeric glycoprotein in the TNF receptor family that inhibits the actions of RANKL. Although the osteoblast lineage is responsible for the majority of OPG production in the bone marrow, other cell types in the marrow can also generate this decoy receptor (a soluble receptor functioning as antagonist) for RANKL (28-30). Bone resorption is controlled by OPG, which prevents osteoclasts from fully differentiating and activating and instead causes them to self-destruct. Because OPG does not become part of the bone matrix, its effects on bone resorption are temporary.

(b) Regulation of Bone Remodelling

The regulation of bone integrity seems to be under the influence of various hormones and proteins that are released by both "hemopoietic bone marrow cells and bone cells". In the realm of bone biology, it is pertinent to acknowledge the existence of both systemic and local regulatory mechanisms governing the intricate functionality of bone cells.

(i) **Systemic Regulation:** Parathyroid hormone (PTH) serves as the primary regulator of calcium homeostasis. The mechanism of action involves the stimulation of bone resorption, enhancement of renal tubular calcium reabsorption, and promotion of renal calcitriol production in order to regulate serum calcium concentrations. According to the cited source (31), intermittent administration of PTH has been found to promote bone formation, while continuous secretion of PTH has been associated with bone resorption. Calcitriol has a crucial role in facilitating the absorption of calcium and phosphorus in the intestines, hence promoting the process of bone mineralization. Furthermore, it is worth noting that vitamin D3 has a significant role in promoting bone anabolism, hence exerting a dual impact on bone remodeling (32). Pharmacologic administration of calcitonin induces the removal of the ruffled border, halts the movement of osteoclasts, and suppresses the release of proteolytic enzymes via binding to its receptor on osteoclasts. Nevertheless, the impact of this phenomenon is constrained by dosage and its physiological significance is negligible within the skeletal structure of adult individuals. The growth hormone/insulin-like growth factor-1 (IGF-1) system, together with insulin-like growth factor-2 (IGF-2), plays a crucial role in skeletal growth, particularly in the cartilaginous end plates and throughout the process of endochondral bone formation. The regulation of bone production and resorption is influenced by these factors, which play a significant role in determining adult bone mass (33). Glucocorticoids have the ability to elicit both stimulatory and inhibitory effects on bone cells. Osteoblast maturation is facilitated by their crucial role in promoting differentiation from mesenchymal progenitors; nevertheless, their impact on osteoblast activity is inhibitory. In addition, it has been observed that glucocorticoids enhance the responsiveness of bone cells to factors that regulate the process of bone remodeling, and they

also increase the recruitment of osteoclasts (34). Thyroid hormones have the capacity to boost both the process of bone resorption and bone growth. Therefore, it may be inferred that hyperthyroidism leads to an elevation in bone turnover, thereby resulting in the occurrence of bone loss (35). Estrogens have been found to reduce the sensitivity of osteoclast progenitor cells to RANKL, leading to the inhibition of osteoclastogenesis (36). In addition, apart from the observed reduction in osteoclast life span (37), estrogens have been found to promote the proliferation of osteoblasts and inhibit their death. They exert an influence on the genetic coding of enzymes, bone matrix proteins, hormone receptors, and transcription factors. Additionally, they enhance the local synthesis of OPG, IGF I, IGF II, and TGF-β (38). Androgens play a crucial role in the growth and maintenance of the skeletal system by exerting their influence on the androgen receptor, which is ubiquitously expressed in several bone cell types (39).

(ii) **Local Regulation:** Regarding the regulation of bone cell function at the local level, the recent finding of the OPG/RANKL/RANK system has provided a more comprehensive understanding of the mechanisms governing osteoclastogenesis and overall bone remodeling. The receptor activator of nuclear factor kappa-B ligand (RANKL), which is present on the surface of preosteoblastic/stromal cells, interacts with the receptor activator of nuclear factor kappa-B (RANK) on osteoclastic precursor cells. This interaction plays a crucial role in various processes including differentiation, fusion into multinucleated cells, activation, and survival of osteoclastic cells (27). Osteoprotegerin (OPG) exerts its inhibitory action on the whole system by effectively impeding the activities of receptor activator of nuclear factor kappa-B ligand (RANKL) (28). The necessity of macrophage colony-stimulating factor (M-CSF) for osteoclast development is attributed to its binding to the receptor c-Fms on preosteoclastic cells. This interaction serves as the key determinant for the pool of precursor cells involved in osteoclast formation (40). The contrasting phenotypes observed in mice with overexpression of OPG or deletion of RANKL (resulting in osteopetrosis) and mice deficient in OPG or with overexpression of RANKL (resulting in osteoporosis) have given rise to the hypothesis that OPG and RANKL may serve as mediators for the

stimulatory or inhibitory effects of various systemic hormones, growth factors, and cytokines on the process of osteoclastogenesis. The phenomenon described in recent literature is commonly known as "the convergence hypothesis." It suggests that the actions of resorptive and antiresorptive agents come together or "converge" at the level of two mediators. The relative balance between these mediators ultimately determines the extent of osteoclast development, activation, and apoptosis (41). Several cytokines, including TNF-_ and IL-10, play a role in modulating this system predominantly through the stimulation of M-CSF synthesis and the direct increase in RANKL expression (42). Furthermore, several other cytokines and hormones modulate the process of osteoclastogenesis by modulating the production of Osteoprotegerin (OPG) and receptor activator of nuclear factor kappa-B (RANK). In addition, IL-6, a multifunctional cytokine produced by osteoblasts, osteoclasts, and stromal cells, seems to play a significant role in regulating bone remodeling. It does so by enhancing osteoclastic bone resorption (43) and facilitating the formation of osteoblasts under situations characterized by increased bone turnover (44). According to recent research, it has been proposed that PTHrP produced from osteoblasts plays a crucial role in facilitating the recruitment of osteogenic cells and inhibiting the apoptotic demise of osteoblasts. Consequently, it serves as a significant regulator of bone cell functionality (45). Various skeletal problems can arise from abnormalities in the process of bone remodeling. Recent advancements in the understanding of systemic and local regulating mechanisms involved in bone remodeling have paved the way for novel diagnostic and therapeutic strategies in the field of skeletal disorders. The recent advancements in molecular and cellular biology have been important in elucidating the aberrations present in cells belonging to the osteoblastic and osteoclastic lineages, which are responsible for the development of bone diseases. Furthermore, these advancements have facilitated the formulation of novel therapeutic strategies by enhancing our comprehension of the underlying pathogenetic pathways. The methodologies encompass the generation of recombinant cytokine molecules and their soluble receptors, the formulation of inhibitory peptides, and the targeted suppression of critical signalling pathways.

[D] RHEUMATOID ARTHRITIS

Rheumatoid arthritis, often known as RA, is a chronic autoimmune illness that affects multiple body systems. Rheumatoid arthritis is characterized by the development of persistent inflammatory synovitis, which typically affects peripheral joints in a symmetrical manner. In certain circumstances, patients also experience extra-articular and systemic signs. Rheumatoid arthritis affects between approximately 0.5 and 1% of the general population in developed nations (46). Although disease can strike people of any age, women are nearly three times more likely to be affected than males, and the onset of symptoms typically occurs when a person is in their 40s or 50s, even though disease can strike people of any age. In patients with RA, cardiovascular disease (also known as CVD), which develops as a result of chronic inflammation, is regarded as one of the primary reasons for patient mortality (47).

The pathophysiology of RA is governed by both T cells and B cells, with the structured participation of pro-inflammatory cytokines. CD4+T cells that have been activated can start a wide variety of immunological responses. Traditional theory holds that T helper 1 (Th1) cells are responsible for controlling cellular immunity, whereas Th2 cells are in charge of humoral immunity. Tbet and signal transducer and activator of transcription-4 (STAT-4) are both transcription factors that are expressed by Th1 cells. STATs are members of a family of transcription factors that were very recently discovered. These transcription factors are responsible for activating gene transcription in response to a variety of cytokines (48). Research has shown that Th2 cells are positive for the GATA binding protein 3 (GATA-3) and the STAT-6 gene. A transacting transcription factor that is particular to t cells is called GATA-3. Before the identification of the Th17 subset, autoimmune disorders like rheumatoid arthritis and other conditions were thought to be caused by the Th1 subset of the immune system. Th17 cells, which are now thought to have a significant role in autoimmune illnesses (49), are responsible for the production of the cytokines IL-17, IL-21, and IL-22. Regulatory T cells, also known as Treg cells, are a different type of T cell that are thought to play a protective role against bacterial and fungal infections as well as a role in suppressing the body's autoimmune response. They generate TGF and IL-10, as well as the forkhead box P3 (FoxP3), CD4, and CD25 surface markers (50). The ratio of Th17 cells to Treg cells, often known as the "Th17/Treg balance," plays a critical part in defining the clinical course of a variety of autoimmune and inflammatory conditions.

At this point in time, it is not particularly apparent if RA is a condition that is mediated by the Th1 or the Th17 immune system.

Pathogenesis of RA

Although the exact cause of RA is still unknown, it does appear that environmental and genetic risk factors play a significant role in the disease's development. According to the findings of a study that was carried out by MacGregor and colleagues, genetic variables are responsible for between 50 and 60 percent of the development of RA. The concordance rate for monozygotic twins' ranges between 12.3% and 15.4%, whereas it is only 3.5% for dizygotic twins (46). It is generally agreed that the class II major histocompatibility complex (MHC) allele HLA-DR4 (DR1*0401) and a few related alleles (DRB*0404, DRB*0101, and DRB*1402) are the most important genetic risk factors for rheumatoid arthritis (RA). The glutamine-leucine arginine-alanine-alanine (QKRAA) or QRRAA sequence, which is located in the third hypervariable region of DR beta chains, is the susceptibility epitope (51). It is also known as the shared epitope (SE). Patients with RA who have SE are more likely to generate antibodies against citrullinated vimentin, which in turn can have an effect on the specificity of anticitrullinated peptide antibodies (ACPA). Other genes that have been linked to RA include PTPN22, PADI4, STAT4, TRAF1-C5, and TNFAIP3. The Human Genome Project and the International HapMap project both contributed data that made it possible to create single-nucleotide polymorphism (SNP) chips. These chips cover variants that occur throughout the whole genome. As a result of genome-wide association studies (GWAS), around sixty genetic markers have been linked to RA (52). A high number of HLA-DRB1 alleles is one of the genetic risk factors for ACPA-positive RA. On the other hand, HLA-DR3, interferon regulatory factor 5, and neuropeptide S receptor gene polymorphisms are some of the genetic risk factors for ACPA-negative RA. The finding of numerous genetic risk factors for ACPA-positive RA (in comparison to ACPA-negative RA) does not necessarily imply that genetic risk factors contribute more to ACPA-positive RA than they do to ACPA-negative RA. However, a recent study found that the heritability of RA among twins is practically identical for ACPA-positive cases (68%) and ACPA-negative cases (66%). Protective effects against RA are conferred by HLADRB1* 13:01(46).

Environmental variables, in addition to one's genetic predisposition, are also contributors to the development of rheumatoid arthritis (RA). Cigarette smoking is the environmental component that poses the greatest threat out of all the possible environmental triggers. In addition, pathogenic pathogens such as Porphyromonas gingivalis, which is linked to periodontal disease, have been discovered in a significant number of individuals with RA (53). In RA, epigenetic factors, which are defined as those that influence gene activity without affecting the basic DNA sequence, serve as bridges between the environmental risk factors and the genetic risk factors. Acylation of histones and methylation of DNA have both been linked to rheumatoid arthritis (RA). These post-translation alterations lower the compactness of the chromatin structure, making the DNA more accessible to transcription factors and allowing for more control over gene expression. In a recent investigation that was carried out on RA synovial fibroblasts, a significant number of sites that were differentially methylated (both hypo- and hyper-) were discovered. It was discovered that the majority of the genes that are affected are involved in inflammatory processes, the recruitment of leukocytes, matrix remodeling, and immunological responses (54). A recent study found that arthritic joints have a preference for histone acetylation over deacetylation, which results in an increase in gene expression. Patients with rheumatoid arthritis and mice with arthritic conditions were shown to have elevated levels of several members of the histone acetyl transferase (HAT) family. In RA, epigenetic factors and enzymes that promote transcription are given a boost, whereas factors and enzymes that inhibit transcription are given a push in the opposite direction. As a direct consequence of this, the pathways that promote inflammation become more robust, while the pathways that suppress inflammation become less robust. Numerous epigenome modifiers can have a direct or indirect effect on the activity of nuclear factor kappa-light-chain-enhancer of activated B cells (NFB), which is a significant regulator of the transcription of inflammatory genes (55). This is the case whether the epigenome modifiers act on the chromatin directly or via histone modifications.

Micro RNAs, commonly known as miRNAs, are non-coding RNAs that are very small and have recently been linked to rheumatoid arthritis (RA). They form complementary base pairs with the 3' untranslated region of their intended mRNA targets, resulting in either mRNA degradation or translation inhibition. Recent research using a bioinformatics method showed that miRNA dysregulation affects the expression of genes

linked to rheumatoid arthritis (56). Increased expression of miRNAs (miRNA 115 and miRNA 203) has been reported in RA fibroblast-like synoviocytes (FLS) as compared to osteoarthritis FLS. Increased levels of IL-6 (interleukin-6) and matrix metalloproteinase-1 (MMP-1) are correlated with this greater expression of miRNAs (52).

The pathophysiology of rheumatoid arthritis (RA) is characterized by the involvement of numerous signaling pathways and immune-modulating agents. The condition under discussion is distinguished by the occurrence of microvascular damage, an elevation in the quantity of synovial lining cells (including macrophages and neutrophils), the infiltration of lymphocytes within the synovium, and the generation of diverse inflammatory cytokines. These aforementioned factors collectively contribute to the stimulation of fibroblast and synoviocyte proliferation, as well as the development of neo-angiogenesis. The aforementioned processes culminate in the occurrence of hyperplasia and hypertrophy within the synovial lining cells, as well as the subsequent differentiation and activation of osteoclasts, ultimately resulting in the detrimental phenomenon of bone destruction. Furthermore, the secretion of proteolytic enzymes, such as matrix metalloproteinases (MMPs), by synoviocytes and chondrocytes results in the degradation of cartilage (57). The production of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) is heightened, thereby leading to an augmentation in the production of prostaglandin E2 and nitric oxide, both of which are recognized as pro-inflammatory mediators. One of the notable laboratory characteristics observed in individuals with rheumatoid arthritis (RA) is the generation of autoantibodies, specifically rheumatoid factor and anti-citrullinated peptide antibody. These autoantibodies target antigens that are not limited to the joints, but rather are found throughout various regions of the body (58). The clinical manifestations of rheumatoid arthritis (RA) encompass a range of symptoms that can be observed in affected individuals. These include but are not limited to fatigue, anorexia, morning stiffness, joint swelling and tenderness, as well as pain and disability. The presence of extra-articular manifestations in this context encompasses a range of conditions, such as rheumatic lung, rheumatic nodules, keratoconjunctivitis sicca, uveitis, rheumatoid pericarditis, and vasculitis. The production of acute-phase proteins, as well as the presence of osteoporosis, anemia, cardiovascular disease, fatigue, and depression, are among the various systemic manifestations that have been identified (59).

Significance of B-lymphocytes in the rheumatoid arthritis:

Recent research findings have elucidated the pivotal role of B cells in the pathogenesis of rheumatoid arthritis (RA), as demonstrated by the notable improvement in symptomatology observed in RA patients upon administration of B cell depleting therapies, such as rituximab, an anti-CD20 antibody. It is widely acknowledged that B cells are responsible for the production of rheumatoid factor, which are auto-antibodies that target IgG. However, the diagnostic specificity of rheumatoid arthritis (RA) has been significantly enhanced to a range of 90-98% after the identification of B cell-mediated production of anti-CCP. The aforementioned agents elicit the production of pro-inflammatory cytokines, specifically tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6), as supported by reference (60). Additional functions encompass the process of antigen presentation and the subsequent activation of T cells. Furthermore, there exists an intricate interplay between these cells and chemokines, which serves to facilitate the formation of TNF-α. Consequently, the activation of macrophages is induced through the aforementioned cascade of events.

Significance of T-cell sub-populations:

The significance of the association between HLA-DR4 (as well as DR1) alleles and the severity of disease in the context of rheumatoid arthritis (RA) implies the critical role played by T cells in the pathogenesis of this condition. They play a crucial role in the propagation of inflammation and the infliction of tissue damage in rheumatoid arthritis. The presentation of arthritis-associated antigens to T cells is facilitated by various immune cells, including activated B cells, macrophages, and dendritic cells, which are classified as antigen-presenting cells. Upon activation, these entities proceed to activate the innate immune cells, provide assistance in B-cell activation, release a multitude of cytokines (which are accountable for the inflammation of the synovial tissue), and induce the activation of chondrocytes and osteoclasts, thereby promoting their destructive behaviour (61). CD4+ T-cells, which are the predominant subset of T cells, are commonly observed near dendritic cells and macrophages that express HLA-DR. Within the synovial tissue, it has been observed that CD4+ T-cells primarily undergo differentiation into Th1-like effector cells. These particular cells play a crucial role in the generation of pro-inflammatory cytokines, including IFN γ and TNFα. Conversely, it appears that the differentiation of CD4+ T-

cells into Th2-like effector cells, responsible for the production of anti-inflammatory cytokines such as IL-4, IL-10, and IL-13, is noticeably deficient (62). The Th17 subtype of CD4+ T-cells is widely recognized as the principal contributor to synovial inflammation and subsequent bone erosion. This is primarily attributed to their ability to produce interleukin-17 (IL-17) and interleukin-23 (IL-23) (50). Both Th1 and Th17 subtypes are responsible for regulating the distinctive inflammation observed in rheumatoid arthritis (RA). Nevertheless, the ongoing discourse revolves around the contentious issue of determining the relative impact exerted by each individual subtype.

Tregulatory cells, also known as Tregs, exert their suppressive effect through a multitude of mechanisms. These include the secretion of inhibitory cytokines, such as transforming growth factor beta (TGFβ) and interleukin-10 (IL-10). Additionally, Tregs inhibit immune activation by expressing a protein called Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4). This protein competes with co-stimulatory ligands on T cells, thereby dampening their activation (63). In the context of inflammation, it has been observed that the presence of pro-inflammatory cytokines, specifically IL-6 and IL-21, elicits a response whereby TGFβ facilitates the upregulation of retinoic acid receptor-related orphan nuclear receptor (ROR-c). Conversely, the expression of FoxP3 is repressed as a consequence of STAT-3 activation (64). On the contrary, if inflammation is not present, the process of Treg cell differentiation takes place as a result of the inhibitory effects mediated by FoxP3 on RORγt. Consequently, the expression of IL-17 and IL-21 is suppressed (65). In the context of rheumatoid arthritis (RA), there is a notable decrease in the quantity of regulatory T cells (Treg cells) or, at the very least, a disruption in the proper functioning of these cells. Consequently, the activation of Th17 cells ensues, thereby precipitating a pronounced inflammatory response in the context of arthritis.

Effects of cytokines in rheumatoid arthritis:

Cytokines, as a class of signaling molecules, are responsible for the execution of numerous vital biological processes, including but not limited to cell growth, proliferation, differentiation, inflammation, tissue repair, and regulation of the immune response (66). It is widely acknowledged that these entities play a significant role in the pathogenesis of rheumatoid arthritis (RA) and are accountable for the inflammatory processes and subsequent joint deterioration that manifest during the arthritic condition.

The underlying cause of the characteristic inflammation observed in rheumatoid arthritis (RA) can be attributed to the prevailing presence of pro-inflammatory cytokines, which surpasses the levels of anti-inflammatory cytokines.

Role of Tumor Necrosis factor- alpha (TNF- α) in rheumatoid arthritis

The cytokine known as TNF-α assumes a prominent role in the pathogenesis of rheumatic arthritis (RA), serving as one of the primary mediators of inflammation within this condition. The production of this substance primarily stems from activated macrophages; however, it is noteworthy that monocytes, fibroblasts, mast cells, and NK cells can also contribute to its production. The subject under discussion exhibits a duality in its receptor composition, specifically consisting of two distinct receptors. The first receptor, denoted as p55 or TNF-R1, possesses a molecular weight of 55 kilodaltons. The second receptor, referred to as p75 or TNF-RII, exhibits a molecular weight of 75 kilodaltons. The p55 receptor, widely expressed in various tissues, exhibits the ability to bind both the membrane-bound and soluble trimeric forms of tumor necrosis factor (TNF). In contrast, the p75 receptor is specifically expressed on immune cells, and exclusively interacts with the membrane-bound TNF (67). The serum of patients with rheumatoid arthritis (RA) has been observed to exhibit heightened levels of TNF-α, a cytokine that plays a pivotal role in the initiation and perpetuation of inflammation and joint deterioration in individuals afflicted with RA (68). The aforementioned substance serves to induce the proliferation and differentiation of B lymphocytes, T lymphocytes, and natural killer cells. Furthermore, it elicits the synthesis of additional pro-inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and matrix metalloproteinases (MMPs). Furthermore, synovial cells have the capacity to induce upregulation of stromelysin, collagenase, prostaglandins, and granulocyte monocyte-colony stimulating factor (GM-CSF). Furthermore, it has been observed that this particular stimulus elicits the expression of adhesion molecules, specifically intracellular adhesion molecule 1 (ICAM-1), by fibroblasts (67). The systemic effects encompass a variety of consequences, such as an elevated propensity for cardiovascular ailments, the generation of acute phase protein, and the manifestation of fatigue and depression resulting from the dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis.

Significance of Interleukin-1 in rheumatoid arthritis

The members of the IL-1 family that are most important are IL-1, IL-1, 1L-18, and IL-33. IL-1 produces its biological action by acting on other cells, whereas IL-1 is expressed on the same cell surface or is kept within the cell. The action of IL-1α and IL-1β can be effectively impeded through the intervention of an endogenous inhibitor known as IL-1 receptor antagonist (IL-1Ra). In the realm of interleukin-1 (IL-1) receptors, we encounter a dichotomy consisting of two distinct types: IL-1RI and IL-1RII. The process of IL-1 binding to IL-1RI leads to the transmission of intracellular signals, and this binding is further enhanced by the presence of IL-1R-AcP, an auxiliary protein. In contrast, it is worth noting that IL-1RII functions as a decoy receptor. This is due to the fact that the binding of IL-1 to IL-1RII does not have the capability to transduce signals. The reason behind this is the presence of a very short cytoplasmic domain, as elucidated by previous studies (69).

In patients diagnosed with rheumatoid arthritis (RA), an observable variation arises in the levels of interleukin-1 receptor antagonist (IL-1Ra) and interleukin-1 (IL-1) (70). A significant elevation of interleukin-1β (IL-1β) levels has been observed in both plasma and synovial fluid (SF) in numerous instances, and this finding correlates with several disease activity indicators, such as the duration of morning stiffness. The presence of IL-1 has been observed to have a stimulating effect on the production and release of various substances by synovial fibroblasts. These substances include cytokines, chemokines, inducible nitric oxide synthase (iNOS), prostaglandins (PGs), and matrix metalloproteinases (MMPs). In numerous instances, it has been observed that there is a presence of osteoclast activation and an expression of endothelial cell adhesion molecules (71). The anti-inflammatory effects of Apolipoprotein A1, an acute-phase protein, have been observed to effectively suppress the expression of IL-1. However, it is important to note that this suppression does not extend to the expression of IL-1Ra. Furthermore, it should be noted that the expression of IL-1Ra can be augmented by the presence of IFN-β, TGF-β, IL-4, and IL-13, whereas the production of IL-1 can be attenuated by these aforementioned cytokines (72).

Polymorphism has been observed within the genetic sequences responsible for encoding interleukin-1 alpha (IL-1α), interleukin-1 beta (IL-1β), and interleukin-1

receptor antagonist (IL-1RA). The polymorphism of IL-1α, specifically at position -889 C/T and +4845 G/T in exon 5, is correlated with a heightened vulnerability to rheumatoid arthritis (RA) and modified production of IL-1 (46). The IL-1β gene polymorphisms, specifically rs16944 (-511 C/T) and rs1143634 in exon 5 at +3953 C/T, have been found to exert an influence on IL-1 expression. Furthermore, these polymorphisms have been associated with the occurrence of erosive damage in patients with rheumatoid arthritis (73). The presence of polymorphism at position -1464 C/G has also been discovered to exhibit a protective effect. I would like to discuss the topic of climate change and its impact on global ecosystems. It is a In the context of Black South Africans, it has been observed that there exists polymorphism in the IL-1RA gene (rs419598) at position +2018 (C/T), which has been associated with a pro-inflammatory effect (46, 73).

Significance of Interleukin-4 in rheumatoid arthritis

Interleukin-4, a prominent cytokine, plays a pivotal role in fostering the development of Th2 cells through the process of differentiating naïve T cells. Through the mechanism of a positive feedback loop, the active Th2 cells facilitate the formation of additional IL-4. The receptor for interleukin-4 (IL-4), known as IL-4Rα, is present in multiple isoforms within the human body. Initially, the prevailing report indicated the absence or exceedingly minimal presence of said substance within the joint fluid of patients afflicted with rheumatoid arthritis (RA). However, recent findings have unveiled its detection in a subset of individuals suffering from RA. The aforementioned substance can be classified as an anti-inflammatory cytokine due to its ability to impede the synthesis of pro-inflammatory cytokines, namely TNFα, IL-1β, and IL-6, within rheumatoid synovial fluid, synovial tissue, and peripheral blood mononuclear cells (PBMCs). The results of in vitro investigations have demonstrated that IL-4 possesses the ability to diminish bone resorption through its direct impact on osteoclasts. Additionally, IL-4 can impede the formation of MMPs (74).

The experimental animal models have also revealed the presence of a suppressive effect exerted by IL-4. The administration of IL-4 has been discovered to exhibit suppressive effects on proteoglycan-induced arthritis through the inhibition of pro-inflammatory cytokines, specifically IL-1 and IL-6, within the joints (75). In stark

contrast, one of the studies revealed that the administration of IL-4 did not exhibit any suppressive effects on collagen-induced arthritis (CIA) in DBA/1 mice. The observed outcome may potentially be attributed to the varying dosage of IL-4 administered and the specific route through which it was delivered (76).

The presence of a polymorphism in the IL-4 gene, specifically the (-590 T/C) variant, has been observed to elevate the susceptibility to rheumatoid arthritis (RA) in both European and Chinese populations. Furthermore, this genetic variation can serve as a valuable marker for assessing the predisposition and intensity of RA (77).

Significance of Interleukin-6 in rheumatoid arthritis

The interleukin-6 (IL-6) is a glycoprotein with a molecular weight ranging from 22 to 29 kilodaltons. It exhibits a dual nature, possessing both pro-inflammatory and anti-inflammatory properties. The production of this substance is facilitated by a diverse array of cellular entities, including but not limited to B cells, T cells, fibroblasts, endothelial cells, monocytes, macrophages, keratinocytes, chondrocytes, and certain neoplastic cells. The IL-6 receptor is composed of two distinct chains, namely the IL-6-specific receptor (IL-6Ra) and a signal transducer known as gp130. Both of the subunits are present in a soluble state. In the context of the classic signaling pathway, it is observed that interleukin-6 (IL-6) exhibits its action by binding to the transmembrane IL-6 receptor (IL-6R). Following this binding event, a complex is formed between IL-6 and IL-6R, which then associates with a signal transducing molecule known as gp130. This association with gp130 subsequently triggers a cascade of downstream signaling events within the target cells, facilitated by the involvement of Janus Kinase. The transmembrane IL-6R is selectively expressed on certain cell types, including hepatocytes and a subset of leukocytes. In contrast, the gp130 receptor is present on all cells throughout the body [37]. In the context of IL-6 trans-signalling, it is observed that the soluble form of IL-6R (known as sIL-6R), which lacks the transmembrane and cytoplasmic region, exhibits an affinity for the membrane bound gp130 subunits. The mounting body of evidence indicates that classic signalling exhibits anti-inflammatory properties, while trans-signalling, on the other hand, demonstrates pro-inflammatory characteristics (78).

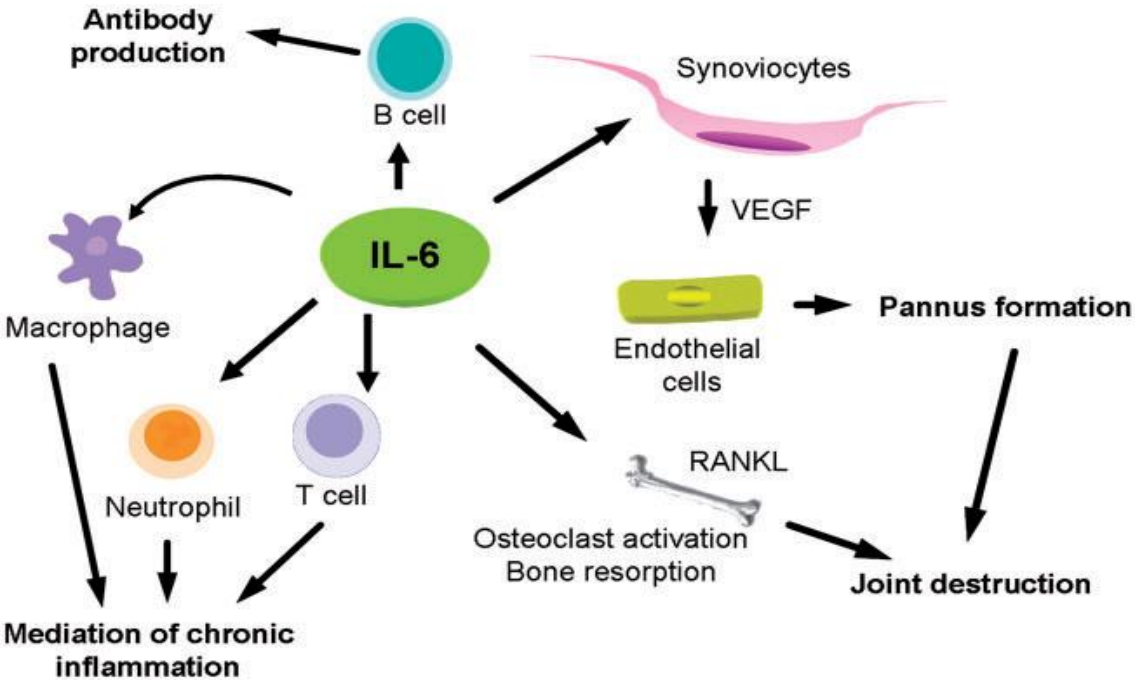

Figure.3: Inflammatory mechanisms induced by IL-6 (79)

A significant elevation in the concentration of interleukin-6 (IL-6) has been observed in both the bloodstream and synovial fluid (SF) of numerous individuals diagnosed with rheumatoid arthritis (RA). IL-6, or interleukin-6, is a cytokine that plays a significant role in the inflammatory response and subsequent joint destruction. It accomplishes this by exerting its effects on neutrophils, which are immune cells responsible for combating infections. Specifically, IL-6 stimulates neutrophils to secrete reactive oxygen intermediates and proteolytic enzymes. These reactive oxygen intermediates and enzymes contribute to the inflammatory process and can lead to tissue damage in the joints. This information is supported by reference (80). Additionally, it elicits the differentiation of osteoclasts through either a mechanism dependent on the receptor activator of NF-kappa B ligand (RANKL) or a mechanism that is independent of RANKL (81). Furthermore, it is worth noting that there exists a notable synergistic relationship between interleukin-1β (IL-1β) and tumor necrosis factor alpha (TNF α) in the facilitation of vascular endothelial growth factor (VEGF) production. VEGF, being a pivotal cytokine, plays a crucial role not only in the establishment but also in the sustenance of pannus, an inflammatory vascular tissue (80). The laboratory findings observed in patients with rheumatoid arthritis (RA) encompass certain notable aspects, including an increase in CRP (C-reactive protein) levels, a decrease in albumin levels (known as hypoalbuminemia), and an augmented

tendency for blood clot formation (referred to as hypercoagulability). These particular manifestations are believed to be influenced by the actions of interleukin-6 (IL-6). Furthermore, it has been observed that there exists a positive correlation between the elevation of IL-6 concentration and the heightened probability of experiencing a myocardial infarction (81).

The IL-6 gene polymorphism, specifically rs1800795, which is located at position -174 (G/C), has been correlated with heightened vulnerability and a greater propensity for erosive rheumatoid arthritis (RA). The polymorphism located at position -572 G/C (rs1800796) has been observed to exhibit polymorphic behaviour. It is worth noting that this polymorphism has been found to be correlated with an elevated susceptibility to rheumatoid arthritis (RA) within the Chinese Hans population. This association has been documented in previous studies (73, 82).

Significance of Interleukin-7 in rheumatoid arthritis

Interleukin 7, a glycoprotein with a molecular weight of 25 kilodaltons, belongs to the IL-2 family. Originally referred to as lymphopoietin (LP-1) or pre-B cell factor, this protein holds significance in the field of immunology. The aforementioned substances are generated by a variety of cellular entities, namely epithelial cells, fibroblasts, stromal cells, endothelial cells, smooth muscle cells, and keratinocytes. The receptor for interleukin-7 (IL-7) is a heterodimeric complex comprised of two distinct subunits, namely IL-7Rα and a common γ chain (γc). The presence of 7R can be observed on various immune cell types, including CD4+ T cells, CD8+ T cells, natural killer (NK) cells, and monocytes. The binding of interleukin-7 (IL-7) to its corresponding receptor, IL-7R, initiates a cascade of phosphorylation events mediated by Janus Kinases (JAK1 and JAK3), Signal Transducers and Activators of Transcription (STAT-5a and 5b), and src family kinases, among others (83). The JAK (Tyrosine-protein kinase) family comprises a group of intracellular tyrosine kinases that function as nonreceptor proteins. These kinases play a crucial role in transmitting signals initiated by cytokines through the JAK-STAT pathway. Within the realm of monocytes, it has been observed that the presence of IL-7 elicits a notable response in terms of the secretion of various cytokines and chemokines. Specifically, IL-7 has been found to stimulate the secretion of IL-1α, IL-1β, IL-6, IL-8, TNFα, and macrophage inflammatory protein (MIP)-1β (84). Nevertheless, it is worth noting that the

expression of IL-7R on monocytes is significantly lower compared to other cell types. Consequently, the threshold of IL-7 concentration necessary for the secretion of the aforementioned cytokines is considerably higher in monocytes than the amount required for T cell activation.

In 2003, J A G van Roon et. al. conducted a study to explore the impact of interleukin 7 (IL7) on proinflammatory cytokine production in synovial fluid mononuclear cells (SFMC) and to understand the mechanism by which IL7 influences CD4+ T cell activity in rheumatoid arthritis (RA) patients. The research involved a cross-sectional cohort of individuals diagnosed with RA, comparing IL7 levels with those of a healthy control group. Additionally, IL7 levels in RA patients were correlated with the observed disease activity. The study investigated the effect of IL7 on cytokine production by conducting experiments on RA SFMC and SF CD4+ T cells in the presence of mononuclear cells (MC). Tumor necrosis factor α (TNFα), interleukin 1β (IL1β), interferon γ (IFNγ), and interleukin 4 (IL4) were quantified using enzyme-linked immunosorbent assay (ELISA) and single-cell fluorescence-activated cell sorting (FACS) analysis. The study also assessed the expression of the IL7 receptor α chain on CD4+ T cells, a crucial component for IL7 signaling. The research specifically explored the direct impact of IL7 on CD4+ T cells isolated from synovial fluid (SF) by analyzing cytokine levels. Moreover, it investigated the indirect impact of IL7 on T cells via accessory cells, neutralizing IL12 in MC cultures. Elevated IL7 serum levels were found in RA patients compared to healthy controls, and a positive correlation was established between IL7 serum levels and C-reactive protein levels. Stimulation of IL7 resulted in the production of TNFα by SFMC and notably stimulated IFNγ and TNFα production by SF CD4+ T cells. The study suggested that the IL7 receptor α chain, highly expressed on synovial fluid CD4+ T cells, played a role in these observed effects. Besides its direct stimulation of T cell cytokine production, IL7's action was partially dependent on IL12, indicating its involvement in stimulating accessory cell function, ultimately activating T cells. In conclusion, the study demonstrated that IL7 exposure led to the stimulation of proinflammatory cytokine production in intra-articular CD4+ T cells and accessory cells from RA patients. The observed correlation with disease activity measures suggests that IL7 may contribute significantly to Th1 and TNFα-mediated proinflammatory responses in individuals with rheumatoid arthritis (85).

Chizzolini C et. al. did a research study in 2006, the aim of the study was to investigate the potential of dermal fibroblasts to generate inflammatory chemokines that may play a role in fibrosis resulting from interaction with polarized human T cells, our study aims to delve into this capacity. The findings suggest that the regulation of chemokine production by fibroblasts is contingent upon the specific T-helper (Th) cell subset employed for their activation. Hence, it can be observed that Th1 and Th2 cells exhibit a distinct preference for the production of IFN-γ inducible protein (IP)-10 and IL-8, respectively. However, it is noteworthy that monocyte chemoattractant protein (MCP)-1 is equally induced by both subsets, as evidenced by the levels of mRNA and protein expression. The findings from the neutralization experiments have provided valuable insights into the mechanisms underlying the induction of IL-8 and MCP-1 by Th1 and Th2 cells. It has been observed that membrane-associated tumour necrosis factor-α and IL-1 exert a significant influence in this process. Conversely, the presence of membrane-associated IFN-γ, which is exclusive to Th1 cells, has been found to contribute, albeit partially, to the differential production of IL-8 and IP-10 by Th1 cells. The veracity of the contributions made by tumour necrosis factor-α, interleukin-1, and interferon-α was substantiated through the process of culturing fibroblasts in isolation, utilizing a semipermeable membrane, while concurrently subjecting living T cells to activation via CD3 cross-linking. Upon further investigation, notable distinctions were observed during our exploration of signal transduction pathway utilization in fibroblasts. The pharmacological inhibition of c-Jun N-terminal kinase and nuclear factor-κB led to the inhibition of IL-8 mRNA transcription that was induced by Th1 cells, but not by Th2 cells. Conversely, the inhibition of MEK/ERK and nuclear factor-κB resulted in the inhibition of MCP-1 mRNA induced by Th2 cells, but not by Th1 cells. Upon careful examination, it was found that there were no discernible variations in the production of chemokines between systemic sclerosis and normal fibroblasts, regardless of whether the responses were triggered by T cell contact or prototypic Th1 and Th2 cytokines. The aforementioned findings suggest that fibroblasts possess the capacity to engage in the modulation of the inflammatory response by means of activating adaptable chemokine production programs, contingent upon the specific Th subset that elicits their reaction (86).

Significance of Interleukin-10 in rheumatoid arthritis

Interleukin 10, usually referred to as human cytokine synthesis inhibitory factor (CSIF), is a homodimer with a molecular weight of 39-KD. It is synthesized by various immune cells, including monocytes, macrophages, T-lymphocytes, and B-lymphocytes. The receptor for interleukin-10 (IL-10) is classified as a type II cytokine receptor, which is composed of both α and β chains. The aforementioned substance can be classified as an anti-inflammatory cytokine, as it possesses the ability to reduce the production of pro-inflammatory cytokines, including but not limited to TNFα, IL-1α, IL-1β, IL-8, IL-6, IL-12, and GM-CSF (74). Another evaluating study was done by Mottonen M. et al. to investigate the implications of interleukin (IL)-10, IL-4 in conjunction with granulocyte/macrophage colony-stimulating factor (GM-CSF), as well as tumour necrosis factor alpha (TNF-alpha), on the phenotype and antigen-presenting capacity of synovial fluid (SF) macrophages derived from individuals afflicted with rheumatoid arthritis. The investigation involved the examination of the impact of IL-4, IL-10, GM-CSF, and TNF-alpha on the expression of surface antigens on SF macrophages through the utilization of flow cytometry. The investigation sought to examine the impact of these cytokines on the ability of synovial fluid (SF) macrophages to stimulate T cells, employing the allogeneic mixed lymphocyte reaction (MLR) as the experimental approach. The administration of IL-10 resulted in a significant decrease in the expression levels of CD40, CD86, and HLA-DR, while concurrently leading to an increase in the expression level of CD14, specifically on the surface of synovial fluid (SF) macrophages. The administration of IL-10 did not elicit any discernible impact on the expression levels of CD80. Significantly, it is of utmost importance to note that the effects exerted by IL-10 on the phenotype of synovial fluid (SF) macrophages seem to yield functional consequences. This is evidenced by the fact that cells subjected to incubation with IL-10 displayed a markedly diminished ability to activate T cells in the context of mixed lymphocyte reaction (MLR). The observed effects of IL-4, GM-CSF, and TNF-alpha were generally found to be in contrast to the outcomes observed in relation to IL-10. The utilization of IL-4 and GM-CSF, two cytokines with a well-established capacity to stimulate the differentiation process of dendritic cells, resulted in an augmentation of CD40, CD80, and CD86 expression, while concurrently leading to a reduction in CD14 expression on macrophages found within the synovial fluid. In accordance with the experimental findings, it was observed

that the combination of IL-4 and GM-CSF resulted in an enhanced ability of synovial fluid (SF) macrophages to stimulate T cells in a mixed lymphocyte reaction (MLR). The inhibitory effects of IL-10 on SF macrophages were observed when exposed to IL-4 and GM-CSF (87).

Joel AGRo. et al. published another article in 2001 that observed that even though it is true that both IL-4 and IL-10 have been observed to independently impede inflammatory responses in vitro among patients with rheumatoid arthritis (RA) and in vivo in experimental arthritis models, it is important to note that the clinical efficacy of either cytokine in the management of RA remains constrained. It is plausible that the reason for this phenomenon lies in the dual nature of IL-4 and IL-10, which possess not only anti-inflammatory attributes, but also exhibit pro-inflammatory properties. In various research endeavors that exploit the unique and complementary attributes of cytokines, a noteworthy dampening of inflammatory reactions has been observed, surpassing the extent achievable through the individual administration of either cytokine. The presence of this phenomenon has been documented in both the inflammatory cells of patients with rheumatoid arthritis (RA) and in experimental models of arthritis (88).

Van Roon JAG. et al. conducted a research study into the manner by which these cytokines exert their influence on activated mononuclear cells (MNC) derived from patients with rheumatoid arthritis (RA), with specific regard to the degradation of human articular cartilage in an in vitro setting. The mononuclear cells (MNCs) derived from both the synovial fluid and peripheral blood of patients with rheumatoid arthritis (RA) were subjected to stimulation using a bacterial antigen. Following this, the MNCs were treated with interleukin-10 (IL-10) and/or interleukin-4 (IL-4). The activation of type 1 T cells and subsequent induction of proinflammatory IL-1/TNF alpha-dependent cartilage damage is a well-documented consequence of bacterial antigen exposure. The investigation encompassed the determination of cytokine production and the effects of conditioned media. Additionally, the impact of IL-10 and IL-4 on proteoglycan (PG) turnover, serving as an indicator for cartilage damage, was assessed. It resulted that the production of proinflammatory cytokines by stimulated rheumatoid arthritis (RA) mononuclear cells (MNC) was inhibited by L-10 and IL-4. Furthermore, the inhibition of cartilage proteoglycan (PG) synthesis induced by these stimulated RA MNC was completely reversed by L-10 and IL-4. In this particular context, it is noteworthy to

observe that IL-10 exhibited a greater degree of potency when compared to IL-4. Furthermore, it is of interest to highlight that the simultaneous presence of IL-10 and IL-4 resulted in an additive effect. Furthermore, it is worth noting that IL-10 has the ability to directly stimulate the synthesis of cartilage proteoglycans (PG). They concluded that the reversal of the breakdown of cartilage induced by antigen-stimulated mononuclear cells (MNC) can be achieved through the administration of IL-10. Additionally, it is worth noting that IL-4 has been observed to have an additive effect on this particular process. Moreover, it is worth noting that interleukin-10 (IL-10) exerts a direct stimulatory impact on prostaglandin (PG) synthesis. Additionally, interleukin-4 (IL-4), acting as a growth factor for type 2 T cells, possesses the ability to diminish the ratio of type 1 to type 2 T cell activity. The aforementioned findings furnish compelling evidence supporting the utilization of a synergistic amalgamation of the two aforementioned cytowas kines in the therapeutic intervention of rheumatoid arthritis (RA) (89).

The research, led by Apparailly F. et al. in 1998, aimed to assess the effectiveness of adenoviral-mediated gene transfer of interleukin-10 (IL-10) in the context of murine collagen-induced arthritis (CIA). Male DBA1 mice, previously immunized with collagen II, underwent a treatment regimen involving the systemic delivery of 10(9) plaque-forming units of a replication-defective adenoviral vector expressing viral IL-10 (vIL-10). This treatment commenced on day 30, coinciding with the onset of arthritis-related clinical symptoms. The experimental results demonstrated that the introduced transgene effectively hindered the initiation of collagen-induced arthritis (CIA), reduced its severity, and exerted a significant suppressive impact on overall joint histopathology. The injected animals' serum displayed notable concentrations of IL-10 for a duration of 7 days. The inhibition of arthritis was enhanced by administering escalating doses of adenovirus-vIL-10. Additionally, it is noteworthy that the immunosuppressive effect of locally administered gene-delivered viral interleukin-10 (vIL-10) could be counteracted by the application of a monoclonal anti-vIL-10 antibody. The symptoms observed in the group receiving the same construct expressing inactive vIL-10 (vIL-10 mut) were comparable to those in untreated animals. Our data indicate that a single systemic administration of an adenoviral vector encoding vIL-10 holds promising potential as a viable candidate for arthritis suppression (90).

Significance of Interleukin-12 in rheumatoid arthritis

IL-12, or interleukin-12, is a noteworthy cytokine that exists as a hetero dimeric structure. Originally identified as natural killer cell stimulatory factor (NKSF), IL-12 exhibits pleiotropic properties. The protein in question consists of two distinct subunits, namely p40 and p35. The receptor associated with this protein is comprised of IL-12Rβ1 and IL-12Rβ2. Individually, none of the subunits exhibit noteworthy biological activity. However, it is the p70 form of IL-12, which exists as a heterodimer, that plays a crucial role in mediating significant biological functions (91). Antigen-presenting cells, such as monocytes, macrophages, and dendritic cells, facilitate the synthesis of this substance. The production of IFNγ is recognized to prompt the subsequent transformation of naïve T cells into Th1 cells. This differentiation is induced either by the sole presence of IL-12 or through a synergistic effect when combined with IL-18. Additionally, it works to enhance the cytotoxic effects of natural killer (NK) cells and cytotoxic T lymphocytes (CTL) (71).

Petrovic-Rackov L. et al. conducted a research study in the year 2006, the primary objective of this investigation was to assess the clinical relevance of serum (S) and synovial fluid (SF) interleukin (IL)-18, IL-15, IL-12, and tumor necrosis factor alpha (TNF-α) measurements in conjunction with laboratory and clinical indicators of disease activity among individuals afflicted with active rheumatoid arthritis (RA). In this study, a cohort of sixty-four patients diagnosed with rheumatoid arthritis (RA) and twenty-five patients diagnosed with osteoarthritis (OA) were selected for inclusion. The quantification of RA activity was ascertained through the utilization of the Disease Activity Score (DAS) 28 index. The concentrations of IL-18, IL-15, IL-12, and TNF-α were quantified using the enzyme-linked immunosorbent assay (ELISA) technique. The levels of Serum C-reactive protein (CRP) were also determined. The cross-sectional correlations were computed between the levels of cytokines S and SF, and the values of the DAS 28 index. The findings have revealed that the concentrations of IL-18, IL-15, IL-12, and TNF-α in the synovial fluid (SF) and serum (S) of individuals diagnosed with rheumatoid arthritis (RA) were markedly elevated compared to those observed in patients with osteoarthritis (OA) ($p<0.01$). The concentrations of IL-18, IL-15, and TNF-α were observed to be markedly elevated in the synovial fluid (SF) when compared to the serum (S) of individuals afflicted with rheumatoid arthritis (RA) ($p<0.01$). RA patients with high disease activity (DAS 28>5.1) exhibited markedly

elevated levels of all four cytokines (S and SF) as well as serum CRP values, in comparison to individuals with mild (DAS 28>3.2) and low disease activity (DAS 28>2.6) ($p<0.01$). The concentrations of all four cytokines, both in the serum and synovial fluid, exhibited a positive correlation with the DAS 28 index values, which is indicative of disease activity. In this study, it was observed that there exists a weak correlation between S and SF IL-12. However, it is noteworthy that the strongest coefficient of correlation was identified between SF IL-18 ($r=0.879$, $p<0.01$) as well as SF TNF-α ($r=0.827$, $p<0.01$) and disease activity. A robust correlation has been observed between the levels of SF TNF-α and SF IL-18, with a correlation coefficient of 0.732 and a significance level of $p<0.01$. In summation, the levels of SF IL-18 and TNF-α in patients diagnosed with rheumatoid arthritis serve as commendable indicators of the activity of the disease. The findings acquired substantiate the utilization of the Disease Activity Score (DAS) within the realm of clinical practice as a dependable approach for evaluating the level of disease activity in patients diagnosed with rheumatoid arthritis (RA) (92).

In 2015, En-Yin W. et al. conducted a research study with the primary aim of investigating the relationship between prevalent polymorphisms in the interleukin (IL)-12A and IL-12B genes and rheumatoid arthritis (RA) among the Chinese Han population. The study included 412 patients diagnosed with rheumatoid arthritis (RA) and 279 control subjects. Genetic variations, specifically single nucleotide polymorphisms (SNPs), within the IL-12 genes were analyzed. The genotypes of these SNPs were determined using the polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) technique. The study's data indicates a significant association between the single nucleotide polymorphisms (SNPs) rs3212227 and rs6887695 in the IL-12B gene and an increased susceptibility to rheumatoid arthritis (RA). The minor allele (C) frequency at loci rs3212227 and rs6887695 in the IL-12B gene was found to be linked to a higher risk of rheumatoid arthritis (RA). Individuals carrying the rs3212227/rs6887695 C/C haplotype demonstrated a notably increased susceptibility to rheumatoid arthritis (RA). Moreover, RA patients with the C allele of the IL-12B gene rs6887695 showed a protective effect against the development of erosive arthropathy. Those with the C allele of the IL-12B gene rs3212227 exhibited a significantly higher likelihood of being rheumatoid factor (RF)-positive. Notably, there was a lack of substantial correlation between the genetic variant rs2243115 in the IL-

12A gene and rheumatoid arthritis (RA), likely due to limitations in statistical power. These findings suggest a potential association between common variants in the IL-12B gene and the onset of rheumatoid arthritis (RA) within the Chinese population (93).

Shen L. et al. conducted research in year 2015, the main objective of the investigation was to evaluate the correlation between polymorphisms of interleukin 12 (IL-12) and biomarkers associated with rheumatoid arthritis (RA) within a Chinese cohort. The results of the study indicate that there was a potential correlation between the IL-12A rs2243115 GG genotype, a specific type of functional single nucleotide polymorphism (SNP), and an elevated susceptibility to rheumatoid arthritis (RA) in patients who test negative for rheumatoid factor (RF). Additionally, the IL-12B rs3212227 AC and AC+CC genotypes demonstrate an association with an increased risk of RA in older patients, RF positive patients, and patients who test negative for anti-citrullinated protein antibodies (ACPA). The potential influence of the IL-12A rs2243115 T/G and IL-12B rs3212227 A/C alleles on the inflammatory response of IL-12 in individuals diagnosed with rheumatoid arthritis (RA) should also be considered (94).

Importance of bone remodeling in Rheumatoid Arthritis:

The maintenance of bone homeostasis is achieved through the equilibrium between the osteoblasts and osteoclasts, which operate within the parameters of normal physiological conditions. An alteration in the balance between osteoblast and osteoclast activity leads to either a positive or negative net bone remodeling outcome. In the context of rheumatoid arthritis (RA), persistent inflammation of the joints leads to an increased development and activity of osteoclasts, while impairing the differentiation and function of osteoblasts. This imbalance ultimately favours bone loss and erosion (95). Osteoclasts are formed through the fusing of cells derived from the monocyte/macrophage lineage. Proteins such as tartrate-resistant acid phosphatase (TRAP), nuclear factor of activated T cells, cytoplasmic 1 (NFATc1), and the Calcitonin receptor are expressed by osteoclasts, enabling their detection both in vitro and in tissue sections. Osteoclasts adhere to the bone surface and generate an acidic microenvironment underneath their ruffled border, facilitated by proton pumps. This acidification process enables the demineralization of bone. Osteoclasts produce enzymes, including matrix metalloproteinases and cathepsin K, which breakdown the

organic bone matrix and initiate bone resorption (96). In contrast, osteoblasts undergo differentiation from mesenchymal stromal cells. The expression of crucial transcription factors, such as Runx2 (97) and osterix (98), is observed in committed osteoblast progenitors. The differentiation of these progenitors is predominantly triggered by two osteogenic pathways, namely the bone morphogenetic protein route (99) and the classical Wingless signaling pathway (100). During the course of their development, osteoblasts acquire the ability to generate bone matrix and mineralize it, and can subsequently undergo further differentiation into osteocytes, which are cells embedded inside the bone matrix and responsible for detecting mechanical strain. The mechanosensors transmit signals in a feedback loop to both osteoblasts and osteoclasts in order to regulate bone homeostasis (101). An essential self-regulatory connection is observed between osteoblasts and osteoclasts. Immature osteoblasts demonstrate the expression of receptor activator of NF-κB ligand (RANKL), while its corresponding receptor, RANK, is predominantly expressed on osteoclast precursor cells. The interaction between RANKL and RANK facilitates the process of osteoclast development and enhances its functionality (102). Furthermore, in order to maintain bone homeostasis, fully developed osteoblasts and other cellular components release Osteoprotegerin, which acts as an inhibitor and soluble decoy receptor for RANKL. The binding of OPG to RANKL results in the inhibition of its interaction with the RANK receptor, hence impeding the process of osteoclastogenesis and the subsequent resorption of bone (103). Mice that are defective in RANKL exhibit an absence of fully developed osteoclasts and demonstrate a protective effect against bone degradation in a mouse model of inflammatory arthritis (104). The significance of this discovery is exemplified in a separate animal model with osteoclast deficiency, namely the cFos-deficient mouse. When mice are provoked with TNF-dependent arthritis, they also exhibit protection against articular bone destruction (105). In the context of rheumatoid arthritis (RA), the presence of certain cytokines such as TNF, IL-1, IL-6, and IL-17 has been observed to upregulate the expression of receptor activator of nuclear factor kappa-B ligand (RANKL). This upregulation subsequently promotes the differentiation and activity of osteoclasts, resulting in the erosion of bone tissue. A number of these cytokines also exhibit synergy with RANKL in facilitating the differentiation of osteoclasts (106).

In 2002, Kurt Redlich et.al. conducted a study to investigate the significance of osteoclasts in the development of arthritis. Transgenic mice expressing human TNF (hTNFtg) with severe arthritis were crossbred with osteopetrotic, c-fos– deficient mice (c-fos –/–), which lack osteoclasts entirely. The resulting mutant mice (c-fos –/– hTNFtg) developed TNF-induced arthritis even in the absence of osteoclasts. Both groups, with and without osteoclasts, exhibited comparable worsening of arthritis symptoms, such as increased paw size and weakened grips, at a similar rate. Microscopic examination of joint slices revealed no differences in synovial inflammation, cell types (except for the absence of osteoclasts), or levels of matrix metalloproteinase-3 (MMP-3) and MMP-13. The study concluded that reducing osteoclasts might be a beneficial approach in treating rheumatoid arthritis, particularly when combined with anti-inflammatory drugs (107).

Crotti TN et. al. conducted a study in 2002 and concluded that the certain patients diagnosed with SpA and those diagnosed with RA with active synovitis exhibited the most elevated concentrations of RANKL. Produced by infiltrating activated T cells and macrophages, an increase in RANKL in the inflamed joint of patients with rheumatoid arthritis is likely a significant contributor to joint erosions in RA (108).

[E] Current Treatment Therapies of Rheumatoid Arthritis and various issues they cause:

Recent advancements in RA pharmaceutical therapy have allowed many patients to achieve remission improving their quality of life and reducing late consequences from RA. Early intervention in disease management helps in reducing damage along with continuous care. However, a significant issue with traditional RA therapy is their high cost, which is mostly due to disease-modifying anti-rheumatic medications “DMARDs” and “selective synthetic DMARDs”. Clinicians should take into account treatment costs when deciding on a course of action (109). However, a number of problems arise, including the likelihood of an elevated risk of cardiovascular disease and the occurrence of disease flare-ups (110,111). The discovery and acceptance of biological DMARD-like generic substances has been the subject of another recent discussion. Some studies have indicated that biosimilars are as effective as the originals. They represent a significant alternative to cutting costs, expanding

treatment options, and reducing access disparities between wealthy and developing countries to care (112). Additionally, Smolen et al. (113) noted that therapy failure is a common occurrence in RA patients. Therefore, even after all therapeutic options have been explored, it is still critical to find novel treatments and understand the mechanisms underlying therapy toxicity and failure in many patients who don't experience remission. One of the main issues with treatment is adverse effects and dangers, particularly in individuals with concurrent comorbidities. In addition to the high cost, side effects impede patient adherence to the medications (109).

(a) **Non-steroidal anti-inflammatory drugs (NSAIDs)** The majority of patients use NSAIDs for self-medication prior to seeing a doctor and getting a diagnosis. NSAIDs are adjuvant drugs that can be used to manage the symptoms of RA, allow quick analgesia, and reduce inflammation. Continuous use must be avoided because it is linked to a wide range of side effects such as nausea, abdominal pain, liver damage (114,115), increased risk of heart attacks (116), and gastrointestinal problems like ulcers and blending (117). These effects can vary depending on the kind of "non-steroidal anti-inflammatory drugs (NSAIDs)", and they may be controlled by other pharmaceuticals, such as antacids & inhibitors of proton pumps, or dietary adjustments (118,114).

(b) **Glucocorticoids:** Glucocorticoids are better as compared to NSAIDs in conjunction with DMARDs and in severe systemic RA, and they are routinely advised. According to Strehl et al. (119), a number of negative consequences might happen. The severity of the symptoms frequently depends on the dosage and duration of treatment and the individual patient (120,121) are documented in the literature. The medications may frequently lead to an increase in the frequency of cardiovascular events, however there are insufficient results in the literature (122). Careful patient monitoring are required throughout the use of glucocorticoids (123), and patients with comorbidities such as diabetes, hypertension, and dyslipidaemia (119), need to be given special consideration.

(c) **TNF-alpha inhibitors:** TNF-alpha is produced and initiates an immune response in T-lymphocytes, monocytes, and activated macrophages. Elevated TNF-alpha levels contribute to bone degradation and have led to the development of medications for the treatment of rheumatoid arthritis (RA) (124)**.** Infliximab, the first chimeric monoclonal antibody, consists of a human antibody framework

combined with a mouse idiotype. It neutralizes the effects of TNF-alpha by binding to all forms of this cytokine (125). It has a long-term safety profile and is administered as an intravenous infusion. In individuals receiving infliximab, significant drops in cytokines like "IL-8, IL-6, MCP-1, and IL-1" have been observed. Infliximab exhibits substantial adverse effects, including malignancies, lymphoma, and the reactivation of hepatitis B or T, even after the safety profile (126). Adalimumab, a totally humanised antibody and another illustration of a TNF-alpha inhibitor that is administered subcutaneously, is one more option. When administered, it has a lesser potential for toxicity and has effects similar to those of MTX. Commonly harmful outcomes include cardiac arrest, cutaneous responses, and latent reactions (127).

(d) T-cell targeted therapies: A large number of T-cells infiltrate the synovium, with a smaller portion entering deeper into the tissue. There, they enhance the production of proinflammatory cytokines, leading to cartilage damage and bone degradation. Several T-cell-specific therapies are proposed which inhibit this pathway e.g., Abatacept, a T-cell activation modulator (128), it blocks signalling between CD 80 and CD 86. Both injectable and infusion versions are offered. The most common adverse effects include a sore throat, headache, cold, infection, and nausea (129).

(e) Targeted treatments for IL-6: IL-6 stimulates Pannal development and ultimately heightens bone resorption in RA by increasing VEGF expression. The "human-based antibody" tocilizumab targets IL-6 in particular. It has a lower immunogenicity and can be given as intravenously and subcutaneously (130). Sirukumab, Clazakizumab, Alacizumab, and Sarilumab are other similar examples. They have common side effects such as headaches, hypertension and respiratory tract infections. However, to establish the therapeutic effectiveness of these drugs against RA, additional clinical investigations are also required (131).

(f) Targeted treatments for IL-1: Once-daily injection of Anakinra, which functions as an IL-1 receptor antagonist has been used for treatment. The activity of IL-1a and IL-1b is inhibited by directly inhibiting the action of "IL-1 receptors". These formulations have the immediate side effects of causing itchy rashes, asthma, gastrointestinal tract infections and respiratory tract infections (132,133).

[F] THYMOSIN ALPHA-1 (Ta-1)

Thymosin alpha 1, initially derived from thymus tissue as a natural substance, is a synthetic peptide consisting of 28 amino acids with an acylated amino-terminal. While early studies utilized a thymic preparation containing approximately 1% thymalfasin, most subsequent research employed synthetically produced Ta-1 using solid-phase peptide synthesis. Detectable levels of endogenous thymalfasin in serum range from 0.1 to 1 ng/mL in healthy adults, as measured by immunoassays. Interestingly, diseased individuals tend to exhibit lower circulating concentrations (134), while levels are higher during pregnancy. Despite these observations, the source, release mechanisms, and regulation of circulating thymalfasin remain unknown. Thymalfasin is inherent in the sequence of prothymosin, a 126-amino-acid peptide primarily located in the cell nucleus, which has been investigated for its potential impact on cell proliferation (135-136). Thymalfasin, most concentrated in the thymus, is also present in various tissues such as the spleen, lung, kidney, brain, blood, and others.

Mechanism of action:

Research on Ta-1's cellular mechanism of action has revealed both direct-acting and immune-modulating properties. Due to its ability to adopt a structured helix in organic solvents, Ta-1 might have the capability to pass through the membrane independently, entering the cell and engaging with intracellular Toll-Like Receptors (137).

Ta-1 effects on the immune system:

Thymosin alpha 1, a peptide hormone, exerts its biological effects by promoting the proliferation and differentiation of stem cells, leading to increased production of natural killer (NK) cells, cluster of differentiation 4 (CD4) cells, and cluster of differentiation 8 (CD8) cells. When Ta-1 was introduced in the setting of human CD34 stem cells cultured in vitro, a noticeable enhancement in thymopoiesis was observed, resulting in a significant increase in the overall quantity of CD3 T cells. This was accompanied by the concurrent production of interleukin-7 (IL-7), a crucial cytokine essential for the maturation process of thymocytes. The specific subpopulation experiencing a marked increase due to Ta-1 administration primarily consisted of

helper T cells, particularly those expressing the CD4 surface marker (138). Ta-1 has been shown to heighten the activity of natural killer (NK) cells in various animal models (139-142) and in individuals infected with the human immunodeficiency virus (HIV) (143). This effect is particularly significant in the context of combating viral infections, considering the observed decrease in natural killer (NK) cell activity resulting from hepatitis C infection (144).

Ta-1 serves to enhance the production of Th1 cytokines:

Ta-1 demonstrates the ability to enhance the synthesis of IFNγ, IL-2, IL-3, and the expression of the IL-2 receptor following activation by mitogens or antigens (141-143,145). The observed increase in cytokine production, particularly IFNγ and IL-2, provides evidence that Ta-1 effectively promotes a Th1-oriented immune response. It is crucial to emphasize the significance of a Th1 response in establishing a robust antiviral defense, in contrast to a predominantly Th2 response, which is associated with the persistence of infections (146). It is noteworthy that the release of Th2 cytokines may potentially serve as a viral strategy aimed at evading immune surveillance (147).

In patients with chronic hepatitis C infection, the introduction of Ta-1 was found to significantly increase the synthesis of interleukin-2 (IL-2) in peripheral blood mononuclear cells. When these cells were treated with IFNα, an elevation in IL-2 levels was observed, but it is important to note that the rise induced by Ta-1 was notably more pronounced. Moreover, combining interferon alpha (IFNα) and Ta-1 during the incubation of peripheral blood mononuclear cells (PBMCs) demonstrated an additive or potentially synergistic effect on IL-2 synthesis. Notably, this study revealed that Ta-1 administration can decrease the levels of Th2 cytokines, specifically IL-4 and IL-10. In contrast, the administration of IFNα stimulated the production of these cytokines. Consequently, when administered alongside IFNα, Ta-1 could offer dual benefits to patients with viral infections. It not only enhances crucial T-cell subsets essential for effectively eliminating HCV but also inhibits the Th2 response triggered by IFNα (148).

Moreover, it has been established that Ta-1 can increase the expression of proteins situated on the outer membrane of cells infected by viruses or affected by tumors. These proteins, namely Major Histocompatibility (MHC) Class I, MHC Class II, and beta-2 microglobulin, play a vital role in facilitating antigen presentation (149-

150). Additionally, Ta-1 can enhance the presence of antigens specific to tumors (151-152). The observed immune escape phenomenon in both virally-infected and tumor cells has been linked to the down-regulation of antigen-presenting molecules, as suggested by prior studies (153). Ta-1, classified as a peptide hormone, demonstrates significant anti-inflammatory properties. It plays a crucial role in regulating immune responses, establishing tolerance, and modulating inflammatory processes (154-155). The regulation of immune responses is achieved through a primary impact on the cells of the immune system that are inherent to immunity, thus serving as an endogenous regulator of both the inflammatory and adaptive immune response.

Clinical applications of Ta-1:

The initial clinical investigations into Ta-1, as part of the compound known as thymosin fraction 5, were conducted in trials sponsored by medical professionals in the United States. These trials focused on individuals with primary immune deficiency disorders, including DiGeorge syndrome (156,157). The research effectively demonstrated improved lymphocyte function in certain individuals, coupled with noticeable enhancements in clinical manifestations believed to be directly linked to thymosin administration.

As a potent biological response modifier (BRM), Ta-1 has a broad range of clinical applications. In the first randomized double-blind Phase II trial conducted by Schulof et al. in 1985 (158), the administration of synthetic Ta-1 to patients with non-small cell lung cancer who had undergone radiotherapy showed significant improvements in both relapse-free and overall survival. These improvements were particularly pronounced among patients with non-bulky tumors. Currently, Ta-1 is undergoing global clinical trials for the treatment of various cancer types and infections caused by the hepatitis B virus (HBV) and hepatitis C virus (HCV), both closely associated with hepatocellular carcinoma (HCC) (159).

In 2009, Yumin L. et al. conducted a study to explore the impact of immunomodulatory therapy using ulinastatin in combination with Ta-1 on patients with sepsis. A cohort of 56 individuals diagnosed with sepsis was randomly assigned to two groups. The treatment group received immunomodulatory therapy, while the placebo

group received a placebo substance. Acute Physiology and Chronic Health Evaluation II scores, relevant clinical data, lymphocyte subsets, immunological indexes, and coagulation parameters were assessed before admission and at specific intervals thereafter (on the 3rd, 8th, and 28th day after admission to the Intensive Care Unit). The experimental group demonstrated a cumulative survival rate of 78%, whereas the control group exhibited a cumulative survival rate of 60%. This disparity in survival rates was reflected in Acute Physiology and Chronic Health Evaluation II scores, along with the accelerated improvement in leukocyte counts, lymphocyte counts, coagulation parameters, and cytokine levels observed in the treatment group (160).

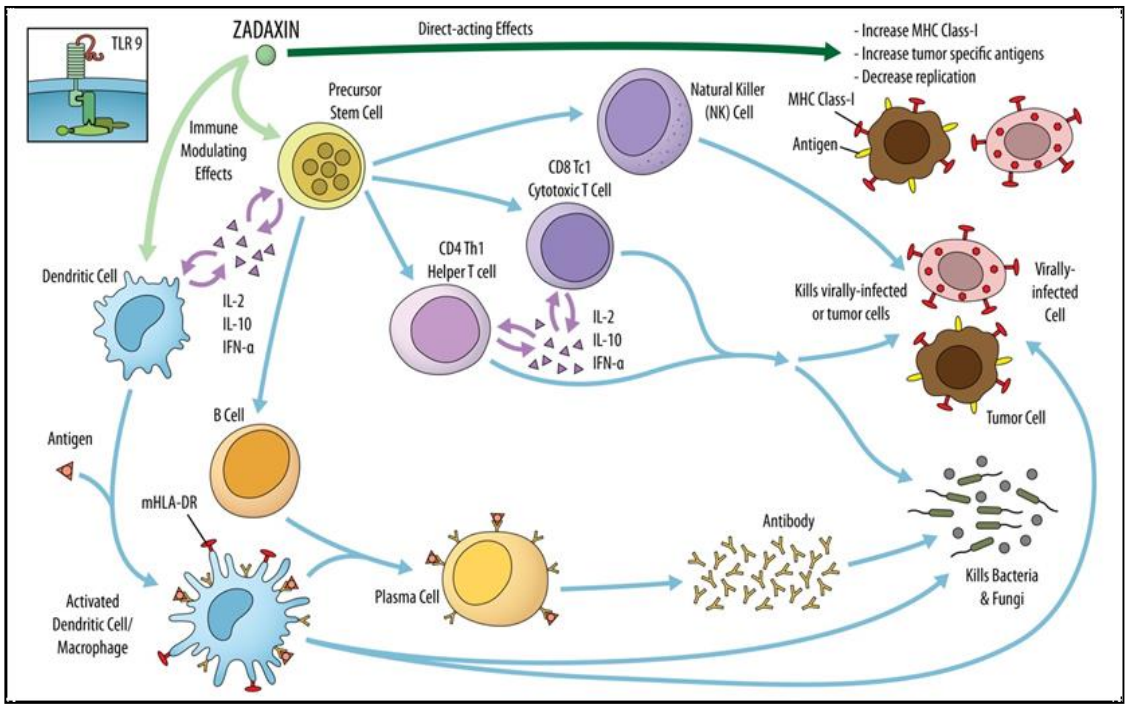

Fig. 4: The immune-stimulating mechanism of Ta-1 involves a dual mode of action. Ta-1 exhibits immune-modulating effects that enhance the body's ability to combat infections and cancer. Additionally, it directly influences infected or tumor cells (161).

Zhang Q. et. al. conducted a study in year 2010 and the findings of the study indicate that Ta-1 exhibited antagonistic properties towards joint symptoms in individuals who have previously battled breast cancer. Furthermore, it was observed that Ta-1 possessed the ability to regulate immune action. The specific mechanisms responsible for the analgesic effects of Ta-1 are not fully understood. It is hypothesized that the immunomodulation, achieved by adjusting the ratios of TH1/TH2 cytokines

IFNγ and IL-4, may serve as a plausible pharmacological mechanism contributing to the anti-inflammatory properties of Ta-1 (15). Pica F. et. al. did a research study in the year 2016 and the outcomes of a study reveal that individuals suffering from chronic inflammatory autoimmune diseases exhibit a notable inclination towards diminished levels of Ta-1 serum in comparison to their healthy counterparts. Specifically, individuals diagnosed with Psoriatic Arthritis (PsA) exhibit the most diminished levels of Ta-1 when compared to Healthy Controls (HC). Notably, these levels are not only substantially lower than those observed in patients with Rheumatoid Arthritis (RA) and Systemic Erythematosus Lupus (SLE), but also significantly lower than the aforementioned groups (16).

Hypothesis:

Our hypothesis asserts the pivotal role of Ta-1 in the process of bone remodeling, primarily through its inhibitory influence on osteoclastogenesis and bone resorption. This assertion is substantiated by the extensively documented immunomodulatory attributes of Ta-1, suggesting its capacity to regulate the activities of osteoclast precursors. Moreover, considering the inherent immunomodulatory nature of Ta-1, we posit that its impact extends to inflammatory pathways associated with bone remodeling. In this regard, we anticipate that the administration of Ta-1 may effectively mitigate inflammation, thereby fostering a microenvironment conducive to optimal bone regeneration. The combination of inhibiting osteoclast formation and having anti-inflammatory effects makes Ta-1 a promising candidate for more research in the development of therapies aimed at improving bone health and regeneration. We also predict that Ta-1 administration will change the levels of specific markers associated with bone remodeling, such as RANKL (Receptor Activator of Nuclear Factor Kappa-B Ligand) and OPG (Osteoprotegerin).

OBJECTIVES OF THESIS

1. Evaluation of the role of Ta-1 on proliferation and differentiation of osteoclasts.
2. Evaluate the synergistic effect of Ta-1 with Glucocorticoids and Estrogen
3. Estimate the effect of the role of Ta-1 on Biochemistry and Histology of bone tissue in animal model.

MATERIAL AND METHODS

CHAPTER-3
MATERIAL AND METHODS

Cell line study:

A.) Cell culture study

1. Evaluation of cytotoxic effects of Ta-1 against RAW264.7 cells by MTT assay.
2. Evaluation of Anti-inflammatory activity of Ta-1 by assessing the NO production in RAW264.7 cells.

B.) Animal study: -

1. Acute toxicity study Ta-1 in Wistar Rats followed by Intraperitoneal administration.
2. *In vivo* effect of Ta-1 in arthritis vs control rats.
3. Perform tissue histology of bone sample of arthritis vs control rats.
4. To check the effect of Ta-1 on:
    - **Bone formation markers: -** Bone-specific alkaline phosphatase and Osteocalcin
    - **Bone resorption markers: -** TRAP and Cathepsin K.
    - **Regulation markers of bone turnover: -** OPG and RANKL

A.) Cell culture study

1. Evaluation of cytotoxic effects of Ta-1 (Gufic Biosciences Limited, Gujarat**) against RAW264.7 cells by MTT assay.**

Raw 264.7 cells, originating from mouse macrophages, are frequently employed in toxicity studies due to their biological significance and ease of handling. These macrophage-like cells are crucial in the immune response, making them suitable for investigating the immunotoxic effects of various substances. They exhibit a strong response to inflammatory agents, such as lipopolysaccharides (LPS) (0 to 10 g/ml; E. coli serotype Olll: B4, Sigma), which enables the evaluation of pro-inflammatory reactions through the secretion of cytokines like TNF-α and IL-6. Additionally, Raw

264.7 cells have intrinsic phagocytic capabilities, allowing for the assessment of the toxicity of particles or nanoparticles. They are also known to generate reactive oxygen species (ROS) and nitric oxide (NO) when exposed to toxic agents, which is essential for studying cytotoxicity linked to oxidative stress. Furthermore, these cells are easy to culture and proliferate quickly, making them ideal for large-scale toxicological experiments. Their well-established characteristics and relevance to in vivo studies render Raw 264.7 cells a valuable model for initial toxicity screening, especially in research focused on immune responses and inflammatory pathways.

Cytotoxic assay Methodology:

The RAW 264.7 cell line was procured from the National Centre for Cell Science (NCCS) located in Pune, India. The cells were cultivated during the logarithmic growth phase using Dulbecco's modified eagle medium (DMEM) supplemented with 10% (v/v) thermal attenuated fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The MTT test, also known as the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide test, was conducted in order to evaluate the cytotoxicity of the substance under investigation towards the RAW 264.7 cell line. The cells were meticulously placed within 96-well microplates, with a density of 5×10^3 cells per well. These microplates were then subjected to a controlled environment of 37°C, ensuring optimal conditions for cellular growth. The incubation process took place in an atmosphere enriched with 5% CO2, further promoting the cells' development. After a span of two days, the cells were allowed to progress until they reached a desirable state of 70-80% confluence. Subsequently, the medium was substituted, and a 24-hour cultivation period ensued subsequent to the cells' exposure to diverse quantities of substances. The morphological modifications of the untreated (control) and treated cells were evaluated and captured through the utilization of a computerized inverted microscope set at a magnification level of 20X, with the observations and images taken precisely 24 hours post-treatment (162).

$$Cell\ viability\ (\%) = \left(\frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) X100$$

2. Evaluation of Anti-inflammatory activity of Ta-1 by assessing the NO production in RAW264.7 cells.

Background of NO Production Assay:

Nitric oxide (NO) is a volatile and reactive chemical that can easily spread and has temporary effects on the body within the range of picomoles to micromoles. Nitric oxide (NO) has a crucial role in regulating the cardiovascular, neurological, and immunological systems by activating guanylate cyclase. Nitric oxide (NO) can be obtained through two pathways: endogenous production by constitutive or induced NOS enzymes, or ingestion of nitrates or nitrites which are then converted into NO.

Nitric oxide's inherent reactivity enables it to function as a cytotoxic agent when macrophages release it as part of an immune response. The high reactivity of NO enables its conversion into a highly harmful radical, which can cause damage to cells and DNA through nitrosylation. Nitrosylation is a controlled post-translational alteration that occurs in cell signalling. The regulatory and damaging aspects of NO play a crucial role in mitochondrial signalling and malfunctioning. Nitric oxide (NO) is associated with a range of health conditions, including coronary heart disease, endothelial dysfunctions, erectile dysfunction, neurological diseases, diabetes, chronic periodontitis, autism, and cancer.

Anti-inflammatory assay

The RAW 264.7 cells were appropriately accommodated within a controlled environment, specifically a humid chamber, wherein the air composition consisted of 95% humidity and was maintained at a temperature of 37°C. Additionally, the atmosphere within the chamber was supplemented with 5% carbon dioxide (CO2) to ensure optimal conditions for the cells' growth and viability. A population of cells, specifically 1x100,000 cells per millilitre, was subjected to a preliminary incubation period lasting one hour. During this time, various concentrations of the testing item were introduced to the cells. Following the incubation, the cells were stimulated for a duration of 24 hours at a temperature of 37°C, utilizing a medium containing 1 microgram per millilitre of lipopolysaccharide (LPS). The assessment of nitric oxide (NO) production can be accomplished through the quantification of nitrite levels present in the culture media. In order to accomplish this objective, the medium was

effectively incorporated with the Griess reagent system. Following a duration of 10 minutes for incubation, the absorption spectrum was duly recorded at a wavelength of 540 nm. The determination of the nitrite level was conducted by using a sodium nitrite calibration curve as a reference standard (163).

Ethical considerations:

Male wistar rats, 6-8 weeks of age were purchased from Invivo Biosciences animal house facility, Bengaluru, Karnataka 560091 and were acclimatized in the animal house conditions with a 12:12 h light: dark schedule. All the experimental procedures were approved/recommended by the IAEC of Invivo Biosciences in its meeting (approval no.: Invivo/006/2022).

1. Acute toxicity study Ta-1 in Wistar Rats followed by Intraperitoneal administration.

<u>Acute Toxicity study in Wistar rats:</u>

Table 1: Brief details about the study

Species	Rat
Strain	Wistar
No. of groups	04 (01 control, 03 for test substance)
No. of animals per group	05
Treatment Age	8-12 weeks
Identification	By cage card, crystal violet/picric acid body marking
Acclimatization	5 days

Table 2: Classification of the groups as per the treatment dose for acute toxicity study

Group No.	Treatment group	Dose	No. of animals	Animal numbers	
				From	To
1	Control	0 mg/kg	5	R1	R5
2	Low Dose	0.25 mg/kg	5	R6	R10
3	Mid Dose	0.5 mg/kg	5	R11	R15
4	High Dose	1 mg/kg	5	R16	R20

Equipment details:

- Weighing Balance: Sartorius model BT125S
- Weighing Balance: Weigh Well, Model WWTT
- Thermo-Hygrometer: CTH 288
- Magnetic Stirrer: Remi, Model 1-MLH

Husbandry:

Conditions

Animals were housed under standard air-conditioned laboratory conditions.

- Temperature: Maximum 24° C and Minimum 23° C
- Relative humidity: Maximum: 63% and Minimum 48%
- 12 h light and 12 h dark cycle

The maximum and minimum temperature and relative humidity in the experimental room was recorded once daily.

Housing:

Rats: Polypropylene cages with provision for water bottle holder and feed hopper with corn cobs as bedding material.

Diet: *ad libitum*

Pelleted rodent feed (VRK nutrition solution)

Water: *ad libitum*

Deep well bore water passed through charcoal filters and exposed to UV rays and water in polypropylene water bottles were provided to the animals.

Test substance administration:

One animal from each dosage group was administered on the initial days and subsequently observed for a duration of 24 hours. After confirming the absence of noticeable effects in the various doses examined, the remaining four animals from each group were administered the doses collectively and subsequently monitored for a duration of 15 days.

2. *In vivo* effect of Ta-1 in arthritis vs control rats.

Anti-Arthritic Study:

Table 3: Brief about the Anti-arthritic study

Species	Rat
Strain	Wistar
No. of groups	05 (01 control, 01 disease, 03 for test substance)
No. of animals per group	08
Treatment Age	6 weeks
Identification	By cage card, crystal violet/picric acid body marking
Acclimatization	3 days

Experimental Model

The wistar rats, 6-8 weeks of age were purchased from Invivo Biosciences animal house facility, Bengaluru, Karnataka 560091 and were acclimatized in the animal house conditions with a 12:12 h light: dark schedule. Free access to food and water was given ad libitum. Rats were randomly divided into 5 groups: normal control, arthritic control, CIA+ Ta-1 0.25 mg/kg, CIA +Ta-1 0.5 mg/kg and CIA+Ta-1 1 mg/kg. The collagen (CII; Sigma-Aldrich) and lipopolysaccharide (LPS) were administered via the intraplantar route to the experimental rat subjects. Before the administration of collagen and LPS, the initial paw volume was measured, and the body weight was duly documented. The administration of the treatment occurred on the first, third, and fifth days. The brief details about the study are given below in Table 4.

Table 4: Group Allocation of wistar rats as per the dosage for the anti-arthritic study

Group	Type	Treatment
I	Control	Vehicle (I.P.)
II	Arthritis group	Type II collagen on day 0 & LPS on day 3
III	Arthritis + Ta-1 High dose	Type II collagen on day 0 & LPS on day 3 + Ta-1 of 1 mg/kg (I.P.) on (1st, 3rd & 5th days)
IV	Arthritis + Ta-1 Mid dose	Type II collagen on day 0 & LPS on day 3 + Ta-1 of 0.5 mg/kg (I.P.) on (1st, 3rd & 5th days
V	Arthritis + Ta-1 Low dose	Type II collagen on day 0 & LPS on day 3 + Ta-1 of 0.25 mg/kg (I.P.) on (1st, 3rd & 5th days

Equipment Details:

- Weighing Balance: Make: Weigh well, model: WWTT
- Vernier calliper

CIA induction and dosage schedule:

The experimental design of the study included five groups of animal models, each subjected to different treatments to assess the role of Thymosin-α 1 in bone health. Group I served as the control group and received a vehicle treatment administered intraperitoneally (I.P.). Group II, the arthritis group, was induced with arthritis through the administration of Type II collagen on day 0 and lipopolysaccharide (LPS) on day 3. Group III, designated as the arthritis + Thymosin-α 1 (1 mg/kg) group, received the same arthritis-inducing agents as Group II, but with the addition of Thymosin-α 1 at a dose of 1 mg/kg administered I.P. on the 1st, 3rd, and 5th days. Group IV, the arthritis + Thymosin-α 1 (0.5 mg/kg) group, followed a similar protocol, receiving Thymosin-α 1 at a reduced dose of 0.5 mg/kg on the same days. Lastly, Group V, the arthritis + Thymosin-α 1 (0.25 mg/kg) group, also received the arthritis-inducing agents along with Thymosin-α 1 at a dose of 0.25 mg/kg I.P. on the 1st, 3rd, and 5th days. This setup allowed for the evaluation of the effects of different doses of Thymosin-α 1 on arthritis-induced bone changes.

Experimental procedure:

The collagen and lipopolysaccharide (LPS) were administered via the intraplantar route to the experimental rat subjects. Prior to the administration of collagen and LPS, the initial paw volume was measured, and the body weight was duly documented. The administration of the treatment occurred on the first, third, and fifth days.

At the end of the dosing period, which occurred on the 1st, 3rd, and 5th days, the final body weight was recorded on the fifteenth day. The measurements of paw volume, paw weight, paw thickness, and ankle thickness were duly recorded. The blood was obtained via the retro-orbital route, subsequently leading to the separation of the serum. The laboratory analysis encompassed the assessment of serum biochemistry markers

including AST, ALT, ALP, Ferritin, as well as the determination of hematological parameters such as RBC count, Differential count, and platelet counts.

The blood sample for hematological parameters underwent analysis using an automated blood analyser, while all biochemical assays were performed utilizing the RoboniK semi-automated analyser through the utilization of kit-based methods.

Assessment of arthritis and arthritic score:

The evaluation of arthritis and subsequent determination of the arthritic score is a critical undertaking in the realm of medical diagnostics. Arthritis, a condition characterized by inflammation and stiffness of the joints, necessitates a comprehensive assessment to ascertain the severity and impact on the affected individual. The arthritic score,

Grade 0 = No sign of arthritis

Grade 1 = Redness and swelling in paw

Grade 2 = Deformity in paw

Grade 3 = Ankylosis in paw

Grade 4 = Maximal swelling and deformity with ankylosis

The rats underwent regular screening to observe the onset and progression of arthritis. This screening occurred on a daily basis, commencing on day 0 and continuing until the 15th day.

The grading of arthritis severity was determined in accordance with the methodology proposed by Brand et al. (2007) (164). The arthritic score of a rat afflicted with arthritis is determined by calculating the sum of the highest grades of arthritis observed in the affected paws. The data were expressed as the mean ± standard error of the mean (SEM) of eight animals per group.

Assessment of paw volume:

To measure paw volume in a collagen-induced arthritis (CIA) model, a plethysmometer is used to monitor inflammation progression. Prior to each session, the plethysmometer

is calibrated per the manufacturer's instructions, and the chamber is filled with a suitable liquid, typically saline, to a consistent baseline level. After CIA induction, rats are gently restrained, and the affected paw is submerged in the chamber up to a marked anatomical point, such as the ankle joint, ensuring uniformity across measurements. The plethysmometer records the displacement volume, representing the paw's swelling and indicating the degree of inflammation. Measurements are taken at day 0 and day 15, enabling detailed tracking of inflammation changes over time (165). This data is then compared across experimental groups to evaluate the effects of different doses of Ta-1 treatments on arthritis progression, providing reliable insights into treatment efficacy.

3. Perform tissue histology of bone sample of arthritis vs control rats.

Procedure for slide preparation, staining and microscopic evaluation:

1. The joint tissues were washed for formalin clearing over-night in running water
2. The joint tissue was decalcified in decalcification solution (10% EDTA+ citrate–phosphate buffer + saturated ammonium oxalate)
3. The joint tissues were prepared for embedding after serial dilution of alcohol by dehydration method.
4. The tissue joints were immersed in xylene or an alternative clearing agent for several hours which makes it more receptive to embedding media.
5. The tissues were then embedded in paraffin blocks for sectioning in microtome. The sections were made at 4 µm thickness suitable for staining.
6. The tissue fixed slides were deparaffinized using xylene and further rehydrated using serially diluted alcohol.
7. Further the tissue sections were stained using Haematoxylin & Eosin stain.
8. The slides were then dehydrated by serially diluted alcohol.
9. Microscopical examinations of H&E-stained slides were done by research microscope at 40x resolution for better visibility and detailed examination.

Rat Gene Expression Analysis using Real Time PCR

RNA isolation – Using TRIZOL Method

1. A total of 5 rat limb tissue samples were processed for RNA extraction and cDNA conversion. Details of the samples are mentioned below:

Table 5: Groups division for the RNA isolation

S. No.	Sample	Type
1.	G1	Control
2.	G2	Arthritis group
3.	G3	Arthritis + Ta-1 1 mg/kg
4.	G4	Arthritis + Ta-1 0.5 mg/kg
5.	G5	Arthritis + Ta-1 0.25 mg/kg

2. The total RNA was extracted from 20 mg of the samples using TRIZOL method as per the following procedures.
3. 1 ml of TRIZOL reagent was added into 30 mg tissue sample and homogenised using mortar and pistol.
4. The homogenised samples were incubated for 5 minutes at room temperature for complete dissociation of nucleoprotein complexes.
5. After incubation, the samples were centrifuged at 12000 rpm for 10 min to remove cell debris.
6. The supernatants were transferred in a fresh tube and 0.2 ml of chloroform was added to each tube.
7. The samples were vortexed vigorously for 15 seconds and incubated for 3 min at room temperature.
8. After incubation, the samples were centrifuged at 12000 rpm for 15 min at 4°C.
9. After centrifugation, around 0.6 ml of the upper aqueous phase was transferred carefully without disturbing the interphase into fresh tube. Measure the volume of the aqueous phase.
10. 0.5 ml of isopropropanol was added into all the tubes and gently inverted the tubes for mixing. The samples were incubated for further 10 min at room temp and centrifuged for 10000 rpm for 10 min.

11. The precipitated pelleted RNAs were washed by adding 1 ml of 75% ethanol and centrifuged for 10 min at 8000 rpm. The washing step was repeated twice.
12. The RNA pellets were air dried for 10 min and dissolved using 100 µl of RNase free water.
13. RNA was quantified using a spectrophotometer and the purity of the sample was determined using A260/280 readings. Samples with A260/280 readings above 1.8 are free of any protein contamination.
14. The RNA quantification details are as follows:
15. 200ng of sample was run on RNA gel to check for DNA contamination after the samples were treated with DNase I from NEB [Catalogue#: M0303S] as per manufacturers protocol to eliminate any possible DNA contamination.

cDNA CONVERSION

16. All the required kit components and reagents are thawed on ice.
17. Required volumes of each component were mixed as per the below mentioned table

Table 6: Volume of reagents used for the cDNA conversion

S. No.	Reagents	Vol/reaction	Total vol
1	10X Reaction Buffer	2µL	12µL
2	Primer Mix	2µL	12µL
3	100mM dNTP's	0.8µL	4.8µL
4	Reverse Transcriptase	1µL	6µL
5	RNA	variable	variable
6	Molecular Grade water	variable	Variable

18. Master mix prepared for 6 samples was equally distributed into 0.2 PCR ml vials.
19. To each vial 200ng of respective RNA sample was added and final volume was made to 20µL using molecular grade water.
20. The reaction mixture was completely mixed with pipetting the reaction mixture up and down 2-5 times.

21. The samples were then loaded into a thermal cycler Himedia personal and the following program was run.

Table 7: Temperature and time required for cDNA conversion

	Step 1	Step 2	Step 3	Step 4
Temperature(°C)	25	37	85	4
Time	10 min	120 min	5 min	∞

22. After completing the cDNA conversion step the samples were stored at -20ºC until further process.

REAL TIME PCR ANALYSIS

23. Real time assay was performed for 5 samples mentioned above for the following rat genes:

- RANKL
- Bone specific alkaline phosphate,
- Cathepsin-K
- OPG
- TRAP
- Osteocalcin
- β-actin

Table 8: Primer Details for the Biomarkers

Gene	Primer	Primer Sequence
B-Actin	Forward	CGGGAAATCGTGCGTGACAT
		ATCTTCATTGTGCTGGGTGCC
Rat OPG Accession No: XM_008770928.2	Forward	GCTCACCTCACCATCAATGCT
	Reverse	GGTACCAAGAGGACAGACTGACTTTA
Rat RANKL Accession No: NM_012870.2	Forward	GCCAACACTGATGGAGCAGAT
	Reverse	TCTTCATTCCCACCAACTGATG

Rat Cathepsin-K Accession No: XM_032897739.1	Forward	CTTGGCTCGGAATAAGAACA
	Reverse	GAGGCCACAACTCTCAGAAA
Rat ALP Accession No: XM_032895843.1	Forward	TTGCTAGTGGAAGGAGGCAG
	Reverse	GTGTTGTACGTCTTGGAGAGAG
Rat Osteocalcin Accession No: XM_032898082.1	Forward	CTGACAAAGCCTTCATGTCCAAGC
	Reverse	TCCAAGTCCATTGTTGAGGTAGCG
Rat TRAP Accession No: XM_032911039.1	Forward	ACGCCAATGACAAGAGGTTC
	Reverse	AGGTGATCATGGTTTCCAGC
Rat GAPDH Accession No: XM_017604885.1	Forward	AAATTCCATGGCACCGTCAA
	Reverse	AGGGATCTCGCTCCTGGAA

1. Procedure:

- All the components required for the setup were thawed on Ice prior to setup.
- A master mix with each primer set was prepared for required number of samples and 2 additional samples to compensate for the pipetting losses.
- All reactions are performed in duplicates along with NTC (non-template control).
- All the components were calculated as per below tabulated calculation:

Table 9: Components used for the calculation

S. No.	Contents	Volume (µl)
1	SYBR Green mix (2×)	10 µl
2	Forward primer	1.0 µl
3	Reverse primer	1.0 µl
4	Template (cDNA)	1.0 µl
5	50X Rox- Reference Dye	0.4 µl
6	Sterile water	6.6 µl
	Total volume	20.0 µl

- Each gene was tested in duplicate.
- All the sample tubes were covered with Optical film/Optical cap (Agilent: #401425).
- Sample tubes were briefly vortexed and centrifuged to bring down the contents and to eliminate any air bubbles.
- The samples were loaded onto the Real time PCR machine Stratgene Mx3000P and the following program was run.

Program: A two-step real time PCR as described in the below table was performed

Table 10: Two step real time PCR

Steps	**Temperature**	**Time**
Initial Denaturation	95°C	10 minutes
Denaturation	95°C	30 Seconds
Annealing/Elongation	60°C	60 seconds
Repeat	40 cycles	

2. The RT-PCR data was collected and analysed on MxPro Software from Agilent Technologies.
3. All the sample CT values were exported to excel file for final reporting.

At the end of the reaction, the real-time PCR products were loaded on 1.5% agarose gel electrophoresis to confirm amplification specificity.

Synergistic effect of Thymosin alpha 1 with glucocorticoids and Estrogen

Experiment model:

Synergistic effect of Glucocorticoids and Ta-1

We systematically evaluate the therapeutic potential of various dosage combinations of Ta-1 and glucocorticoids in modulating inflammatory responses. The first group of combinations involves administering Ta-1 at a low dose of 0.25 mg/kg alongside glucocorticoid doses of 0.5 μmol/kg, 1 μmol/kg, and 2 μmol/kg. This allows for the assessment of how low levels of glucocorticoids influence the effectiveness of Ta-1 in reducing inflammation.

The second set of combinations increases the Ta-1 dosage to 0.5 mg/kg while maintaining the same glucocorticoid levels. This evaluation aims to determine if higher concentrations of Ta-1 enhance the therapeutic effects when paired with varying glucocorticoid dosages. Finally, the highest combination tested includes Ta-1 at 1 mg/kg with 2 µmol/kg of glucocorticoids, providing insights into the maximum potential effects of this pairing.

Table 11: Dosage Combinations of Ta-1 and Glucocorticoids

S. No.	**Dosage Combination (**Ta-1 **+ Glucocorticoid)**
1.	Ta-1 (0.25 mg/kg) + glucocorticoid (0.5 umol/kg)
2.	Ta-1 (0.25 mg/kg) + glucocorticoid (1 umol/kg)
3.	Ta-1 (0.25 mg/kg) + glucocorticoid (2 umol/kg)
4.	Ta-1 (0.5 mg/kg) + glucocorticoid (0.5 umol/kg)
5.	Ta-1 (0.5 mg/kg) + glucocorticoid (1 umol/kg)
6.	Ta-1 (0.5 mg/kg) + glucocorticoid (2 umol/kg)
7.	Ta-1 (0.25 mg/kg) + glucocorticoid (0.5 umol/kg)
8.	Ta-1 (0.5 mg/kg) + glucocorticoid (1 umol/kg)
9.	Ta-1 (1 mg/kg) + glucocorticoid (2 umol/kg)

Synergistic effect of Estradiol and Ta-1

We examine the effects of various dosage combinations of Ta-1 and estradiol to evaluate their combined impact on inflammatory responses. The first set of combinations features Ta-1 at a dosage of 0.25 mg/kg with increasing estradiol doses of 2.5 µg, 5 µg, and 10 µg. This allows for an analysis of how lower levels of Ta-1 interact with varying estradiol concentrations. The next set utilizes Ta-1 at 0.5 mg/kg paired with the same estradiol dosages, aiming to assess whether higher Ta-1 levels enhance the therapeutic effects when combined with estradiol. Finally, the highest dosage combination includes Ta-1 at 1 mg/kg with estradiol doses of 2.5 µg, 5 µg, and 10 µg. This comprehensive examination helps clarify the potential synergistic effects and optimal dosing strategies for using Ta-1 and estradiol in inflammatory conditions.

Following nine combinations doses were given to animal groups for Ta-1 and estradiol:

Table 12: Dosage Combinations of Ta-1 and Estradiol

S. No.	**Dosage Combination (**Ta-1 + **Estradiol)**
1.	Ta-1 (0.25 mg/kg) + estradiol (2.5µg)
2.	Ta-1 (0.25 mg/kg) + estradiol (5µg)
3.	Ta-1 (0.25 mg/kg) + estradiol (10µg)
4.	Ta-1 (0.5 mg/kg) + estradiol (2.5µg)
5.	Ta-1 (0.5 mg/kg) + estradiol (5µg)
6.	Ta-1 (0.5 mg/kg) + estradiol (10µg)
7.	Ta-1 (1 mg/kg) + estradiol (2.5µg)
8.	Ta-1 (1 mg/kg) + estradiol (5µg)
9.	Ta-1 (1 mg/kg) + estradiol (10µg)

Statistical analysis of data

Average of all the data was compiled and SEM was calculated. All the parameter of treated groups were compared with negative control group by one-way ANOVA followed by Dunnett's multiple comparison tests. Values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

CHAPTER-4
RESULTS AND DISCUSSION

Cytotoxic assay:

The cytotoxicity of the sample was evaluated using the MTT assay. When subjecting the sample to various concentrations of Ta-1, it was noted that the drug concentrations ranging from 15.62 µg/mL to 31.25 µg/mL demonstrated the highest cellular viability. Following the analysis, it was established that the IC50 value of Ta-1 against RAW 264.7 cells was measured at 368.10 µg/mL.

Table 13: Cytotoxic activities of Ta-1 RAW 264.7 cells

Concentrations (ug/mL)	Absorbance			Average	Cell Viability (%)	Inhibition (%)
	I	II	III			
Control	0.42	0.42	0.42	0.42	100	0
15.62	0.41	0.41	0.40	0.41	97.71	2.28
31.25	0.40	0.39	0.40	0.39	94.25	5.74
62.5	0.36	0.37	0.38	0.37	88.50	11.49
125	0.35	0.33	0.34	0.34	81.02	18.97
250	0.27	0.27	0.29	0.28	66.14	33.85

With the increase of the test concentration treatment, there was a noticeable decline in cell density and a concurrent rise in cytotoxicity observed in RAW 264.7 cells, as detailed in the observations. The modifications in morphology are visually depicted in the accompanying images Figure 5.

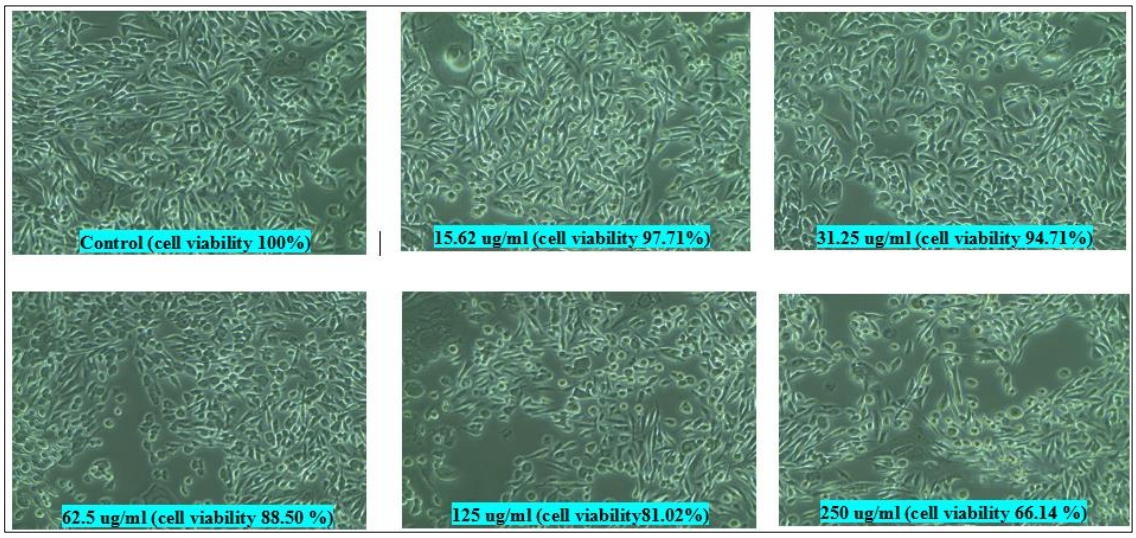

Fig. 5: Dose-dependent cytotoxicity effect of Ta-1 and morphological changes in the RAW 264.7 cells

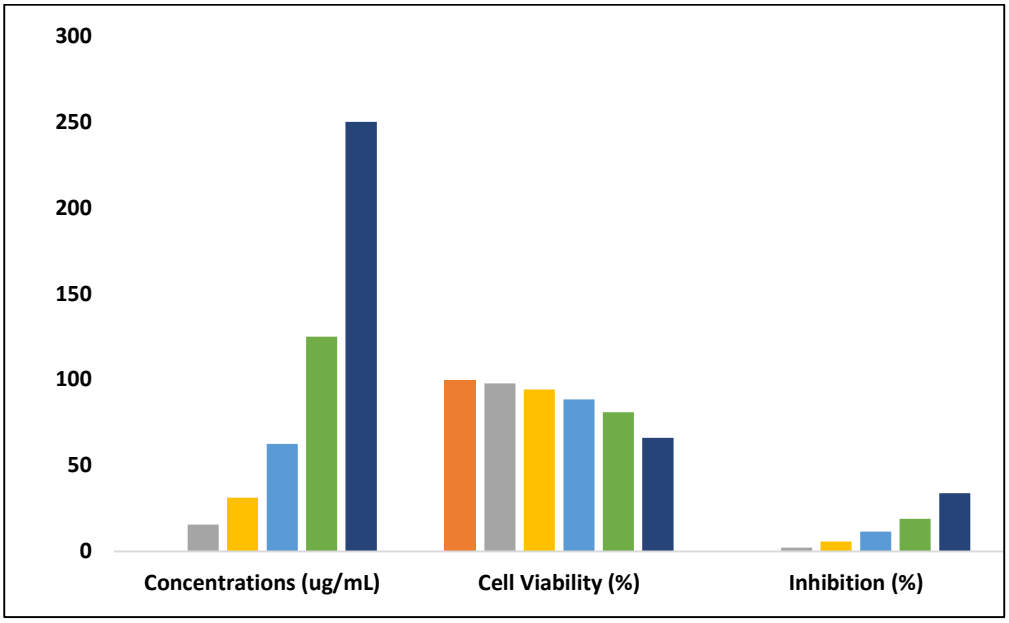

Fig. 6: Cytotoxic activities of Ta-1 RAW 264.7 cells

Anti-inflammatory assay results:

Model organisms commonly utilized for the investigation of macrophage biology and immune function often involve the use of the RAW 264.7 cell line. To ascertain whether T-1 demonstrated anti-inflammatory properties, we measured the release of nitric oxide (NO) from the RAW 264.7 cell line. A pivotal aspect of our study focused on evaluating the anti-inflammatory effects of Ta-1 on NO production in RAW 264.7 cells. Various concentrations of the sample, as outlined in Table 14, were employed to assess nitric oxide production. A dose-dependent reduction in NO production was

observed in the test items at concentrations ranging from 7.81 to 31.25 µg/mL, in comparison to the control.

Table 14: Cytotoxic activities of Ta-1 RAW 264.7 cells

Concentrations (ug/mL)	Absorbance			Average	Nitric Oxide (uM/l)
	I	II	III		
Untreated	0.08	0.08	0.07	0.07	7.76
LPS Induced	0.25	0.26	0.26	0.26	40.04
LPS + 7.81	0.24	0.23	0.23	0.23	35.48
LPS + 15.62	0.22	0.21	0.21	0.21	31.91
LPS + 31.25	0.16	0.15	0.15	0.15	21.80

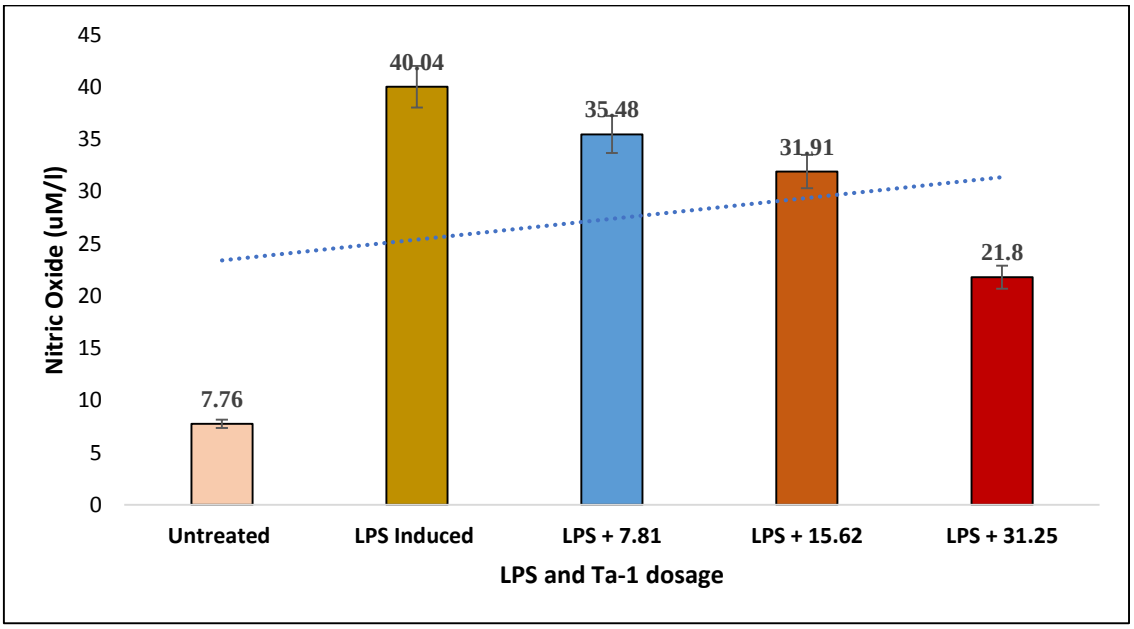

Fig. 7: The Ta-1 concentrations ranging from 7.813 to 31.25 ug/ml showed dose-dependent reduction of NO production compared with control

Nitric oxide (NO) serves as a crucial inflammatory mediator, produced by macrophages in response to various stimuli. Our findings indicate that Ta-1 treatment results in a dose-dependent reduction in NO production in RAW 264.7 cells. From the outcomes of this investigation, it can be deduced that Ta-1 possesses notable anti-inflammatory properties in macrophages, suggesting its potential as a therapeutic intervention for

inflammatory disorders. The potential mechanism through which Ta-1 diminishes NO production may involve its capacity to regulate the activity of inducible nitric oxide synthase (iNOS), the cellular enzyme responsible for NO synthesis. Recent study has furnished evidence supporting the idea that Ta-1 has the capability to inhibit both the expression and activity of iNOS, leading to a decline in NO production.

Rationale for Using RAW 264.7 Cells in Toxicity Analysis

The RAW 264.7 cell line, derived from murine macrophages, is widely used in toxicity analysis due to its macrophage-like properties, including the ability to phagocytose, produce reactive oxygen species (ROS), and secrete cytokines. These characteristics make it highly suitable for evaluating immune response and inflammatory pathways, which are key in understanding toxicity mechanisms (166-167). Macrophages play a central role in inflammation, so RAW 264.7 cells are particularly useful for assessing immunotoxicity and oxidative stress, which are often markers of cellular toxicity (168). Additionally, the continuous nature of this cell line ensures consistent, reproducible results, reducing variability compared to primary macrophages (169). RAW 264.7 cells are widely accepted in preclinical toxicity studies for drugs and other compounds, as their response in assays often predicts in vivo toxicities, thus aiding early-stage drug development (170).

Acute Toxicity experiment results:

Each group of animals was administered a dosage of 250ug/kg, 500ug/kg, and 1000ug/kg body weight, respectively, as an initial dose for observation purposes. The animals were closely monitored for a duration of 30 minutes following the administration, and subsequently at regular intervals within the first 24 hours. However, particular emphasis was given on monitoring the animals during the initial 4-hour period. No clinical signs were observed. Consequently, the remaining quadrupeds from each respective assemblage were administered doses of 250 micrograms per kilogram, 500 micrograms per kilogram, and 1000 micrograms per kilogram of body weight, and their subsequent behaviour was meticulously monitored over a span of 15 days.

After comparing the treated groups with the control group, it was observed that no clinical signs were evident.

Anti-arthritic study results:

The rats, induced with arthritis, underwent a treatment regimen involving the administration of test substances on days 1, 3, and 5, with a consistent timing of test substance application. Throughout the entire study duration, a meticulous daily monitoring process was implemented to assess the overall health of all animals and to identify any discernible clinical changes. Swellings in the hind paw region were consistently observed in both the groups receiving treatment and the induced group.

Upon the culmination of the 15-day study period, specifically on the 15th day, a thorough examination was conducted for all animals. This comprehensive assessment encompassed various parameters such as body weight, paw volume, paw thickness, paw weight, ankle thickness, and an array of biochemical and haematological markers. This multifaceted approach ensured a comprehensive evaluation of the physiological responses and outcomes associated with the administered treatments and the induced arthritic condition.

Change in body weight:

As changes in body weight play a significant role in diagnosing rheumatoid arthritis, our study revealed a significant increase in body weight across all treatment groups compared to the disease control. The presented graph illustrates body weight measurements taken on Day-0, signifying the study's onset, and Day-15, marking its conclusion. The data indicates that initially, on day 0, the values of all groups were comparable. However, on the 15th day, a notable decrease in body weight was observed in the arthritic group compared to the control group. Following treatment with Ta-1, a significant increase in body weight was observed in all treated groups compared to the diseased control group (Figure-8).

The body weight of animals affected by arthritis consistently diminishes throughout the progression of arthritis, as demonstrated by previous studies (171-172). In the current investigation, a substantial decline in body weight was similarly observed in arthritic group compared with control.

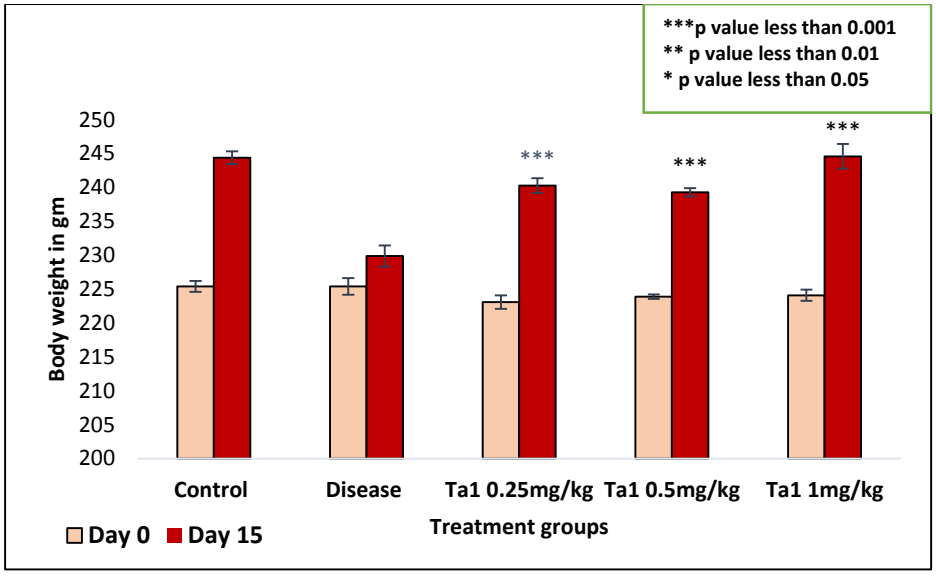

Fig. 8: Graphical representation of Body weight changes in all the groups post Ta-1 treatment

Paw volume:

In this study, the marked increase in paw volume observed in arthritic control rats aligns with findings in previous studies on rheumatoid arthritis models, where inflammation and swelling are hallmarks of disease progression. The increase in paw volume from 1.1±0.02 ml to 1.8±0.02 ml over the 15-day period highlights the ongoing inflammation characteristic of untreated arthritis. This observation concurs with studies where untreated arthritic animals show progressive joint inflammation due to increased pro-inflammatory mediators and immune cell infiltration in the joint tissue (173). In comparison, the normal control rats maintained a stable paw volume (1.1±0.03 ml), indicating an absence of inflammation and corroborating prior studies where non-arthritic animals do not exhibit significant changes in paw size (174).

The significant reduction in paw volume in arthritic rats treated with different doses of Ta-1 (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg) over the treatment period mirrors findings in studies evaluating anti-inflammatory agents in arthritis models. In this study, the highest dose of Ta-1 (1 mg/kg) showed the most substantial effect, reducing paw volume to 1.1±0.03 ml on day 0 and 1.4±0.02 ml on the 15th day, demonstrating a clear dose-dependent efficacy. Comparable results are reported in studies by Patel et al. (2021) and Zhang et al. (2018) (175-176), where anti-arthritic drugs exhibited a dose-dependent reduction in paw volume, which reflects the effectiveness of the drug in

attenuating inflammatory symptoms. The significant difference in paw volume between Ta-1 treatment groups and the disease control group underscores Ta-1's potential as a therapeutic option, as previously documented anti-inflammatory compounds have shown similar efficacy in reducing arthritic inflammation and swelling.

A substantial decrease in paw volume was evident in all treatment groups compared to the disease control group (Figure-9).

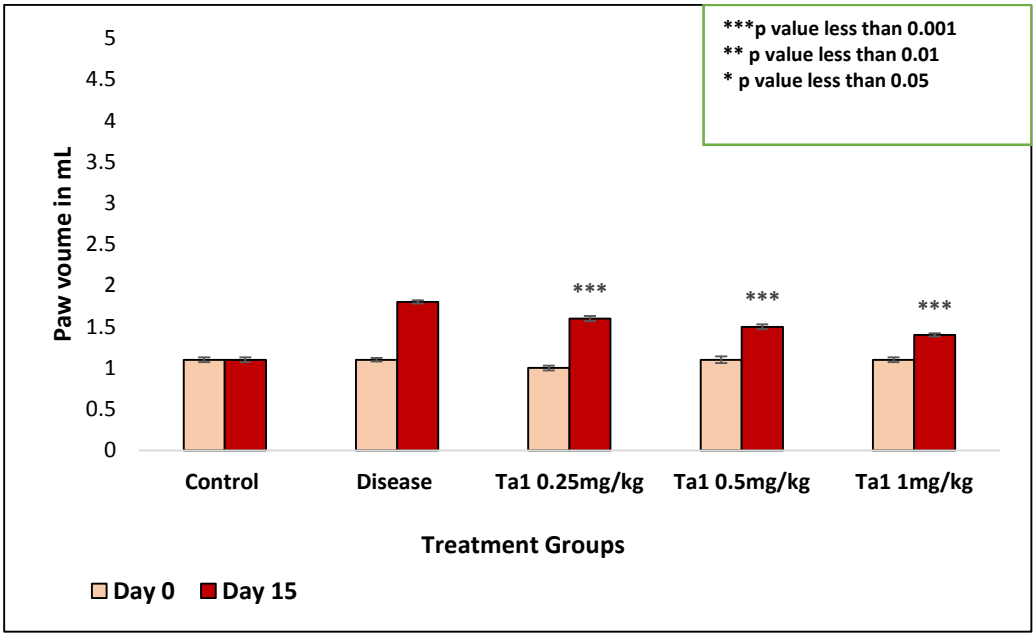

Fig. 9: Changes in paw volume of rats from day 0 and 15th day post Ta-1 treatment

Paw Weight:

A marked increase in paw weight was observed in arthritic control rats as arthritis advanced, with measurements on the 15th day registering at 5.4±0.13g. Arthritic control rats showed a notable difference in paw weight when compared to normal control rats, with measurements taken on the 15th day recorded at 2.6±0.14g. Upon treatment with Ta-1 at varying doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a notable reduction in paw volume in arthritic rats was evident on day 15th. Specifically, the paw weights were 5.1±0.17g at the dosage of 0.25 mg/kg, 4.4±0.23g at the dosage of 0.5 mg/kg, and 3.3±0.13g at the dosage of 1 mg/kg. A notable reduction in paw weight was observed in the mid and high dosage groups when compared to the arthritis control group (Figure-10). The significant alterations in paw weight underscore the efficacy of Ta-1 in mitigating the progression of arthritis in this experimental context.

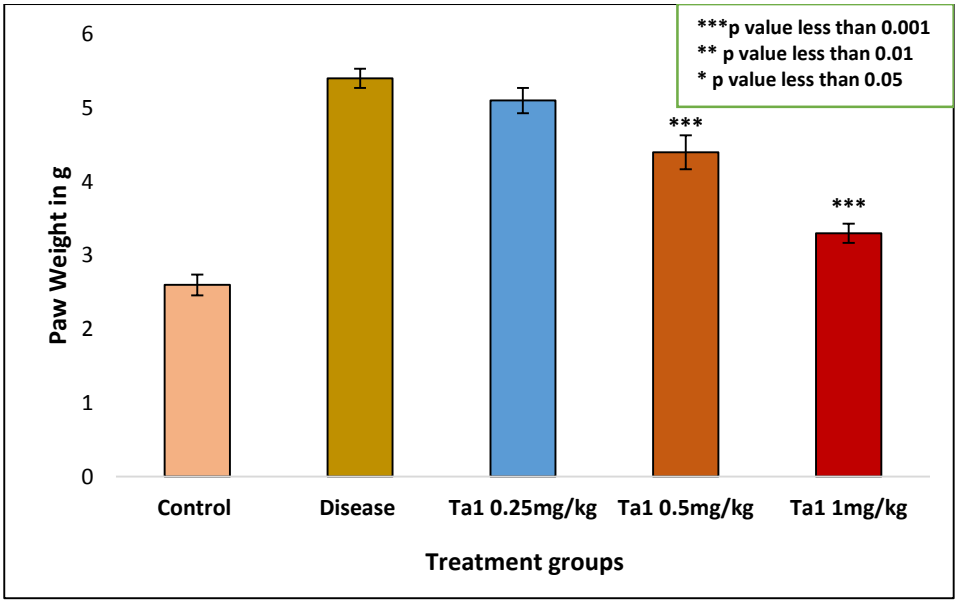

Fig. 10: Graphical representation of paw weight reduction after administration of Ta-1

Arthritic Score:

In arthritic control rats, on day 15th (3.75±0.16) after the arthritis development, a notable arthritic score was observed. However, the treatment of arthritic rats with Ta-1 (0.25mg/kg, 0,5mg/kg and 1mg/kg) all three dosage groups led to a significant decrease in their arthritic score on the 15th day (3.12±0.22, 2.62±0.18 and 0.62±0.18) after arthritis development compared to the diseased control group. There was a significant decrease in the arthritis score of all the treatment groups when compared to disease control (Figure-11).

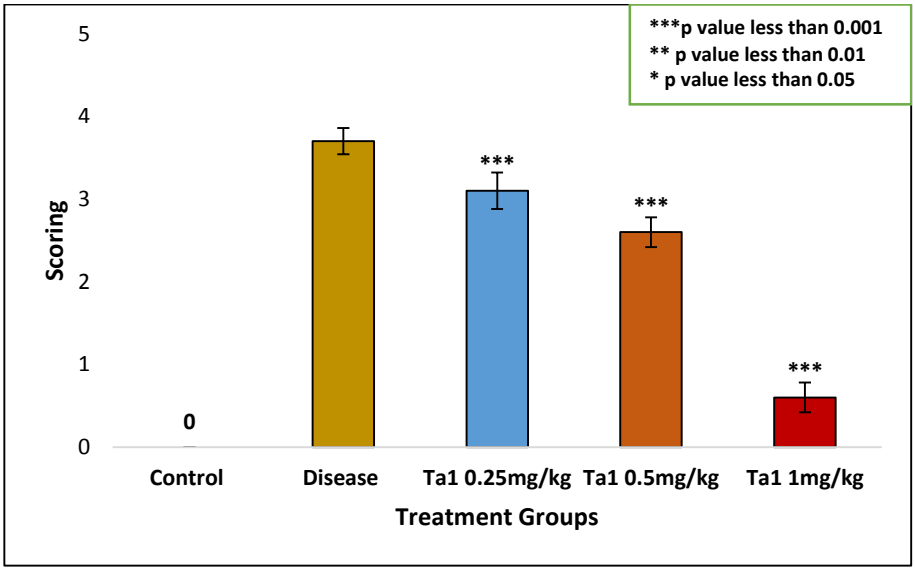

Fig. 9: Arthritic score reduction in all the treated groups compared with diseased control

Paw Thickness:

A noticeable increase in hind paw thickness was observed in arthritic control rats during the progression of arthritis, with measurements on the 0, 2nd, 4th, 8th, 12th and 15th days being 6.4±0.09 mm, 10.5±0 mm, 12.6±0.06, 12 .5±0.05, 12.6±0.04 and 12.7±0.03 mm, respectively. Arthritic control rats exhibited a significant difference in hind paw thickness compared to normal control rats, ranging from 6.5±0.10 mm, 6.5±0.09 mm, 6.6±0.08, 6.6±0.09, 6.5±0.11 and 6.5±0.09 mm on the 0, 2nd, 4th, 8th, 12th and 15th days, respectively. Treatment with Ta-1 at a dosage of 0.25 mg/kg, 0.5 mg/kg and 1 mg/kg resulted in a significant reduction in paw thickness in arthritic rats from the 0, 2nd, 4th, 8th, 12th and 15th day (6.4±0.07mm, 10.8±0.03mm, 11.5±0.03mm, 10.7±0.04mm, 10.5±0.02mm and 10.2±0.03mm at the dosage 0.25mg/kg), 0, 2nd, 4th, 8th, 12th and 15th day (6.4±0.09mm, 10.9±0.07mm, 11.0±0.08mm, 10.2±0.05mm, 8.5±0.04mm and 8.4±0.04mm at the dosage 0.5mg/kg) and 0, 2nd, 4th, 8th, 12th and 15th day (6.4±0.04mm, 10.8±0.03mm, 7.6±0.05mm, 7.5±0.05mm, 7.5±0.05mm and 7.2±0.05mm at the dosage 1mg/kg). A substantial decrease in paw thickness was noted in all treatment cohorts in comparison to the group with the disease as the control (Figure-12).

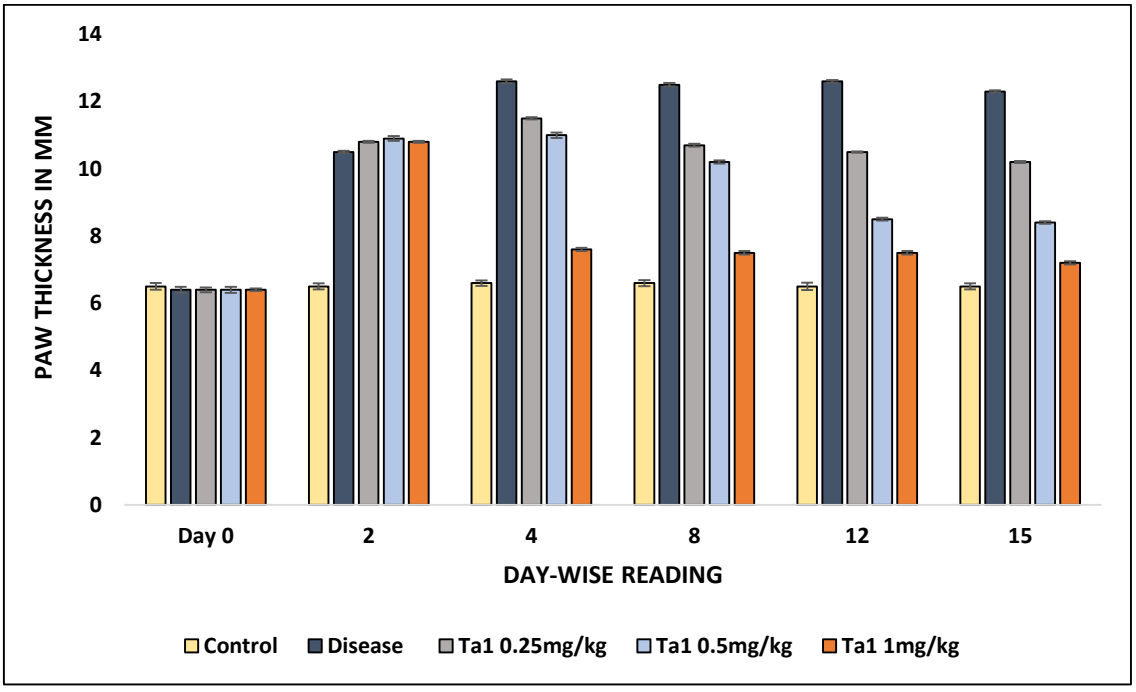

Fig. 12: Paw thickness of rats from day 0 to 15th day post collagen immunization

Ankle Thickness measurement:

A notable increase in the ankle dimensions of arthritic control rats was documented between the day 0 (7.8 ± 0.03 mm), 2nd (11.6 ± 0.04 mm), 5th (11.6 ± 0.03), 9th (11.8 ± 0.03 mm), 13t (11.8 ± 0.03 mm) and 15th day (11.8 ± 0.04 mm), in contrast to the ankle dimensions of their normal control counterparts (Day 0 7.8±0.02, 2nd 7.8±0.01, 5th 7.9±0.02, 9th 7.9±0.02, 13th 7.9±0.02 and 15th 8.1±0.03). Conversely, the administration of Ta-1 at a dosage of 0.25mg/kg, 0.5mg/kg and 1mg/kg respectively to the arthritic rats resulted in a significant reduction in their ankle dimensions (Day0 7.8±0.04mm, 2nd 11.2±0.04mm, 5th 11.2±0.03mm, 9th 10.7±0.03mm, 13th 10.5±0.02mm and 15th 10.5±0.02mm at the dosage 0.25mg/kg), (Day0 7.8±0.02mm, 2nd 11.7±0.03mm, 5th 1100±0.05mm, 9th 10.5±0.04mm, 13th 10.5±0.03mm and 15th 10.0±0.04mm at the dosage 0.5mg/kg) and (Day0 7.7±0.07mm, 2nd 10.7±0.05, 5th 9.8±0.03, 9th 9.6±0.04mm, 13th 9.5±0.04mm and 15th 9.2±0.03mm at the dosage 0.25mg/kg). There was a significant reduction in ankle thickness observed in all the Ta-1 treated groups when compared with the diseased control (Figure-13).

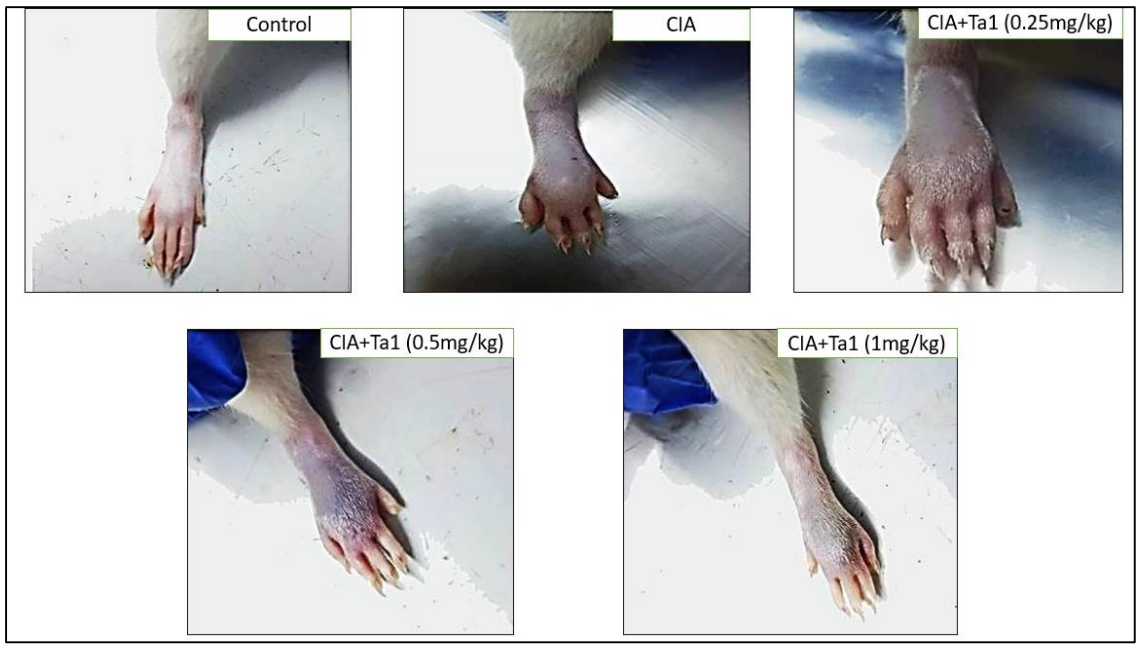

Fig. 13: Photographs of paw swelling in rats showing Front view of hind paws. The paw thickness (mm) of each rat was measured with a Vernier calliper on day 0, 2, 4, 8, 12 and 15 during the establishment of the CIA rat model. Paw thickness is expressed as the sum of the results of both hind paws. CIA, collagen‐induced arthritis

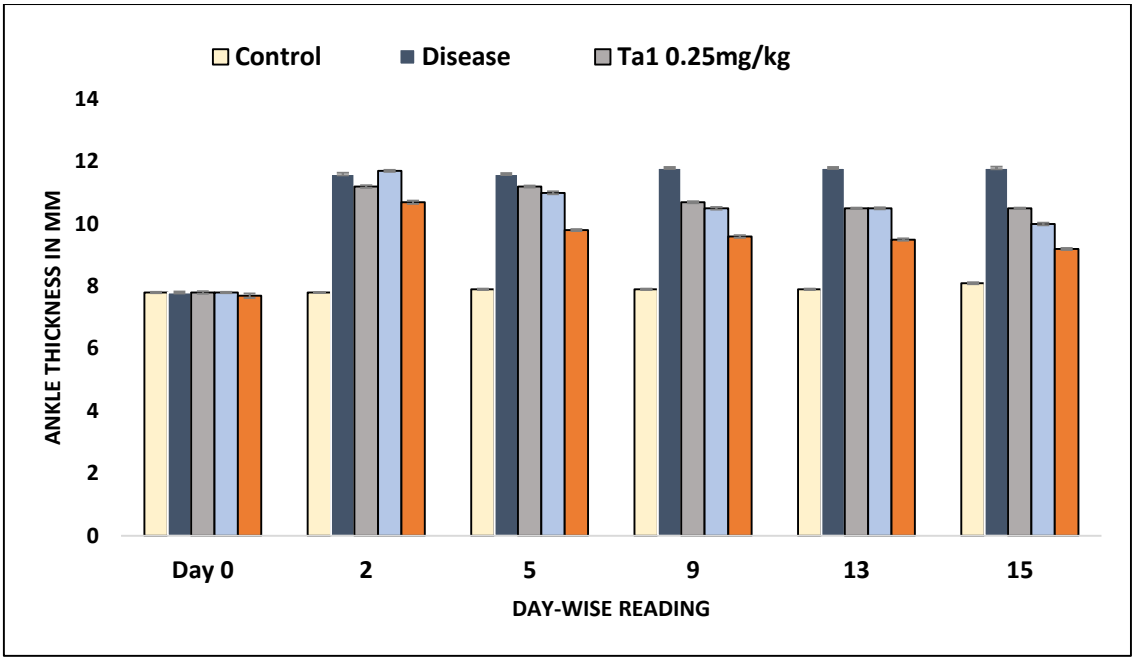

Fig. 14: Graphical representation of significant reduction in ankle dimensions in all the treated groups compared with arthritic control

Biochemical Parameters:

Aspartate transaminase and Alanine Transaminase:

In 2010, Curtis JR. et al. conducted a research study and discovered that irregular ALT/AST levels emerged in 14-35% of patients starting DMARD therapy for RA or PsA. The risks were higher, especially for those with PsA and those taking a combination of MTX (at least 10 mg/day) and LEF. These discoveries can guide the monitoring process for potential liver issues in these patient groups (177).

In earlier studies, many treatment approaches were associated with an increase in liver markers. However, Ta-1 did not exhibit any adverse effects on the liver marker ALT, as observed in those previous studies.

On 15th day, we checked the levels of a substance called serum ALT. It turned out that the levels were pretty much the same in all the groups, whether they had arthritis or not. For the arthritic group, the ALT levels were 94.1±0.58 IU/ml, and for normal rats, it was 94.1±0.80 IU/ml. After giving different amounts of Ta-1 (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), we didn't see any big changes in ALT levels in arthritic rats on the 15th day. Specifically, the ALT levels were 94.8±0.77 IU/ml at 0.25 mg/kg, 95.9±0.81

IU/ml at 0.5 mg/kg, and 95.4±0.84 IU/ml at 1 mg/kg. None of the treatment groups showed significant differences compared to the arthritis control group (Figure-15).

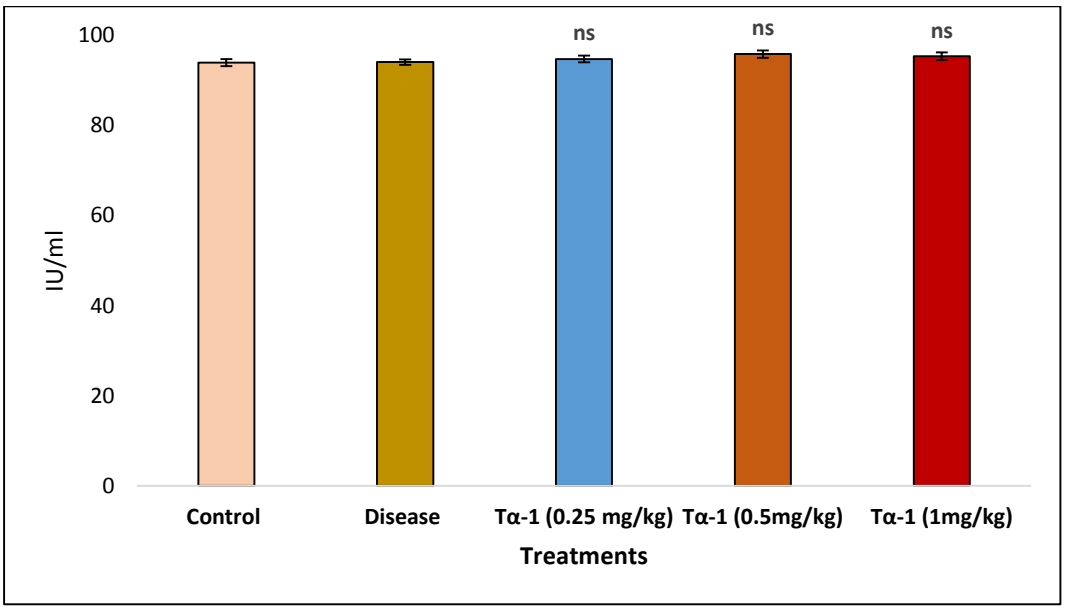

Fig. 15: Graphical representation of Serum ALT levels in all the five groups

Serum AST Levels

On the 15th day, we assessed the levels of a substance called serum AST. Interestingly, these levels were quite consistent across all groups, regardless of whether they had arthritis. The arthritic group exhibited AST levels of 154 ±0.39 IU/ml, while normal rats showed levels of 154.6±0.62 IU/ml.

Following the administration of various doses of Ta-1 (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), we observed no significant changes in AST levels in arthritic rats on the 15th day. Specifically, AST levels were 153.6±0.51 IU/ml at 0.25 mg/kg, 154.1±0.51 IU/ml at 0.5 mg/kg, and 154±0.52 IU/ml at 1 mg/kg. None of the treatment groups demonstrated notable differences when compared to the arthritis control group (refer to the Figure 16).

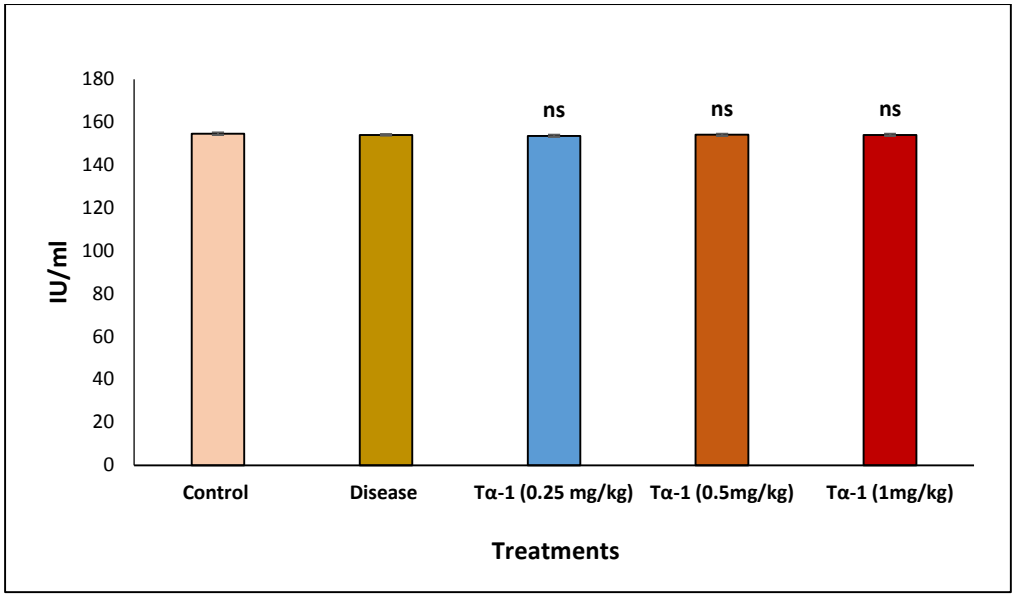

Fig. 16: Graphical representation of Serum AST levels in all the five groups

Serum Alkaline Phosphatase:

A significant rise in the serum levels of Alkaline Phosphatase was observed as arthritis progressed in arthritic control rats, with measurements on the 15th day reaching 211.4±0.95 U/L. Arthritic control rats exhibited a noteworthy difference in serum ALP levels compared to normal control rats, recording values at 112.6±0.41 U/L on the 15th day.

Following the administration of Ta-1 at varying doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a marked decrease in serum levels of ALP in arthritic rats was evident on the 15th day. Specifically, the ALP levels were 197.5±3.75 U/L at the dosage of 0.25 mg/kg, 144.5±0.38 U/L at the dosage of 0.5 mg/kg, and 124.8±1.0 U/L at the dosage of 1 mg/kg. Recent investigations have further unveiled a noteworthy decrease in ALP levels within the low, mid, and high dose groups, as opposed to the arthritis control group (Figure-17). This observed reduction in ALP levels suggests a potential influence of the administered doses on the regulatory mechanisms of ALP, emphasizing the need for a more nuanced understanding of the relationship between Ta-1 treatment and ALP dynamics in the context of RA. Further exploration of this phenomenon could contribute valuable insights into the complex interplay between Ta-1 and the biochemical markers associated with RA pathology.

In 1998, Niino-Nanke Y. et al. conducted a study, determining that out of 123 patients 37.4% of individuals with rheumatoid arthritis (RA) exhibited elevated serum alkaline phosphatase (ALP) values, measuring at 245.2 +/- 91.2 IU/L. These values were notably higher than those observed in osteoarthritis (OA) patients, averaging 192.3 +/- 45.2 IU/L ($P < 0.01$, RA vs. OA). The research findings in RA patients further confirmed a positive correlation between the increase in serum ALP levels and the activity of the disease (178).

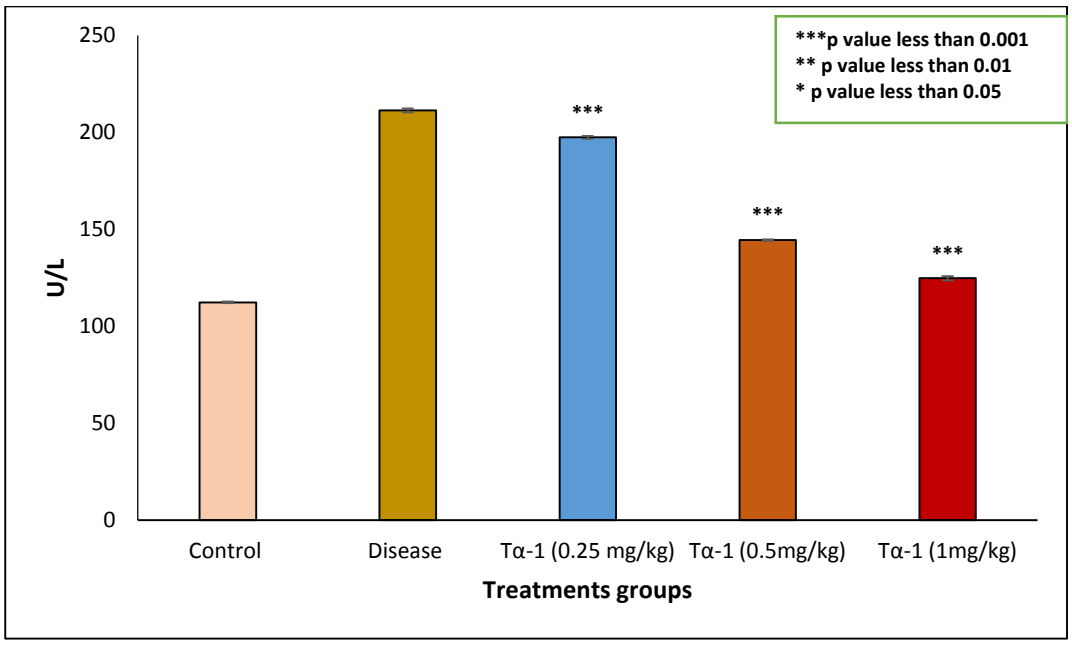

Fig. 17: Graphical representation of reduction in serum ALP levels post-treatment with Ta-1 compared to disease group

Serum Ferritin Levels:

A marked increase in serum Ferritin levels was evident as arthritis progressed in arthritic control rats, peaking at 822.1±36.14 ng/ml on the 15th day. Notably, arthritic control rats exhibited a substantial disparity in serum Ferritin levels compared to their non-arthritic counterparts, registering values of 606±12.9 ng/ml on the same day.

Upon the administration of Ta-1 at varying doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a discernible reduction in serum Ferritin levels in arthritic rats became observable by the 15th day. Specifically, Ferritin levels recorded were 711.1±35.84 ng/ml at the 0.25 mg/kg dosage, 665±25.19 ng/ml at the 0.5 mg/kg dosage, and 610.1±33.65 ng/ml at the 1 mg/kg dosage. This observed decline suggests a potential

therapeutic impact of Ta-1 on the modulation of serum Ferritin levels in the context of arthritis progression. The findings emphasize the importance of investigating the influence of Ta-1 on Ferritin as a crucial facet in comprehending its potential role in the management of arthritis.

A substantial reduction in ferritin levels was evident across all treatment groups when compared to the arthritis control group (Figure-18).

R. S. Rothwell. et al. also confirmed in their research study that in the context of acute rheumatoid disease, serum ferritin serves as an acute-phase reactant, reflecting the intensity of disease activity. Within the cohort of 15 patients, observations reveal notable declines in serum ferritin levels that correspond with a decrease in disease activity, as evaluated through the Ritchie index and ESR. The similarity between our study and Rothwell et al.'s research strengthens the idea that serum ferritin is a reliable sign of how active rheumatoid disease is. This agreement not only supports the trustworthiness of using serum ferritin as an indicator but also shows that this link holds true in various research studies. This shared evidence adds to what we know about how important serum ferritin is in understanding rheumatoid disease better, helping us grasp its clinical significance more fully (179).

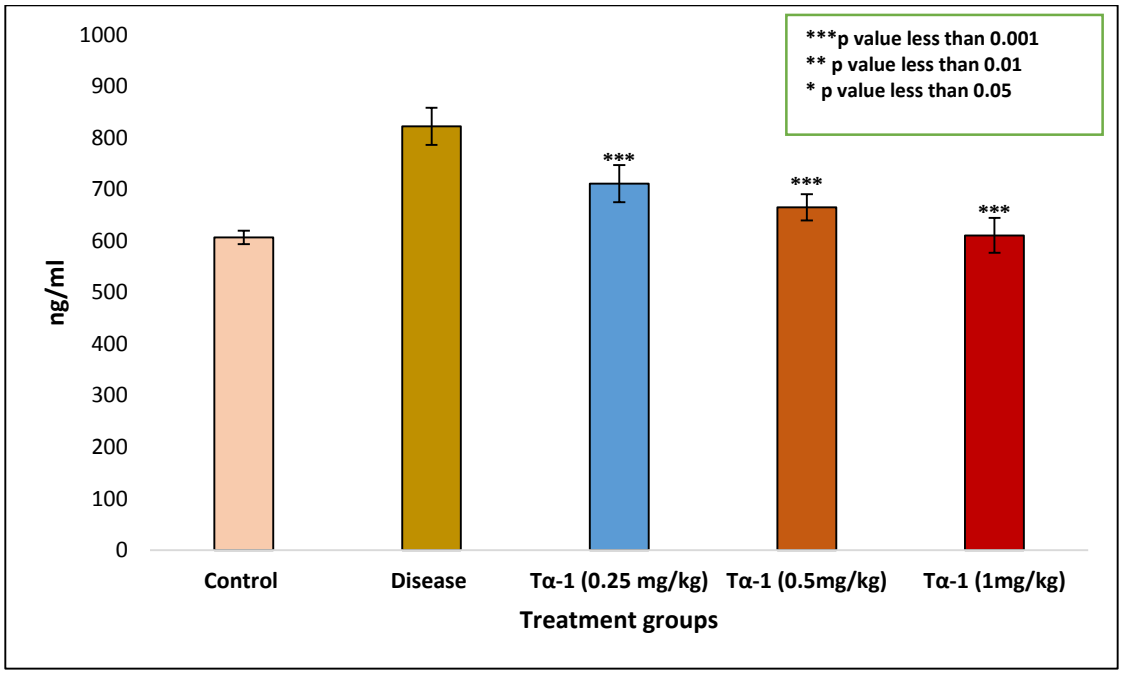

Fig. 18: Graphical representation of significant reduction in the levels of serum ferritin in all the treatment groups

Haematological parameters:

Haematological parameters like RBC, Platelet count, Differential count was measured on day 15th and the values are given below.

Red blood cells:

A noteworthy decrease in the count of red blood cells (RBCs) was noted, measuring 5.4±0.1 million/mm3 on the 15th day, as compared to the control group with a count of 5.8±0.02 million/mm3. Following the administration of Ta-1 at varying doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a substantial reduction in RBCs count in arthritic rats was evident on the 15th day. Specifically, the RBCs count measured 5.6±0.1 million/mm3 at a dosage of 0.25 mg/kg, 5.6±0.1 million/mm3 at a dosage of 0.5 mg/kg, and 5.7±0.05 million/mm3 at a dosage of 1 mg/kg. A marked increase in RBCs count was observed in the high-dosage group when compared to the arthritis control group, as illustrated in the figure-17. These significant changes in RBCs count underscore the effectiveness of Ta-1 in attenuating the progression of arthritis in this experimental context.

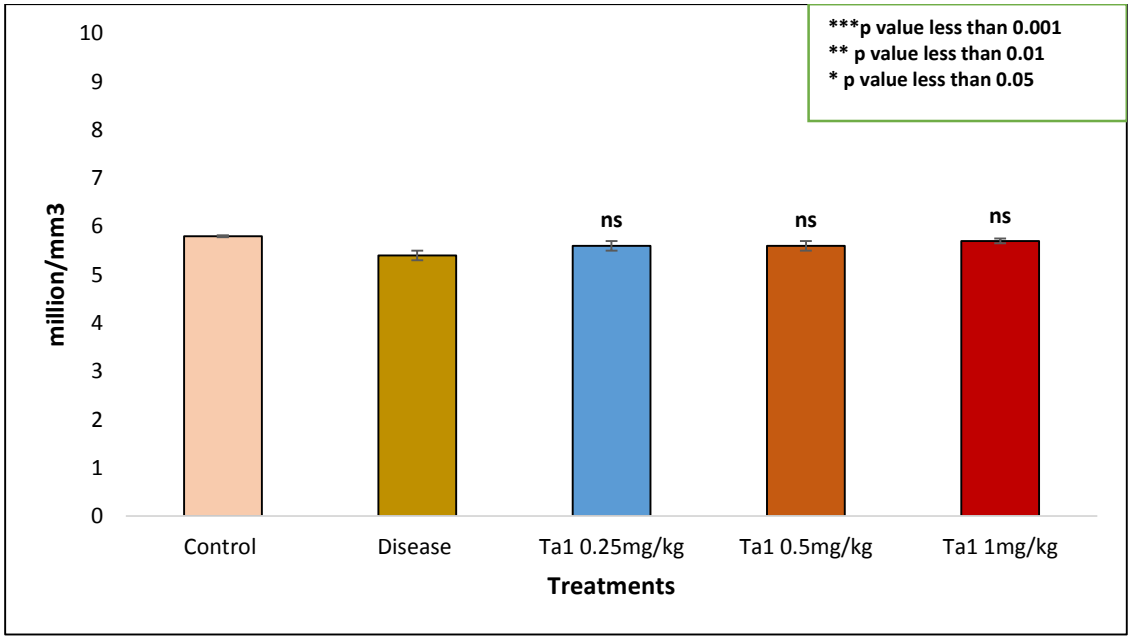

Fig. 19: Graphical representation of RBCs count in all the groups

Platelet count:

On the 15th day, we examined how many platelets were in the blood. Interestingly, the levels were quite similar for all groups, whether or not they had arthritis. The arthritis

group had about 9.3±0.05 lakhs/cumm platelets, while the normal rats had 9.4±0.07 lakhs/cumm.

After giving different amounts of Ta-1 (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), we didn't notice any significant changes in platelet count for the arthritic rats on the 15th day. The platelet counts were approximately 9.4±0.02 lakhs/cumm at 0.25 mg/kg, 9.3±0.02 lakhs/cumm at 0.5 mg/kg, and 9.4±0.04 lakhs/cumm at 1 mg/kg. None of the groups receiving treatment looked significantly different from the arthritis control group, as depicted in the figure-20.

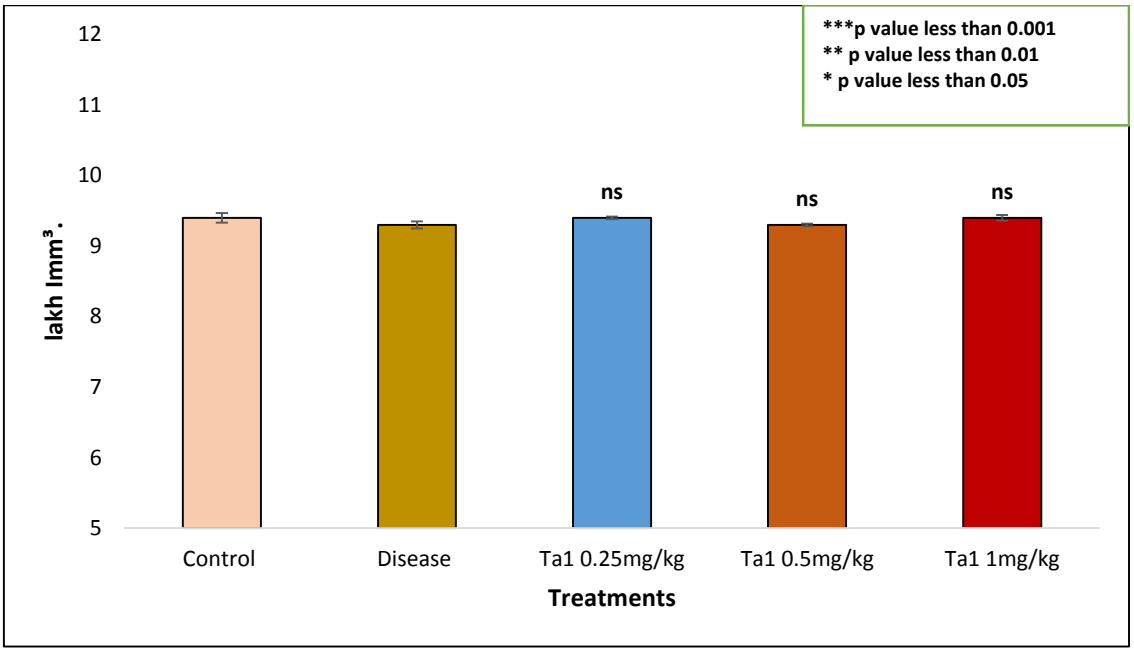

Fig. 20: Comparison of Platelets count in all the groups

Neutrophil count:

A substantial increase in neutrophil count was observed in arthritic group, recording at 18±0.42% on the 15th day, compared to the control group with a count of 10.0±0.38%. Following the administration of Ta-1 at various doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a notable decrease in neutrophil count in arthritic rats was evident on the 15th day. Specifically, the neutrophil count measured 16.6±0.46% at a dosage of 0.25 mg/kg, 16.6±0.46% at a dosage of 0.5 mg/kg, and 16.4±0.46% at a dosage of 1 mg/kg. A considerable reduction in neutrophils was observed in the high-dosage group when compared to the arthritis control group, as depicted in the figure-21.

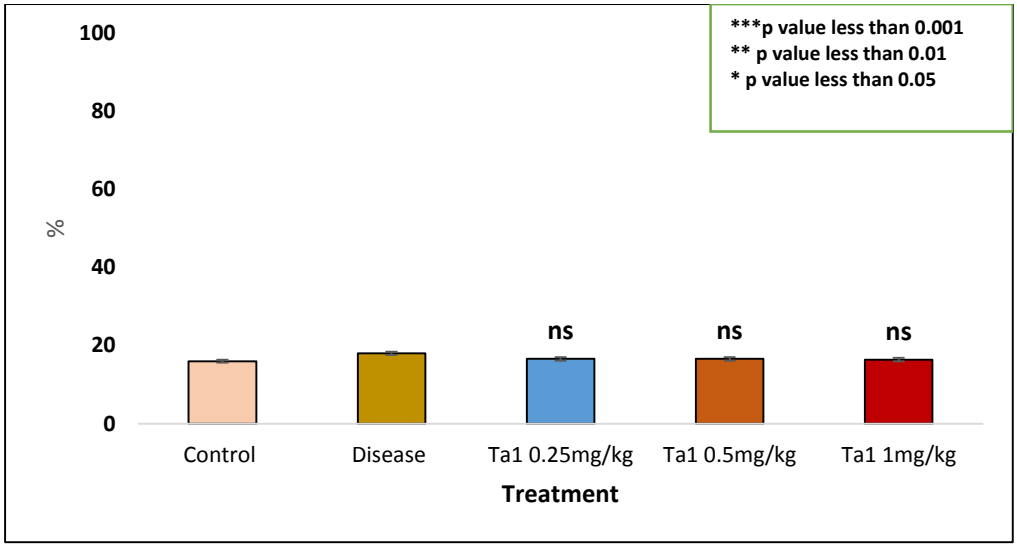

Fig. 21: Graphical representation of neutrophil count in all the groups

Lymphocyte count:

The graph illustrates a significant increase in lymphocyte count within the arthritic group, reaching 87.4±0.78% on the 15th day, in contrast to the control group, which had a count of 78.1±0.72%. After the administration of Ta-1 at different doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a noticeable decrease in lymphocyte count in arthritic rats was evident on the 15th day. Specifically, the count measured 82.1±2.19% at a dosage of 0.25 mg/kg, 80.8±1.99% at a dosage of 0.5 mg/kg, and 79.8±1.87% at a dosage of 1 mg/kg. A considerable reduction in lymphocyte count was observed in the mid and high-dosage groups compared to the arthritis control group, as shown in Figure 22.

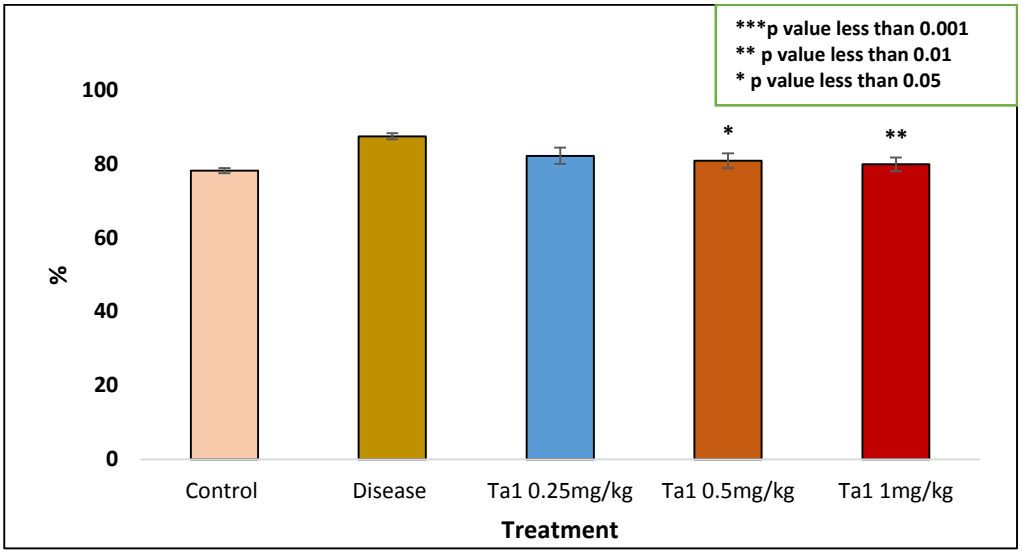

Fig. 22: Graphical representation of lymphocyte count reduction in mid and high dosage comparing to arthritic group

Eosinophil count:

On the 15th day, we assessed the eosinophil count in the blood. Surprisingly, the levels were quite consistent across all groups, whether or not they had arthritis. The arthritis group displayed approximately 2.0±0.19% eosinophils, while the normal rats had a similar count of 2.0±0.19%.

Following the administration of different doses of Ta-1 (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), we did not observe any significant changes in eosinophil count for the arthritic rats on the 15th day. The eosinophil counts were approximately 1.9±0.23% at 0.25 mg/kg, 1.8±0.16% at 0.5 mg/kg, and 2.0±0.19% at 1 mg/kg. None of the treatment groups appeared significantly different from the arthritis control group, as depicted in the figure-23.

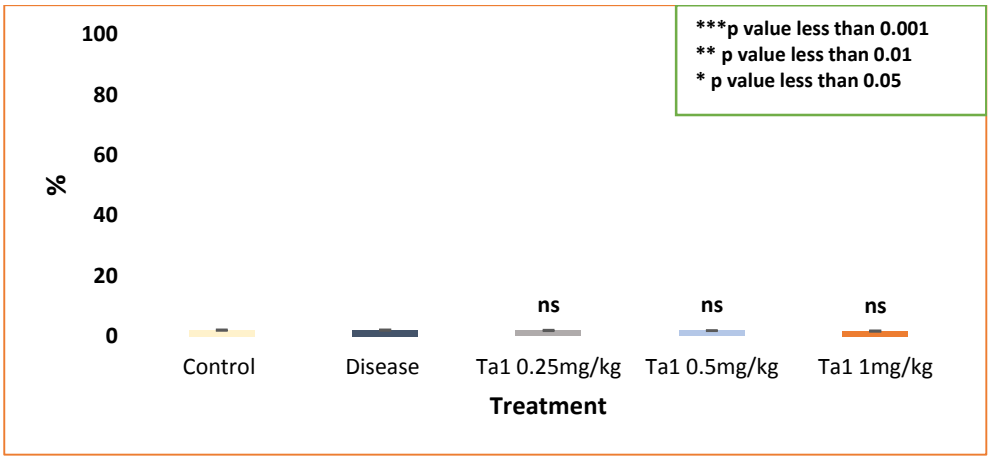

Fig. 23: Graphs show the percentage of eosinophil count in all the groups

Monocyte count:

On the 15th day, we evaluated the monocyte count in the bloodstream. Interestingly, the levels remained consistently similar across all groups, regardless of the presence of arthritis. The arthritis group exhibited approximately 4.8±0.25% eosinophils, while the normal rats showed a comparable count of 5.0±0.27%.

Following the administration of varied doses of Ta-1 (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), there were no notable changes observed in monocyte count for the arthritic rats on the 15th day. The monocyte counts were around 5.0±0.33% at 0.25 mg/kg, 5.0±0.31% at 0.5 mg/kg, and 5.3±0.33% at 1 mg/kg. None of the treatment groups exhibited significant differences from the arthritis control group, as illustrated in the figure-24.

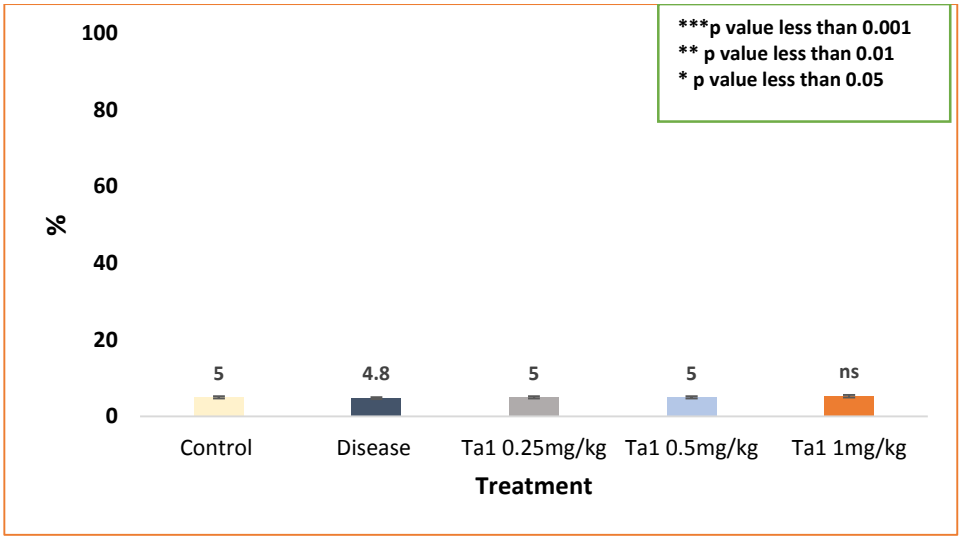

Fig. 24: Graph shows the monocyte count in all the groups

Tissue Histology results:

Histopathological examination unveiled cartilage erosion and the infiltration of mononuclear cells in arthritic rats subjected to castration. The joint tissue was severely damaged, the tissue structure was loose, some areas were necrotic and shed, the cells showed oedema and focal haemorrhage. Infiltration of many inflammatory cells could be seen when compared with normal control the joint tissue structure was intact, and no infiltration of inflammatory cells were observed. Following the treatment with Ta-1 at different dosage 0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg respectively there was a improvement in the histological changes in tissues. At the low dose of 0.25 mg/kg, the joint tissue showed a neat joint structure, and cells were clear and regularly distributed. The infiltration of inflammatory cells was mainly in the outer layer, and rarely in the lower layer, and there were few eosinophilic granulocytes. At the 0.5 mg/kg dose, the joint tissue showed neat tissue structure, tissue cells were clear and regularly distributed, there were few eosinophilic granulocytes and there was less infiltration of inflammatory cells. And at the highest dose 1mg/kg the joint tissue showed neat tissue structure, tissue cells were clear and regularly distributed, and there was less infiltration of inflammatory cells (Figure-25).

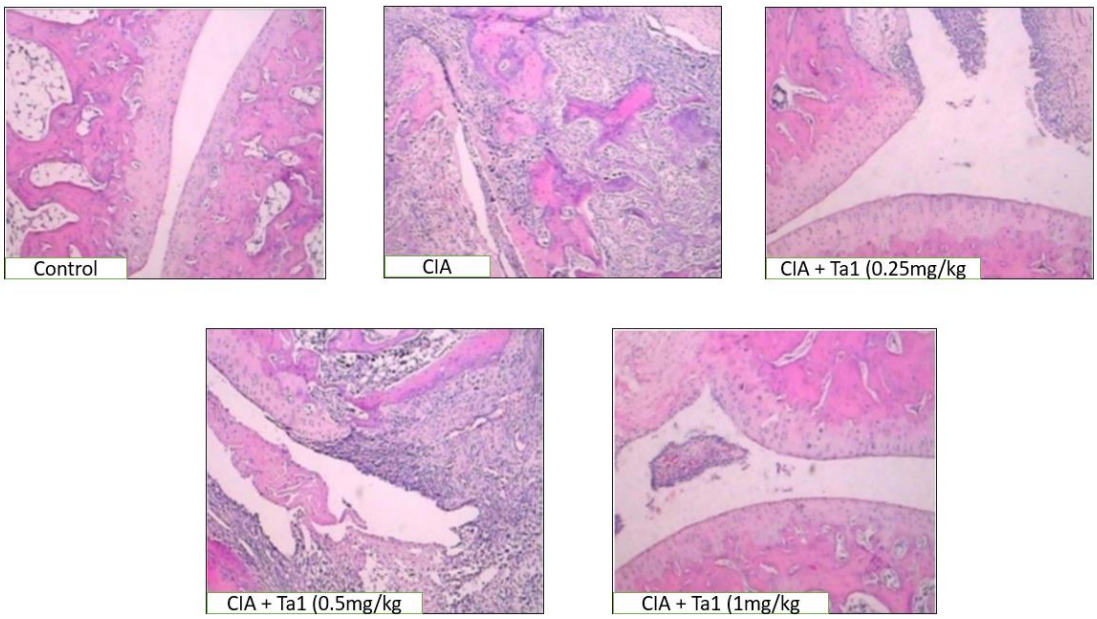

Fig. 25: Rats were sacrificed on day 15, and knee joints were dissected, fixed, decalcified and processed for histopathology. 5 um sections were stained for H&E

Gene Expression Study results:

RANKL Gene expression:

The graphical representation illustrates an elevation in RANKL levels at the gene level within the arthritic control group compared to the normal control group. However, with the administration of Ta-1 across all three dose groups, 0.25mg/kg, 0.5mg/kg and 1 mg/kg respectively, a decrease in RANKL levels at gene level was observed. Particularly noteworthy was the significant reduction in the gene expression of RANKL, specifically in the mid and high dose groups (0.5mg/kg and 1mg/kg) as shown on (figure-26). The activator of nuclear factor kappa B ligand (RANKL), a factor that stimulates the differentiation of osteoclasts, is a member of the tumor necrosis factor superfamily and plays a crucial role in the process of osteoclast formation. In individuals with rheumatoid arthritis (RA), RANKL is notably elevated in synovial tissues, contributing to osteoclast development and subsequent bone damage in RA. Rama Hussein et al. conducted a research study revealing an increase in serum RANKL levels among rheumatoid arthritis (RA) patients. The levels ranged from 168.17 to 870.9 pg/ml, with a mean of 247.92 ± 124.1 pg/ml. In contrast, healthy controls exhibited lower levels ranging from 133.1 to 178.55 pg/ml, with a mean of 166.57 ± 13.6 pg/ml. Importantly, this disparity was determined to be statistically significant (180). In 2020, Tanaka S. et al. reached the conclusion that women

diagnosed with rheumatoid arthritis (RA) exhibited significantly elevated circulating levels of sRANKL before undergoing anti-TNF-α treatment when compared to individuals of the same age in the healthy control group (181).

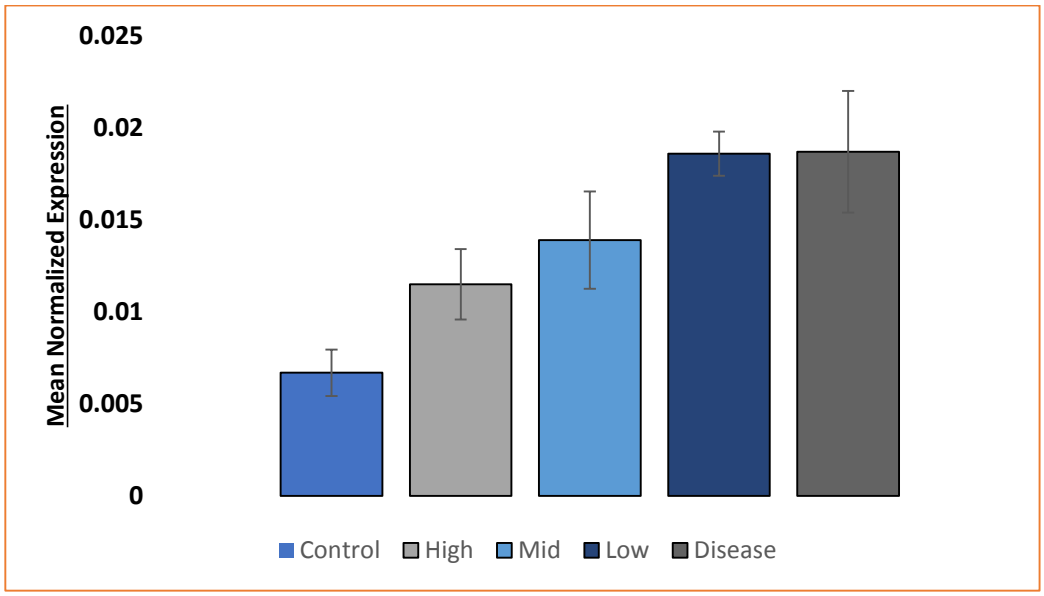

Fig. 26: Graphical representation of RANKL gene expression in the all the groups

ALP gene expression

The depicted graph illustrates a decrease in ALP levels at the gene level within the arthritic control group when compared to the normal control group. However, upon administering Ta-1 across all three dosage groups—0.25mg/kg, 0.5mg/kg, and 1mg/kg, respectively—an increase in ALP levels at the gene level was observed. Particularly notable was the significant reduction in the gene expression of ALP across the low, mid, and high dose groups (0.25mg/kg, 0.5mg/kg, and 1mg/kg) (Figure-27).

Monteagudo L. et al. did a cross-sectional retrospective study in the year 2019 shows discernible trend was noted indicating that individuals with lower ALP levels tended to exhibit seropositive rheumatoid arthritis (RA), with a prevalence of 35% compared to 15% in those with higher ALP levels, and this trend approached statistical significance (p=0.064) (182).

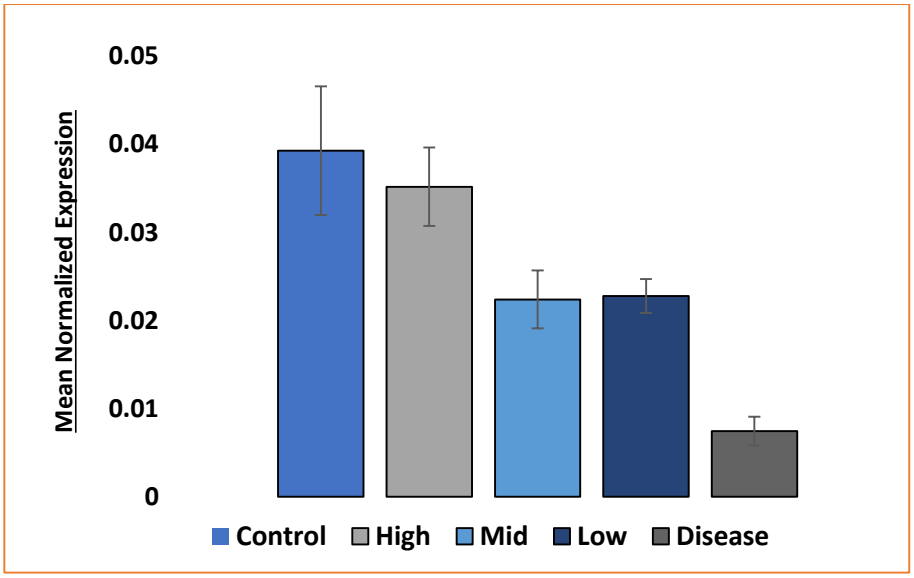

Fig. 27: Graphical representation of ALP gene in all the groups

Cathepsin-K gene expression:

Cathepsin K, known for its potent collagen-degrading activity, plays a crucial role in this system.

Cathepsin K serves as a significant focus for pharmacological application, a concept that has undergone testing and implementation by numerous pharmaceutical companies. Several cathepsin K inhibitors have been synthesized and extensively examined, with a focus on assessing their stability. In addition to stability studies, their efficacy in inhibiting bone resorption has been thoroughly investigated. One notable example is a peptide aldehyde inhibitor, which not only inhibits cathepsin K but also demonstrates the ability to impede osteoclast-mediated bone resorption in both human and animal assays. This inhibition has promising implications for mitigating bone and cartilage loss, presenting a potential avenue for therapeutic interventions in conditions associated with excessive bone resorption (173).

Skoumal M.et al. conducted a study in the year 2008, The study aimed to further understand the interplay between OPG, sRANKL, and cathepsin K in patients with longstanding RA.

Serum levels were measured in 100 individuals with active, longstanding RA, revealing elevated cathepsin K and OPG levels, while sRANKL levels remained normal. The study suggests that despite the compensatory effect of increased OPG levels on

sRANKL, bone degradation, indicated by elevated cathepsin K levels, may persist, contributing to the radiological destruction observed in these patients (183).

Our findings indicate an elevated level of Cathepsin-K at the gene level within the arthritic control group compared to the normal control group. However, following the administration of Ta-1 across all three dosage groups—0.25mg/kg, 0.5mg/kg, and 1mg/kg, respectively—a notable increase in Cathepsin-K levels at the gene level was observed. Particularly noteworthy was the significant reduction in the gene expression of Cathepsin-K across the low, medium, and high-dose groups (0.25mg/kg, 0.5mg/kg, and 1mg/kg), as depicted in the (figure-28).

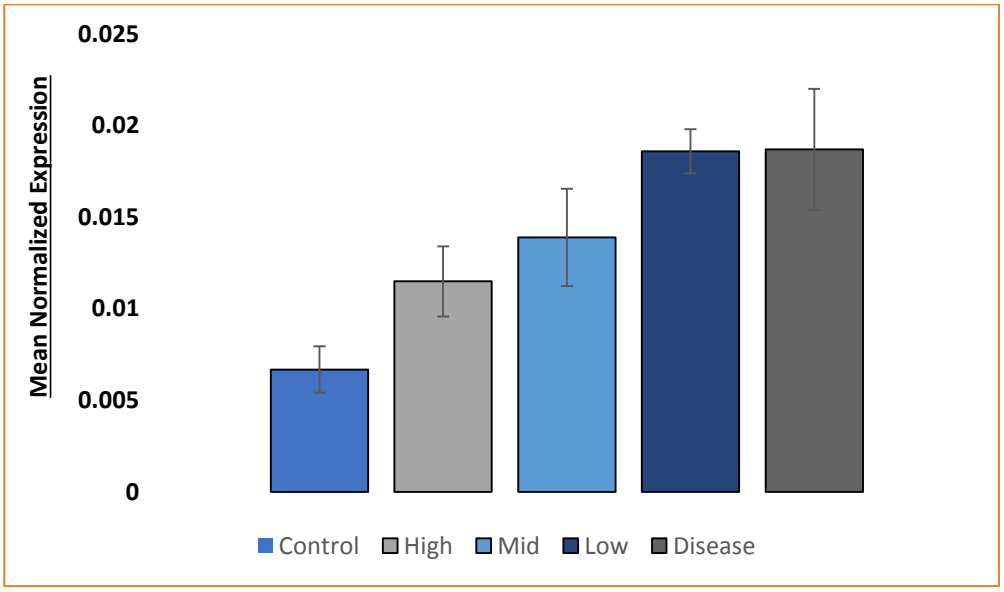

Fig. 28: Graph shows the reduction in Cathepsin-K gene expression in all the treated groups post Ta-1 treatment

OPG gene expression:

In recent years, Osteoprotegerin (OPG) has gained prominence as a highly effective means of inhibiting osteoclast formation. The pivotal signal for osteoclastogenesis relies on the interaction between Receptor Activator of Nuclear Factor Kappa B (RANK) and its ligand (RANKL) (**184).**

Bucay N. et al. did a research study in 1998, the study delves into the physiological role of Osteoprotegerin (OPG), a secreted protein known for inhibiting osteoclast formation. To investigate this, OPG-deficient mice were generated. The results reveal that adolescent and adult OPG −/− mice experience a notable reduction in total bone

density, marked by severe trabecular and cortical bone porosity, significant thinning of the parietal bones in the skull, and a heightened occurrence of fractures. These findings underscore the critical role of OPG as a regulator of postnatal bone mass **(185).**

The presented graph indicates a decline in the gene expression of OPG in the arthritic control group in comparison to the normal control group. However, upon the administration of Ta-1 across all three dosage groups—0.25mg/kg, 0.5mg/kg, and 1mg/kg, respectively—an elevation in OPG levels at the gene level was observed. Notably, there was a significant increase in the gene expression of OPG across the low, medium, and high dose groups (0.25mg/kg, 0.5mg/kg, and 1mg/kg) (Figure-29).

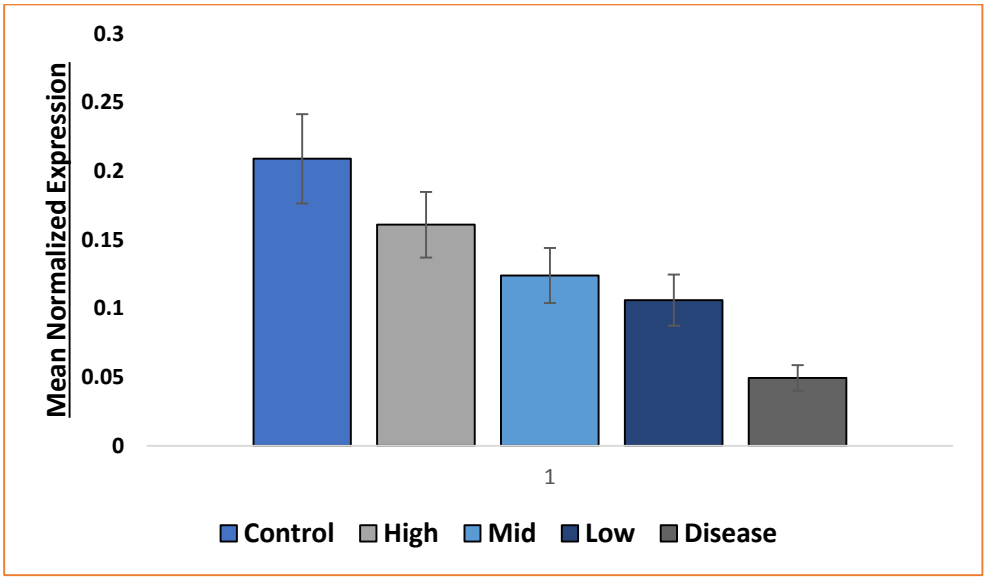

Fig. 29: Graph depicted the increase gene expression of OPG in all the treatment groups post treatment with Ta-1 comparing with arthritic control.

<u>Osteocalcin gene expression results:</u>

In 1989, Gevers G. et al. conducted a study, offering valuable insights into the subject that Serum osteocalcin levels were assessed using radio-immunoassay in 56 rheumatoid arthritis (RA) patients and 50 controls. The average serum osteocalcin levels were notably higher in RA patients. A positive correlation was observed between osteocalcin and the fasting mucopolysaccharide/creatinine ratio in both genders, as well as between osteocalcin and fasting hydroxyproline/creatinine ratio in women. These findings indicate an overall heightened bone turnover in RA, suggesting that serum osteocalcin could offer supplementary insights for assessing bone metabolism in this condition (186).

In contrast to previous studies Our results reveal an elevation in the gene-level expression of osteocalcin within the arthritic group as opposed to the normal control group. Following the administration of Ta-1 at doses of 0.25mg/kg, 0.5mg/kg, and 1mg/kg, a noteworthy reduction in the gene expression of osteocalcin was observed across all three dosage groups. This reduction is evident in the graphical representation provided (Figure-30).

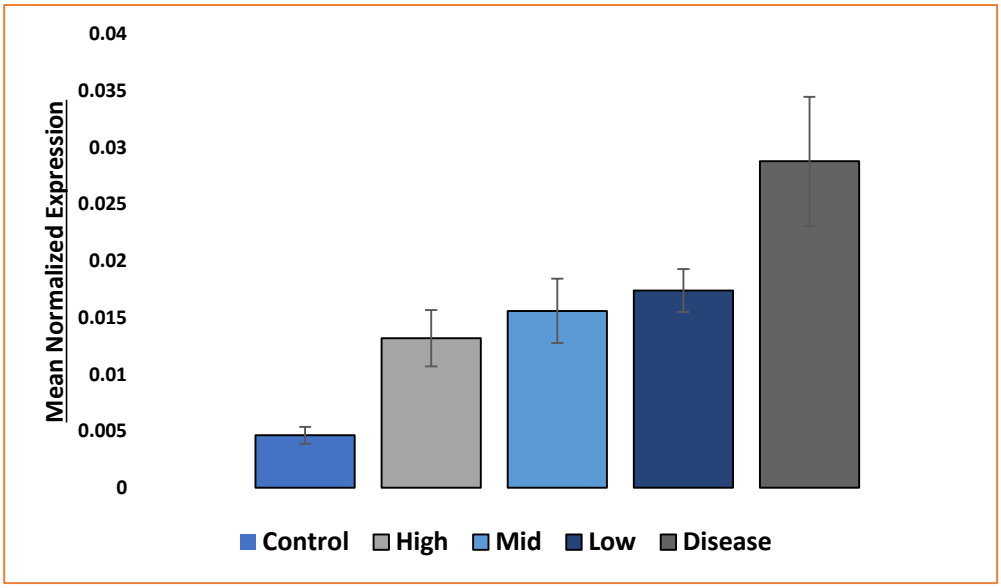

Fig. 30: Graphical representation of reduced gene expression of Osteocalcin in all the treated groups compared with arthritic group post treatment with Ta-1

TRAP gene expression:

In 2004, Kishimoto Y. et al. conducted a research study, the results, derived from both RT-PCR and real-time PCR analyses, indicated the presence of specific single bands for all genes in the synovium. Notably, expression levels of TNF-α, IL-1β, IL-6, RANKL, TRAP, and cathepsin K mRNA showed an increase, while RANK and OPG exhibited unchanged and decreased expression, respectively (187).

In the present study the levels of TRAP gene were enhanced in the arthritic group when compared with the normal healthy control. But with the administration of Ta-1 at doses of 0.25mg/kg, 0.5mg/kg, and 1mg/kg (low, mid and high) the gene expression of TRAP was significantly reduced in all the 3 groups (Figure-31).

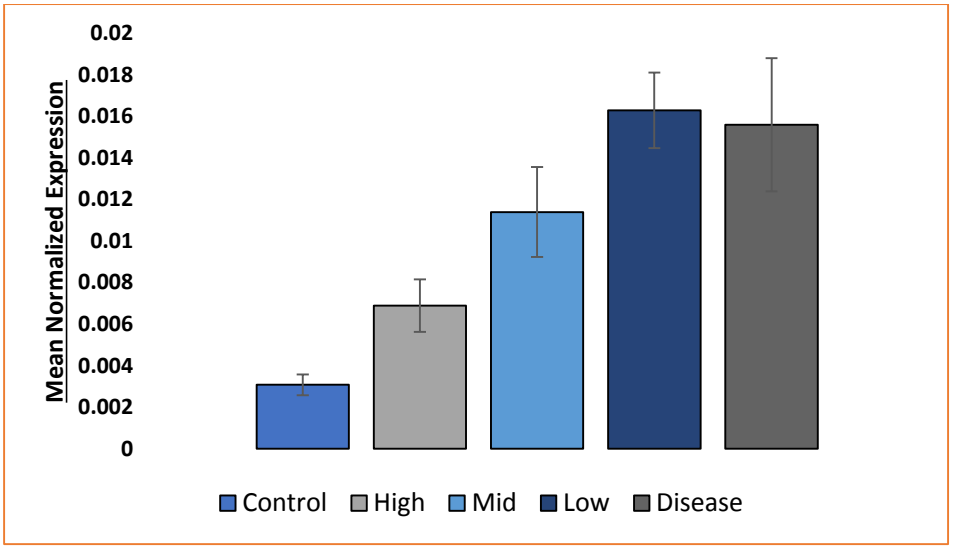

Fig. 31: Graphs shows the reduction of TRAP gene expression in mid and high dose groups compared with arthritic control post-treatment with Ta-1

Combination of Ta-1 and glucocorticoids and Estradiol

We used three different doses of Ta-1 and combined with three different doses of glucocorticoid or estradiol. The doses of Ta-1 were 0.25mg/kg, 0.5mg/kg, and 1.0 mg/kg, the doses of glucocorticoids were 0.5 umol/kg, 1 umol/kg, and 2 umol/kg, and the same for estradiol were 2.5µg, 5µg, and 10µg. Following nine combinations doses were given to animal groups for Ta-1 and glucocorticoids:

The effect of combination therapy of thymosin and glucocorticoids or estradiol were measured in terms of body weight and paw volume measurement. The results of these two are given below. The measurements were taken on zero days and also on 15th days.

We investigated the impact of varying concentrations of Ta-1, glucocorticoids, and estradiol on body weight in a rat model. Our results indicated a trend where body weight increased with higher doses of Ta-1 and glucocorticoids. Specifically, the highest body weight was observed with a combined dosage of Ta-1 at 1 mg/kg and glucocorticoids at 2 µmol/kg (Figure no-35).

When examining estradiol, we also noted an increase in body weight corresponding to higher estradiol doses, as well as increasing doses of Ta-1. However, similar to the findings for glucocorticoids, these increases in body weight were statistically nonsignificant, suggesting that while there is a correlation between hormone dosage and body weight, the effects may not be strong enough to be deemed significant under the experimental conditions used.

Notably, when we compared the body weight changes resulting from combinations of thymosin with glucocorticoids versus those with estradiol, the results revealed a significant increase in body weight in the glucocorticoid combination. This finding indicates that glucocorticoids may exert a more pronounced effect on weight gain compared to estradiol, suggesting a potential differential impact of these agents on metabolism and growth in this model. This observation aligns with previous literature suggesting that glucocorticoids can influence appetite and metabolic rates, thereby contributing to weight changes (188).

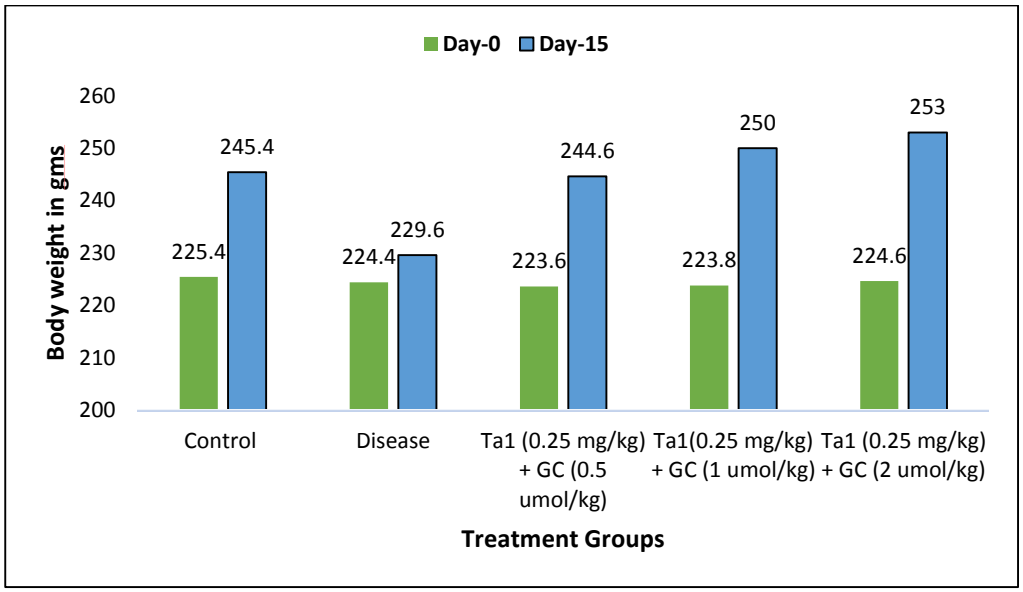

Fig. 32: Effect of Different Dosage Combinations of Glucocorticoids and Ta-1 (0.25mg/kg) on body weight on Day 0 and Day 15

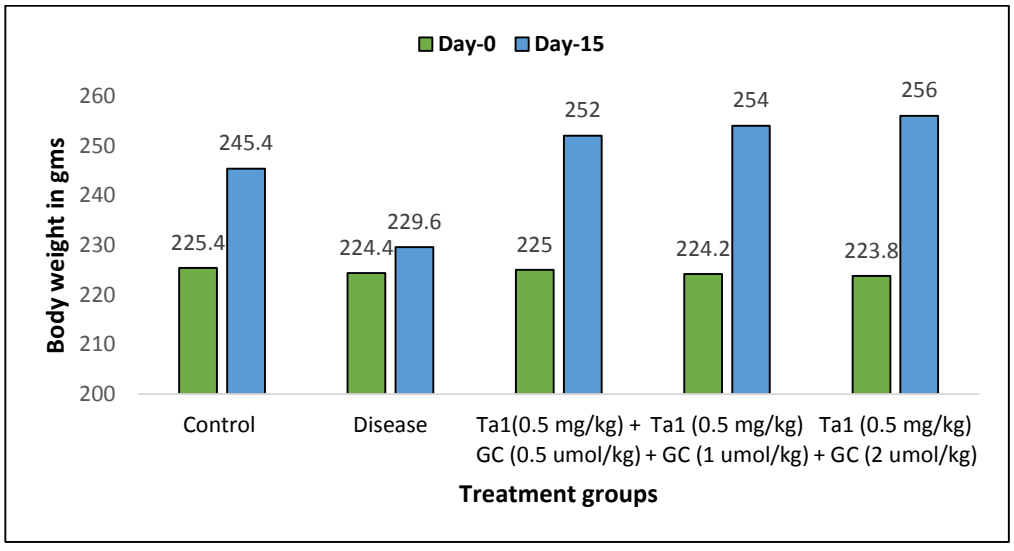

Fig. 33: Effect of Different Dosage Combinations of Glucocorticoids and Ta-1 (0.5mg/kg) on body weight on Day 0 and Day 15

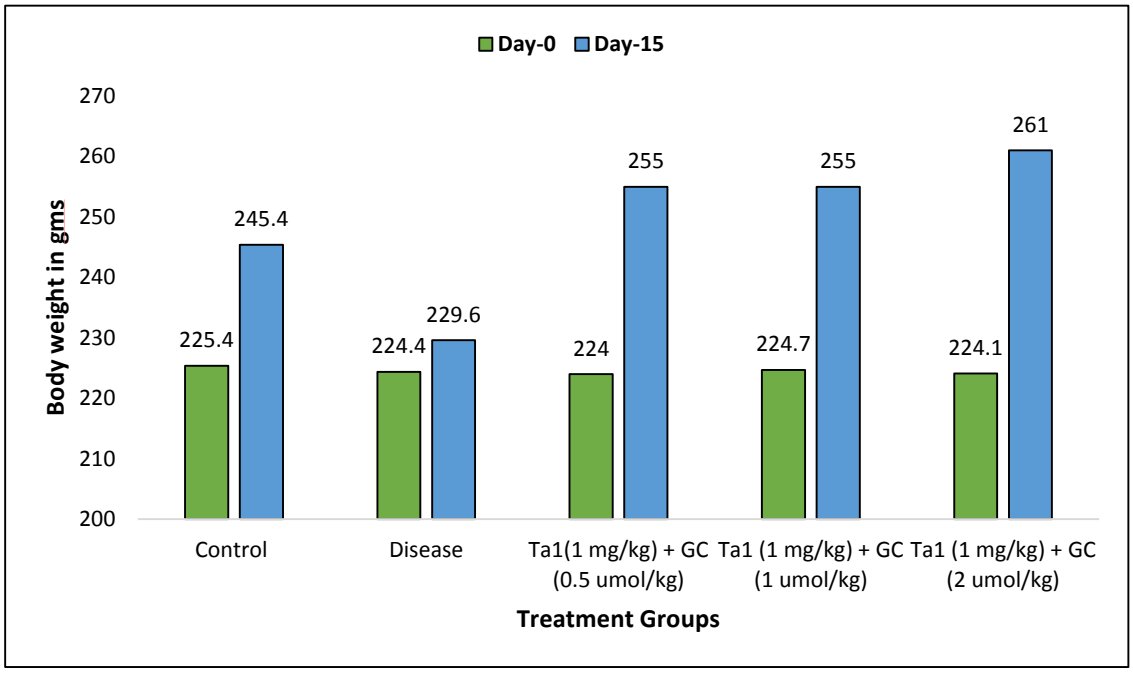

Fig. 34: Effect of Different Dosage Combinations of Glucocorticoids and Ta-1 (1 mg/kg) on body weight on Day 0 and Day 15

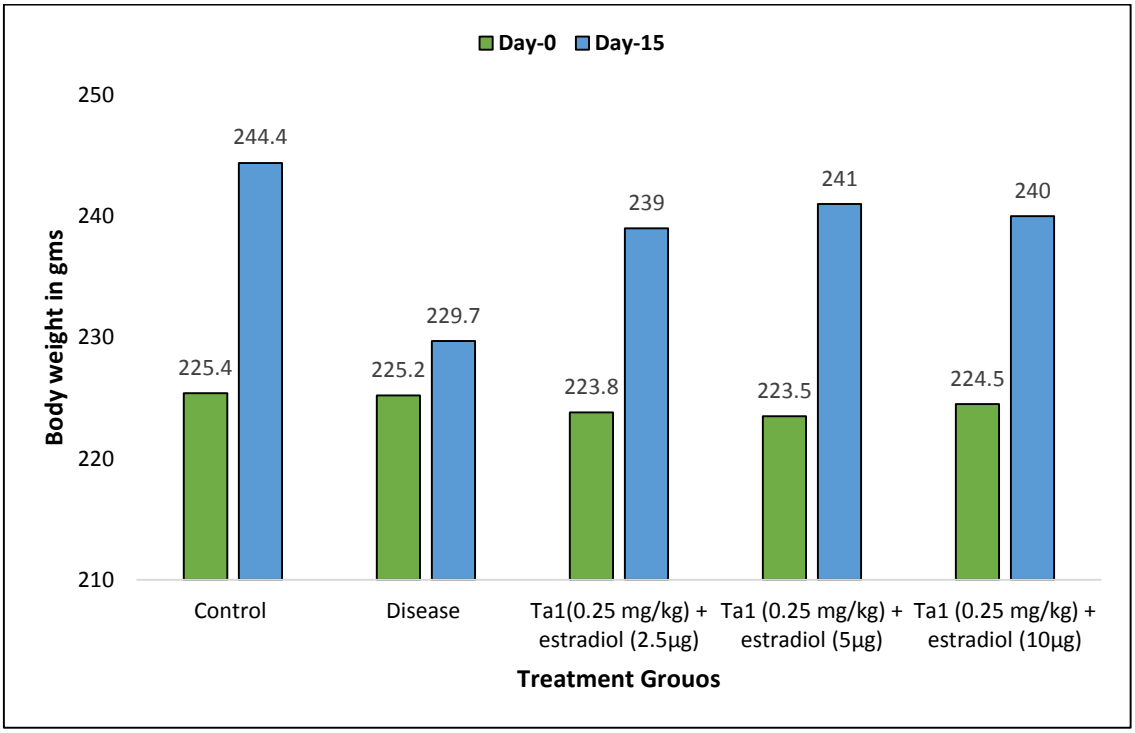

Fig. 35: Effect of Different Dosage Combinations of Estradiol and Ta-1 (0.25 mg/kg) on body weight on Day 0 and Day 15

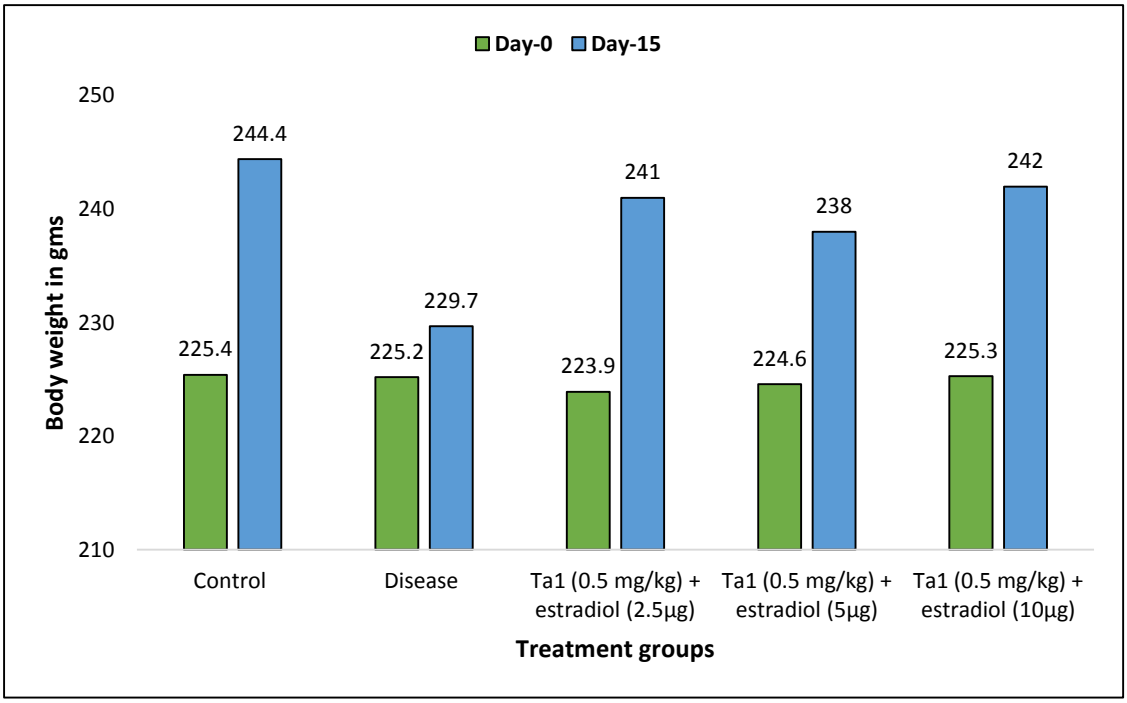

Fig. 36: Effect of Different Dosage Combinations of Estradiol and Ta-1 (0.5 mg/kg) on body weight on Day 0 and Day 15

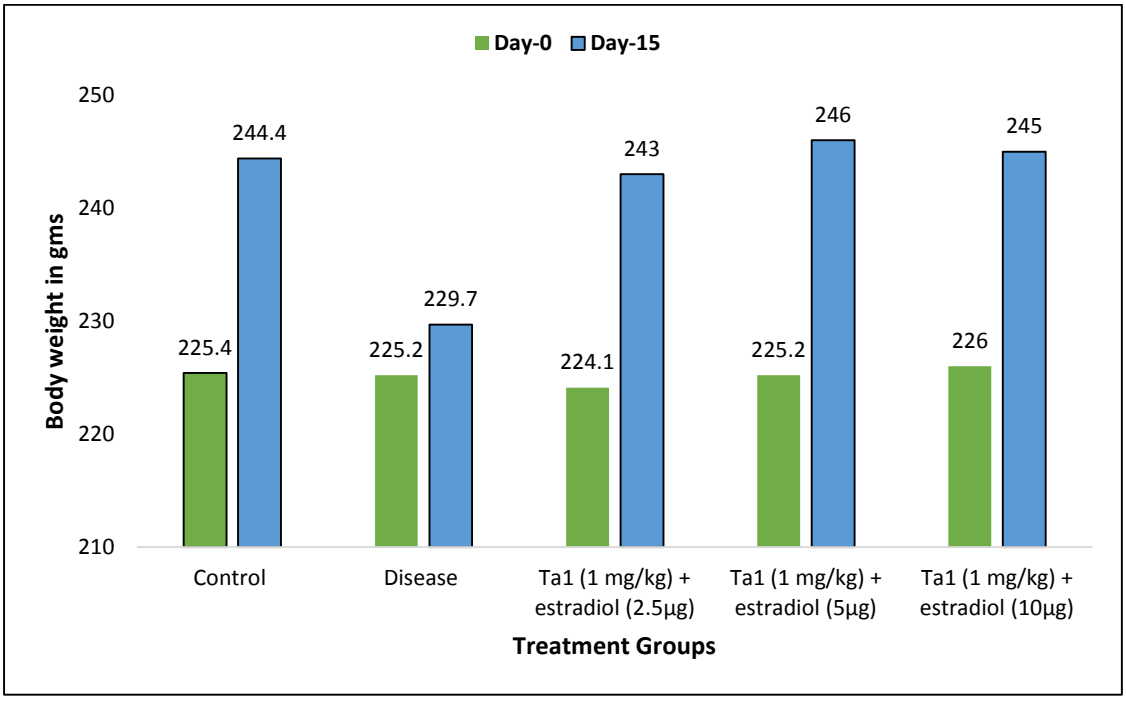

Fig. 37: Effect of Different Dosage Combinations of Estradiol and Ta-1 (1 mg/kg) on body weight on Day 0 and Day 15

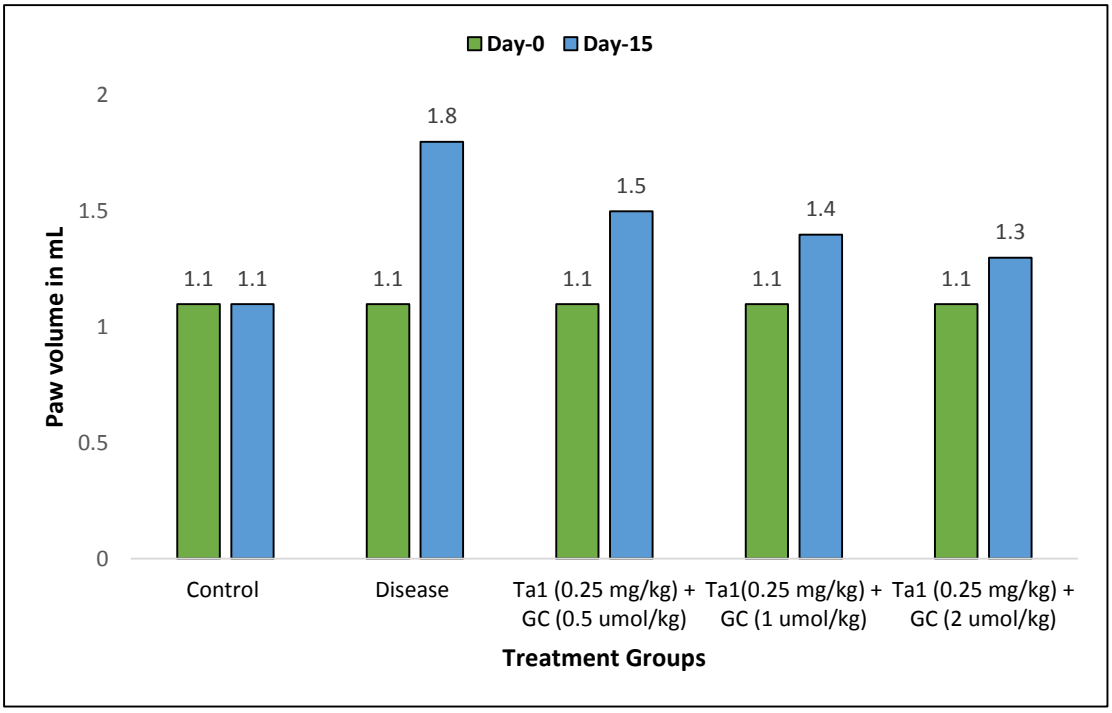

Fig. 38: Effect of Different Dosage Combinations of Glucocorticoids and Ta-1 (0.25mg/kg) on paw weight on Day 0 and Day 15

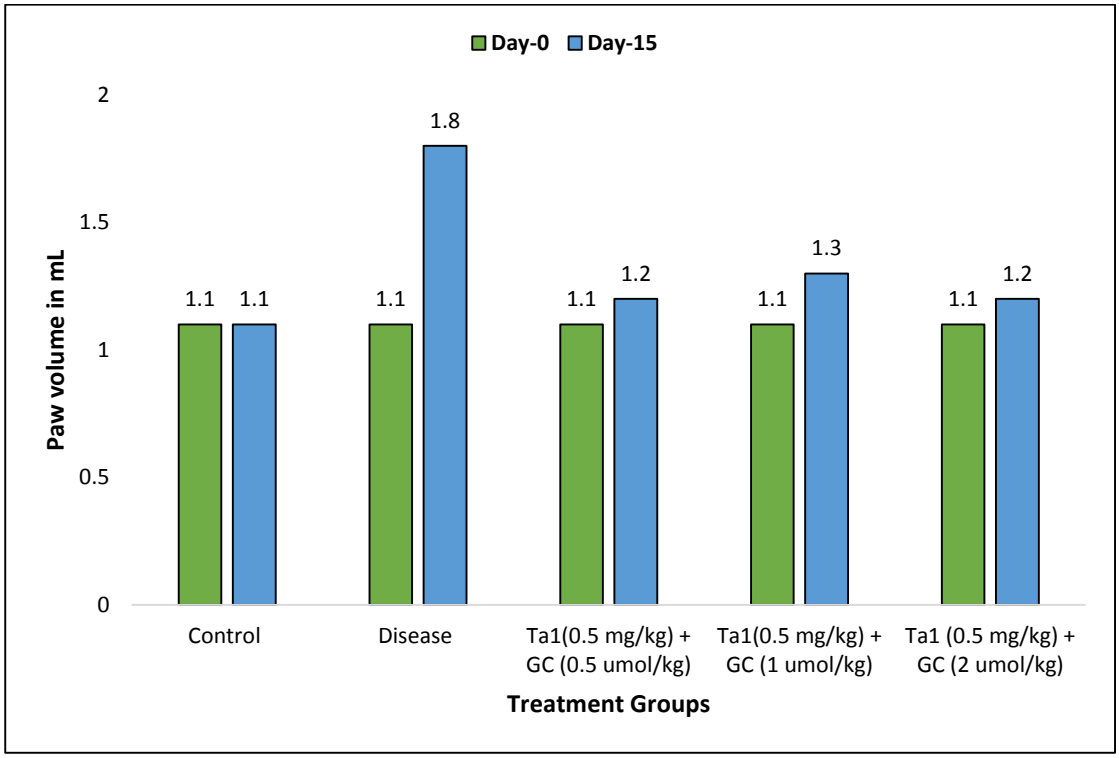

Fig. 39: Effect of Different Dosage Combinations of Glucocorticoids and Ta-1 (0.5mg/kg) on paw weight on Day 0 and Day 15

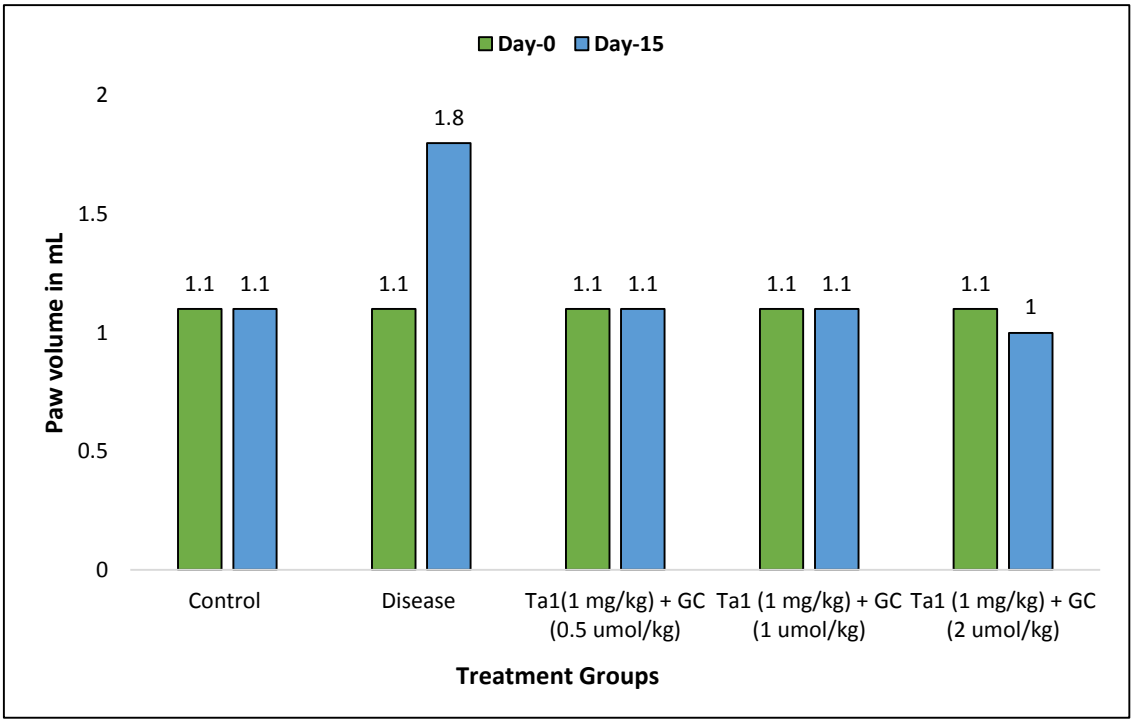

Fig. 40: Effect of Different Dosage Combinations of Glucocorticoids and Ta-1 (1 mg/kg) on paw weight on Day 0 and Day 15

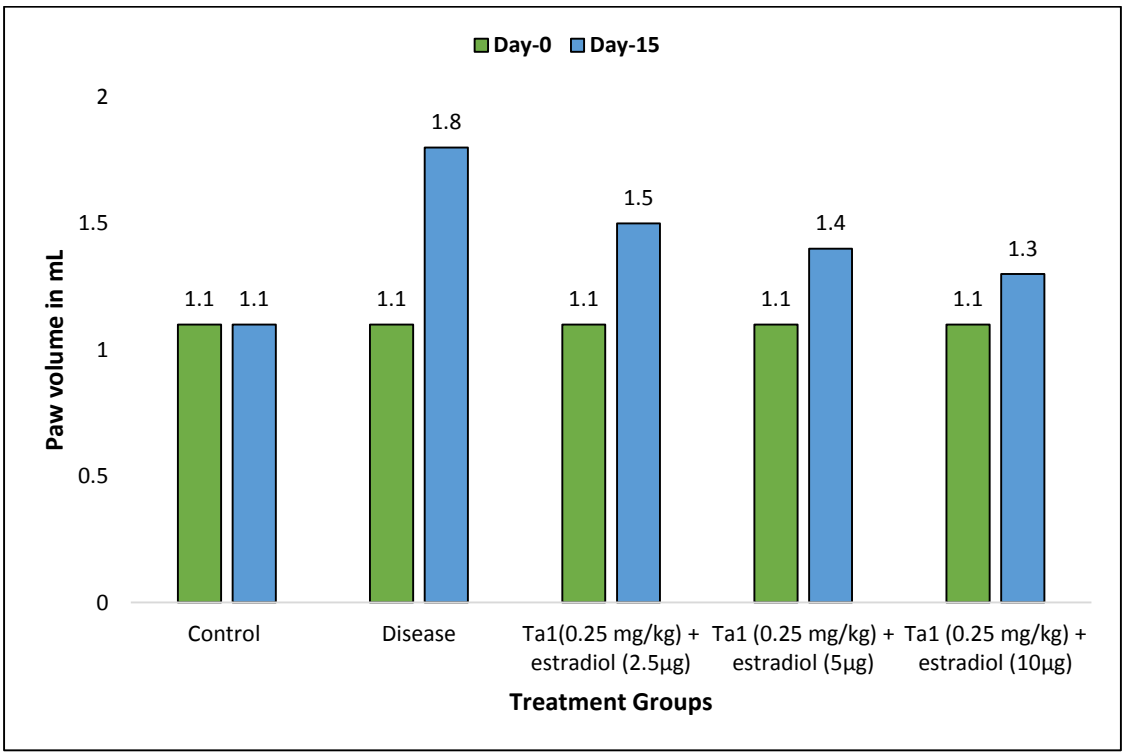

Fig. 41: Effect of Different Dosage Combinations of Estradiol and Ta-1 (0.5 mg/kg) on paw weight on Day 0 and Day 15

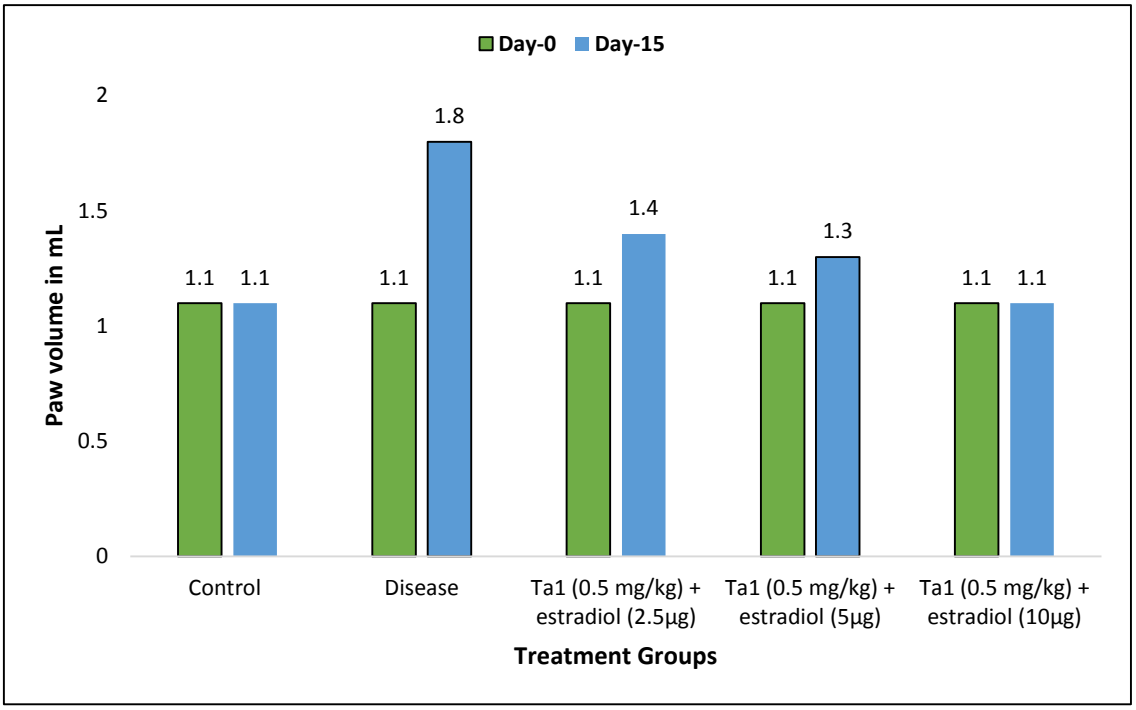

Fig. 42: Effect of Different Dosage Combinations of Estradiol and Ta-1 (0.5 mg/kg) on paw weight on Day 0 and Day 15

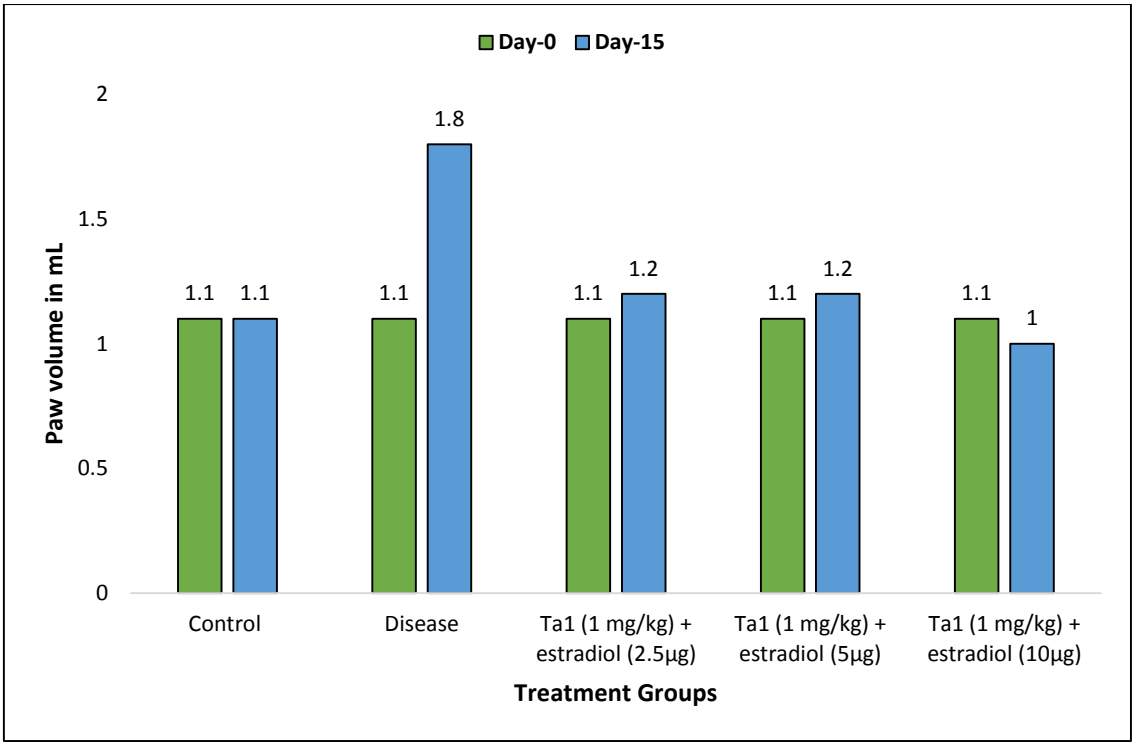

Fig. 43: Effect of Different Dosage Combinations of Estradiol and Ta-1 (0.5 mg/kg) on paw weight on Day 0 and Day 15

The paw volume was found to decrease as the concentration of Ta-1 and glucocorticoids and estradiol increased. The lowest paw volume was found at the combined dose of Ta-1 (1 mg/kg bodyweight) and glucocorticoids of 2 micromol/kg body weight but the difference or the growth was statistically nonsignificant. Similarly, the paw volume increases as the dose of estradiol increases or the dose of Ta-1 increase. But similar to previous on these increases of reduction of paw volume were statistically nonsignificant. When we compared the paw volume increase during the combination of thymosin with glucocorticoids or thymosin with estradiol, the paw volume growth were significantly higher in the case of glucocorticoids combination.

CONCLUSION

CONCLUSIONS

Musculoskeletal issues, characterized by pain and mobility limitations, can have negative repercussions on an individual's work capacity, social engagement, mental well-being, and community health. Common conditions encompass osteoarthritis, back and neck pain, bone fragility-related fractures, traumas, and inflammatory disorders such as rheumatoid arthritis. The Global Burden of Disease (GBD) study highlights the substantial disability caused by musculoskeletal disorders, with lower back pain ranking as a primary global cause of disability since 1990, and musculoskeletal issues securing the second position in global disability in the 2016 GBD research. Approximately 20% to 33% of the global population experiences discomfort due to musculoskeletal ailments.

Bone, a complex structure composed of cells, blood vessels, and calcium compounds arranged in a crystalline pattern, serves multifaceted functions. Firstly, it plays a crucial role in maintaining "calcium homeostasis" and acts as a vital repository for storing essential elements like phosphate, magnesium, potassium, and bicarbonate. Secondly, it provides critical structural support to soft tissues and functions as a lever facilitating muscular movement. Lastly, bones serve as the primary site for the haematopoiesis process, significantly contributing to the formation of blood cells in our body. In essence, bone is a dynamic and integral part of our physiological framework, fulfilling key roles in mineral storage, structural support, and blood cell generation. To maintain optimal health, bones undergo a continuous cycle of growth, repair, and breakdown, a process known as remodeling. Osteoclasts and osteoblasts, specialized cells, play key roles in orchestrating these changes. Bone remodeling unfolds in a four-stage process, involving the breakdown of old or damaged bone through resorption by osteoclasts, followed by the formation of new bone tissue by osteoblasts. This dynamic cycle ensures the constant adaptation and renewal of bone structure, contributing to overall skeletal health.

Local regulation of bone remodeling is influenced by a range of hormones and cytokines, with the delicate balance between new bone growth and degradation being crucial for skeletal health. Conditions like osteoporosis and rheumatoid arthritis (RA) exemplify situations where bone loss exceeds formation. RA, a prominent systemic autoimmune disorder, is characterized by persistent synovial inflammation, leading to

joint and cartilage deterioration. Immunological dysfunctions, particularly an abundance of pro-inflammatory responses, contribute to the chronic nature of arthritis. Both regional and systemic abnormalities in the body's natural defense appear to play a role in the initiation and progression of the disease. Recent advancements in RA pharmaceutical therapy have allowed many patients to achieve remission, enhancing their quality of life and minimizing long-term consequences. Early intervention and continuous care play pivotal roles in reducing damage. However, a notable challenge is the high cost associated with traditional RA treatments, primarily stemming from disease-modifying anti-rheumatic medications (DMARDs) and selective synthetic DMARDs. Despite the benefits, concerns arise, including an elevated risk of cardiovascular disease and disease flare-ups.

A recent focus has been on the exploration and acceptance of biosimilars, generic substances resembling biological DMARDs. Some studies suggest their effectiveness, offering a significant alternative to cut costs, broaden treatment options, and reduce access disparities between affluent and developing nations. Even after exhausting therapeutic options, the common occurrence of therapy failure in RA patients emphasizes the need for novel treatments and understanding the mechanisms behind therapy toxicity and failure in non-remissive cases. Adverse effects, coupled with the high cost, pose significant barriers to patient adherence to medications.

Ta-1, a synthetic peptide consisting of 28 amino acids and originally isolated from thymus tissue, is now produced synthetically. Studies suggest its potential in influencing immunological activity and its contrasting effects on joint complaints. Given the immune-modulating and anti-inflammatory properties of Ta-1, a study was designed to assess its potential role in bone remodeling using the Collagen-induced arthritis model in rats for rheumatoid arthritis (RA). After administering Ta-1 treatment, noticeable improvements were observed in various parameters, including body weight, paw swelling, arthritic score, and both biochemical and hematological parameters. Tissue histology of the hind paw and bone remodeling biomarkers also showed improvement after the administration of Ta-1. Thus, it can be concluded that Ta-1 holds promise for use in the treatment of rheumatoid arthritis and other bone-related disorders. The combination of Ta-1 along with glucocorticoids and estradiol showed

increase in the bodyweight and reduction on the paw volume after 15 days of treatment when compared with day zero. The difference between the combination of glucocorticoids and estradiol were statistically significant. Ta-1 has shown a promising effect of the reduction in the pathophysiology of rheumatoid arthritis and hence it can be a better therapeutic candidate of future for the management of RA. The more detailed analysis of the mechanistic study may provide a better understanding.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. VOS, Theo, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 28 diseases and injuries for 195 countries, 1990–2016: a systematic analysis for the Global Burden of Disease Study 2016. *The Lancet*, 2017, 390.10100: 1211-1259.

2. Bone and Joint Initiative USA. 2016.

3. BRENNAN-OLSEN, Sharon L., et al. Prevalence of arthritis according to age, sex and socioeconomic status in six low- and middle-income countries: analysis of data from the World Health Organization study on global AGEing and adult health (SAGE) Wave 1. *BMC musculoskeletal disorders*, 2017, 18.1: 271

4. Frost, H. M. Dynamics of bone remodeling. Frost, H. M., ed. *Bone Biodynamics*. Boston: Little, Brown; 1964: 315-333.

5. Dempster DW. The impact of bone turnover and bone-active agents on bone quality: focus on the hip. Osteoporosis international. 2002 May 20;13(5):349-52.

6. Parfitt AM. Surface specific bone remodeling in health and disease. Clinical disorders of bone and mineral metabolism. 1989: 7-14.

7. Weyand CM, Goronzy JJ. Pathogenesis of rheumatoid arthritis. Medical Clinics. 1997 Jan 1;81(1):29-55.

8. Toh ML, Miossec P. The role of T cells in rheumatoid arthritis: new subsets and new targets. Current opinion in rheumatology. 2007 May 1;19(3):284-8.

9. Weller FE, Shah U, Cummings GD, Chretien PB, Mutchnick MG. Serum levels of immunoreactive thymosin alpha 1 and thymosin beta 4 in large cohorts of healthy adults. Thymus. 1992 Feb;19(1):45-52.

10. Knutsen AP, Freeman JJ, Mueller KR, Roodman ST, Bouhasin JD. Thymosin-α1 stimulates maturation of CD34+ stem cells into CD3+ 4+ cells in an in vitro thymic epithelia organ coculture model. International journal of immunopharmacology. 1999 Jan 25;21(1):15-26.

11. Serrate SA, Schulof RS, Leondaridis L, Goldstein AL, Sztein MB. Modulation of human natural killer cell cytotoxic activity, lymphokine production, and interleukin 2 receptor expression by thymic hormones. The Journal of Immunology. 1987 Oct 1;139(7):2338-43.

12. Sztein MB, Serrate SA, Goldstein AL. Modulation of interleukin 2 receptor expression on normal human lymphocytes by thymic hormones. Proceedings of the National Academy of Sciences. 1986 Aug 1; 83(16):6107-11.

13. Mutchnick MG, Appelman HD, Chung HT, Aragona E, Gupta TP, Cummings GD, Waggoner JG, Hoofnagle JH, Shafritz DA. Thymosin treatment of chronic hepatitis B: a placebo-controlled pilot trial. Hepatology. 1991 Sep 1;14(3):409-15.

14. Goldstein AL, Goldstein AL. From lab to bedside: emerging clinical applications of thymosin α1. Expert opinion on biological therapy. 2009 May 1;9(5):593-608.

15. Zhang Q, Tang D, Zhao H. Immunological therapies can relieve aromatase inhibitor-related joint symptoms in breast cancer survivors. American journal of clinical oncology. 2010 Dec 1;33(6):557-60.

16. Pica F, Chimenti MS, Gaziano R, Buè C, Casalinuovo IA, Triggianese P, Conigliaro P, Di Carlo D, Cordero V, Adorno G, Volpi A. Serum thymosin α 1 levels in patients with chronic inflammatory autoimmune diseases. Clinical & Experimental Immunology. 2016 Oct;186(1):39-45.

17. Adler CP. Bones and bone tissue. In Bone diseases 2000 (pp. 1-11). Springer, Berlin, Heidelberg.

18. Bianco P, Riminucci M, Gronthos S, Robey PG. Bone marrow stromal stem cells: nature, biology, and potential applications. Stem cells. 2001 May;19(3):180-92.

19. Ducy P, Zhang R, Geoffroy V, Ridall AL, Karsenty G. Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. cell. 1997 May 30; 89(5):747-54.

20. Canalis ER, Pash JA, Gabbitas B, Rydziel SH, Varghese SA. Growth factors regulate the synthesis of insulin-like growth factor-I in bone cell cultures. Endocrinology. 1993 Jul 1;133(1):33-8.

21. Elmardi AS, Katchburian MV, Katchburian E. Electron microscopy of developing calvaria reveals images that suggest that osteoclasts engulf and destroy osteocytes during bone resorption. Calcified tissue international. 1990 Apr 1;46(4):239-45.

22. Lanyon LE. Osteocytes, strain detection, bone modeling and remodeling. Calcified tissue international. 1993 Feb 1;53(1): S102-7.

23. Teitelbaum SL. Bone resorption by osteoclasts. Science. 2000 Sep 1;289(5484):1504-8.

24. Vaananen HK, Zhao H, Mulari M, Halleen JM. The cell biology of osteoclast function. J Cell Sci. 2000 Feb 1;113(3):377-81.

25. Frost HM. Skeletal structural adaptations to mechanical usage (SATMU): 2. Redefining Wolff's law: the remodeling problem. The anatomical record. 1990 Apr;226(4):414-22.

26. FROST, H.M. 1996. Dynamics of bone remodeling. In Bone Biodynamics. H.M. Frost, Ed.: 315–333. Littel Brown. Boston, MA

27. Hsu H, Lacey DL, Dunstan CR, Solovyev I, Colombero A, Timms E, Tan HL, Elliott G, Kelley MJ, Sarosi I, Wang L. Tumor necrosis factor receptor family member RANK mediates osteoclast differentiation and activation induced by osteoprotegerin ligand. Proceedings of the National Academy of Sciences. 1999 Mar 30;96(7):3540-5.

28. Hofbauer LC, Schoppet M. Clinical implications of the osteoprotegerin/ RANKL/RANK system for bone and vascular diseases. Jama. 2004 Jul 28;292(4):490-5.

29. Simonet WS, Lacey DL, Dunstan CR, Kelley MC, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. cell. 1997 Apr 18;89(2):309-19.

30. Steeve KT, Marc P, Sandrine T, Dominique H, Yannick F. IL-6, RANKL, TNF-alpha/IL-1: interrelations in bone resorption pathophysiology. Cytokine & growth factor reviews. 2004 Feb 1;15(1):49-60.

31. Kim CH, Takai E, Zhou H, Von Stechow D, Müller R, Dempster DW, Guo XE. Trabecular bone response to mechanical and parathyroid hormone stimulation: the role of mechanical microenvironment. Journal of bone and mineral research. 2003 Dec 1;18(12):2116-25.

32. Chapuy MC, Arlot ME, Duboeuf F, Brun J, Crouzet B, Arnaud S, Delmas PD, Meunier PJ. Vitamin D3 and calcium to prevent hip fractures in elderly women. New England journal of medicine. 1992 Dec 3;327(23):1637-42.

33. Wang J, Zhou J, Cheng CM, Kopchick JJ, Bondy CA. Evidence supporting dual, IGF-I-independent and IGF-I-dependent, roles for GH in promoting longitudinal bone growth. Journal of Endocrinology. 2004 Feb 1;180(2):247-56.

34. Weinstein RS, Jilka RL, Parfitt AM, Manolagas SC. Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids. Potential mechanisms of their deleterious effects on bone. The Journal of clinical investigation. 1998 Jul 15;102(2):274-82.

35. Britto JM, Fenton AJ, Holloway WR, Nicholson GC. Osteoblasts mediate thyroid hormone stimulation of osteoclastic bone resorption. Endocrinology. 1994 Jan 1;134(1):169-76.

36. Srivastava S, Toraldo G, Weitzmann MN, Cenci S, Ross FP, Pacifici R. Estrogen decreases osteoclast formation by down-regulating receptor activator of NF-κB ligand (RANKL)-induced JNK activation. Journal of Biological Chemistry. 2001 Mar 23;276(12):8836-40

37. Kameda T, Mano H, Yuasa T, Mori Y, Miyazawa K, Shiokawa M, Nakamaru Y, Hiroi E, Hiura K, Kameda A, Yang NN. Estrogen inhibits bone resorption by directly inducing apoptosis of the bone-resorbing osteoclasts. Journal of Experimental Medicine. 1997 Aug 18;186(4):489-95.

38. Manolagas SC. Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocrine reviews. 2000 Apr 1;21(2):115-37.

39. Sato T, Kawano H, Kato S. Study of androgen action in bone by analysis of androgen-receptor deficient mice. Journal of bone and mineral metabolism. 2002 Nov 20;20(6):326-30.

40. Udagawa N, Takahashi N, Akatsu T, Tanaka H, Sasaki T, Nishihara T, Koga T, Martin TJ, Suda T. Origin of osteoclasts: mature monocytes and macrophages are capable of differentiating into osteoclasts under a suitable microenvironment prepared by bone marrow-derived stromal cells. Proceedings of the national academy of sciences. 1990 Sep 1;87(18):7260-4.

41. Hofbauer LC, Khosla S, Dunstan CR, Lacey DL, Boyle WJ, Riggs BL. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. Journal of Bone and Mineral Research. 2000 Jan;15(1):2-12

42. Hot'bauer L, Lacey DL, Dunstan CR. Interleukin—lbeta and tumor necrosis factor-alpha, but not interleukin — 6, stimulate osteoprotegerin ligand gene expression in human osteoblastic cells. Bone. 1999;25(3):255-9.

43. Moonga BS, Adebanjo OA, Wang HJ, Li S, Wu XB, Troen B, Inzerillo A, Abe E, Minkin C, Huang CL, Zaidi M. Differential effects of interleukin-6 receptor activation on intracellular signaling and bone resorption by isolated rat osteoclasts. Journal of Endocrinology. 2002 Jun 1;173(3):395-405.

44. Sims NA, Jenkins BJ, Quinn JM, Martin TJ, Glatt M, Gillespie MT, Ernst M. gp130 regulates bone turnover and bone size by distinct downstream signaling pathways. InJOURNAL OF BONE AND MINERAL RESEARCH 2002 Sep 1 (Vol. 17, pp. S154-S154). 2025 M ST, NW, STE 800, WASHINGTON, DC 20036-3309 USA: AMER SOC BONE & MINERAL RES.

45. Miao D, He B, Jiang Y, Kobayashi T, Sorocéanu MA, Zhao J, Su H, Tong X, Amizuka N, Gupta A, Genant HK. Osteoblast-derived PTHrP is a potent endogenous bone anabolic agent that modifies the therapeutic efficacy of administered PTH 1–34. The Journal of clinical investigation. 2005 Sep 1;115(9):2402-11.

46. Bax M, van Heemst J, Huizinga TW, Toes RE. Genetics of rheumatoid arthritis: what have we learned? Immunogenetics. 2011 Aug; 63:459-66.

47. Dhawan SS, Quyyumi AA. Rheumatoid arthritis and cardiovascular disease. Current atherosclerosis reports. 2008 Apr;10(2):128-33.

48. Boissier MC, Semerano L, Challal S, Saidenberg-Kermanac'h N, Falgarone G. Rheumatoid arthritis: from autoimmunity to synovitis and joint destruction. Journal of autoimmunity. 2012 Sep 1;39(3):222-8.

49. Stockinger B, Veldhoen M. Differentiation and function of Th17 T cells. Current opinion in immunology. 2007 Jun 1;19(3):281-6.

50. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science. 2003; 299: 1057–61.

51. Smolen JS, Aletaha D, Koeller M, Weisman MH, Emery P. New therapies for treatment of rheumatoid arthritis. The lancet. 2007 Dec 1;370(9602):1861-74.

52. Viatte S, Plant D, Raychaudhuri S. Genetics and epigenetics of rheumatoid arthritis. Nature Reviews Rheumatology. 2013 Mar;9(3):141-53.

53. Arvikar SL, Collier DS, Fisher MC, Unizony S, Cohen GL, McHugh G, Kawai T, Strle K, Steere AC. Clinical correlations with Porphyromonas gingivalis antibody responses in patients with early rheumatoid arthritis. Arthritis research & therapy. 2013;15:1-2.

54. Klein K, Gay S. Epigenetics in rheumatoid arthritis. Current opinion in rheumatology. 2015 Jan 1;27(1):76-82.

55. Glant TT, Mikecz K, Rauch TA. Epigenetics in the pathogenesis of rheumatoid arthritis. BMC medicine. 2014 Dec; 12:1-5.

56. Song YJ, Li G, He JH, Guo Y, Yang L. Bioinformatics-based identification of microRNA-regulated and rheumatoid arthritis-associated genes. PLoS One. 2015 Sep 11;10(9):e0137551.

57. Smolen JS, Steiner G. Therapeutic strategies for rheumatoid arthritis. Nature reviews Drug discovery. 2003 Jun 1;2(6):473-88.

58. Rantapää-Dahlqvist S, De Jong BA, Berglin E, Hallmans G, Wadell G, Stenlund H, Sundin U, van Venrooij WJ. Antibodies against cyclic citrullinated peptide and IgA rheumatoid factor predict the development of rheumatoid arthritis. Arthritis & Rheumatism. 2003 Oct;48(10):2741-9.

59. Hochberg MC, Johnston SS, John AK. The incidence and prevalence of extra-articular and systemic manifestations in a cohort of newly-diagnosed patients with rheumatoid arthritis between 1999 and 2006. Current medical research and opinion. 2008 Feb 1;24(2):469-80.

60. Paula FS, Alves JD. Non-tumor necrosis factor-based biologic therapies for rheumatoid arthritis: present, future, and insights into pathogenesis. Biologics: Targets and Therapy. 2013 Dec 9:1-2.

61. Paula FS, Alves JD. Non-tumor necrosis factor-based biologic therapies for rheumatoid arthritis: present, future, and insights into pathogenesis. Biologics: Targets and Therapy. 2013 Dec 9:1-2.

62. Firestein GS. Etiology and pathogenesis of rheumatoid arthritis. In: Firestein GS, Budd RC, Harris EDJ, McInnes IB, Ruddy S, Sergent JS, editors. Kelley's Textbook of Rheumatology. 8th ed. Philadelphia, PA: Saunders; 2009.

63. Wan YY, Flavell RA. TGF-β and regulatory T cell in immunity and autoimmunity. Journal of clinical immunology. 2008 Nov; 28:647-59.

64. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006 May 11;441(7090):235-8.

65. Zhou L, Lopes JE, Chong MM, Ivanov II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF. TGF-β-induced Foxp3 inhibits TH17 cell differentiation by antagonizing RORγt function. Nature. 2008 May 8;453(7192):236-40.

66. Schellekens GA, De Jong BA, Van den Hoogen FH, Van de Putte LB, van Venrooij WJ. Citrulline is an essential constituent of antigenic determinants recognized by rheumatoid arthritis-specific autoantibodies. The Journal of clinical investigation. 1998 Jan 1;101(1):273-81.

67. Vasanthi P, Nalini G, Rajasekhar G. Role of tumor necrosis factor-alpha in rheumatoid arthritis: a review. APLAR Journal of Rheumatology. 2007 Dec;10(4):270-4.

68. Edrees AF, Misra SN, Abdou NI. Anti-tumor necrosis factor (TNF) therapy in rheumatoid arthritis: correlation of TNF-alpha serum level with clinical response and benefit from changing dose or frequency of infliximab infusions. Clinical and experimental rheumatology. 2005 Jul 1;23(4):469.

69. Kay J, Calabrese L. The role of interleukin-1 in the pathogenesis of rheumatoid arthritis. Rheumatology. 2004 Jun 1;43(suppl_3): iii2-9.

70. Arend WP. Cytokine imbalance in the pathogenesis of rheumatoid arthritis: the role of interleukin-1 receptor antagonist. InSeminars in arthritis and rheumatism 2001 Apr 1 (Vol. 30, No. 5, pp. 1-6). WB Saunders.

71. McInnes IB, Schett G. Cytokines in the pathogenesis of rheumatoid arthritis. Nature Reviews Immunology. 2007 Jun;7(6):429-42.

72. Chizzolini C, Dayer JM, Miossec P. Cytokines in chronic rheumatic diseases: is everything lack of homeostatic balance?. Arthritis research & therapy. 2009 Oct;11:1-1.

73. Magyari L, Varszegi D, Kovesdi E, Sarlos P, Farago B, Javorhazy A, Sumegi K, Banfai Z, Melegh B. Interleukins and interleukin receptors in rheumatoid arthritis: Research, diagnostics and clinical implications. World journal of orthopedics. 2014 Sep 9;5(4):516.

74. Isomäki P, Punnonen J. Pro-and anti-inflammatory cytokines in rheumatoid arthritis. Annals of medicine. 1997 Jan 1;29(6):499-507.

75. Finnegan A, Mikecz K, Tao P, Glant TT. Proteoglycan (aggrecan)-induced arthritis in BALB/c mice is a Th1-type disease regulated by Th2 cytokines. The Journal of Immunology. 1999 Nov 1;163(10):5383-90.

76. Joosten LA, Lubberts E, Durez P, Helsen MM, Jacobs MJ, Goldman M, Van Den Berg WB. Role of interleukin-4 and interleukin-10 in murine collagen-induced arthritis. Protective effect of interleukin-4 and interleukin-10 treatment on cartilage destruction. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 1997 Feb;40(2):249-60.

77. Park HK, Kim SK, Kweon HY, Lee KG, Arasu MV, Kim YO. Promoter polymorphism (− 590, T/C) of interleukin 4 (IL4) gene is associated with rheumatoid arthritis: An updated meta-analysis. Saudi Journal of Biological Sciences. 2017 Feb 1;24(2):444-9.

78. Rose-John S. IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. International journal of biological sciences. 2012;8(9):1237.

79. Srirangan S, Choy EH. The role of interleukin 6 in the pathophysiology of rheumatoid arthritis. Therapeutic advances in musculoskeletal disease. 2010 Oct;2(5):247-56.

80. Dayer JM, Choy E. Therapeutic targets in rheumatoid arthritis: the interleukin-6 receptor. Rheumatology. 2010 Jan 1;49(1):15-24.

81. Yoshida Y, Tanaka T. Interleukin 6 and rheumatoid arthritis. BioMed research international. 2014 Oct;2014.

82. Li X, Chai W, Ni M, Xu M, Lian Z, Shi L, Bai Y, Wang Y. The effects of gene polymorphisms in interleukin-4 and interleukin-6 on the susceptibility of rheumatoid arthritis in a Chinese population. BioMed Research International. 2014 Feb 23;2014.

83. Churchman SM, Ponchel F. Interleukin-7 in rheumatoid arthritis. Rheumatology. 2008 Jun 1;47(6):753-9.

84. Hartgring SA, Bijlsma JW, Lafeber FP, Van Roon JA. Interleukin-7 induced immunopathology in arthritis. Annals of the rheumatic diseases. 2006 Nov 1;65(suppl 3):iii69-74.

85. van Roon J A, Glaudemans K A, Bijlsma J W, Lafeber F P. Interleukin 7 stimulates tumour necrosis factor alpha and Th1 cytokine production in joints of patients with rheumatoid arthritis. Ann Rheum Dis 200362113–119.

86. Chizzolini C, Parel Y, Scheja A, Dayer JM. Polarized subsets of human T-helper cells induce distinct patterns of chemokine production by normal and systemic sclerosis dermal fibroblasts. Arthritis research & therapy. 2005 Feb; 8:1-2.

87. Möttönen M, Isomäki P, Saario R, Toivanen P, Punnonen J, Lassila O. Interleukin-10 inhibits the capacity of synovial macrophages to function as antigen-presenting cells. British journal of rheumatology. 1998 Nov 1;37(11):1207-14.

88. van Roon JA, Lafeber FP, Bijlsma JW. Synergistic activity of interleukin-4 and interleukin-10 in suppression of inflammation and joint destruction in rheumatoid arthritis. Arthritis & Rheumatism: Official Journal of the American College of Rheumatology. 2001 Jan;44(1):3-12.

89. Van Roon JAG, van Roy JLAM, Gmelig-Meyling FHJ, Lafeber FPJG, Bijlsma JWJ. Prevention and reversal of cartilage degradation in rheumatoid arthritis by interleukin-10 and interleukin-4. Arthritis Rheum 1996; 39:829–35.

90. Apparailly F, Verwaerde C, Jacquet C, Auriault C, Sany J, Jorgensen C. Adenovirus-mediated transfer of viral IL-10 gene inhibits murine collagen-induced arthritis. The Journal of Immunology. 1998 Jun 1;160(11):5213-20.

91. Pope RM, Shahrara S. Possible roles of IL-12-family cytokines in rheumatoid arthritis. Nature Reviews Rheumatology. 2013 Apr;9(4):252-6.

92. Petrovic-Rackov L, Pejnovic N. Clinical significance of IL-18, IL-15, IL-12 and TNF-α measurement in rheumatoid arthritis. Clinical rheumatology. 2006 Jul;25:448-52.

93. En-Yin W, Qin Y, Zhi-Gang L. Association of polymorphisms in interleukin 12 genes (IL-12A and -B) with rheumatoid 1 arthritis in a Chinese population. Clin Exp Immuno. 2015; 180: 83-9.

94. Shen L, Zhang H, Zhou X, Liu R. Association between polymorphisms of interleukin 12 and rheumatoid arthritis associated biomarkers in a Chinese population. Cytokine. 2015 Dec 1;76(2):363-7.

95. Baum R, Gravallese EM. Impact of inflammation on the osteoblast in rheumatic diseases. Current osteoporosis reports. 2014 Mar 1;12(1):9-16.

96. Teitelbaum SL, Ross FP. Genetic regulation of osteoclast development and function. Nature Reviews Genetics. 2003 Aug;4(8):638.

97. Schroeder TM, Jensen ED, Westendorf JJ. Runx2: a master organizer of gene transcription in developing and maturing osteoblasts. Birth Defects Research Part C: Embryo Today: Reviews. 2005 Sep 1;75(3):213-25

98. Nakashima K, Zhou X, Kunkel G, Zhang Z, Deng JM, Behringer RR, de Crombrugghe B. The novel zinc finger-containing transcription factor osterix is required for osteoblast differentiation and bone formation. Cell. 2002 Jan 11;108(1):17-29.

99. Sánchez-Duffhues G, Hiepen C, Knaus P, ten Dijke P. Bone morphogenetic protein signaling in bone homeostasis. Bone. 2015 Nov 1;80:43-59.

100. Monroe DG, McGee-Lawrence ME, Oursler MJ, Westendorf JJ. Update on Wnt signaling in bone cell biology and bone disease. Gene. 2012 Jan 15;492(1):1-8.

101. Robling AG, Niziolek PJ, Baldridge LA, Condon KW, Allen MR, Alam I, Mantila SM, Gluhak-Heinrich J, Bellido TM, Harris SE, Turner CH. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. Journal of Biological Chemistry. 2008 Feb 29;283(9):5866-75.

102. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. cell. 1998 Apr 17;93(2):165-76.

103. Simonet WS, Lacey DL, Dunstan CR, Kelley MC, Chang MS, Lüthy R, Nguyen HQ, Wooden S, Bennett L, Boone T, Shimamoto G. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. cell. 1997 Apr 18;89(2):309-19.

104. Pettit AR, Ji H, von Stechow D, Müller R, Goldring SR, Choi Y, Benoist C, Gravallese EM. TRANCE/RANKL knockout mice are protected from bone erosion in a serum transfer model of arthritis. The American journal of pathology. 2001 Nov 1;159(5):1689-99.

105. Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, Steiner G, Smolen JS, Wagner EF, Schett G. Osteoclasts are essential for TNF-α–mediated joint destruction. The Journal of clinical investigation. 2002 Nov 15;110(10):1419-27.

106. Brennan FM, McInnes IB. Evidence that cytokines play a role in rheumatoid arthritis. The Journal of clinical investigation. 2008 Nov 3;118(11):3537-45.

107. Redlich K, Hayer S, Ricci R, David JP, Tohidast-Akrad M, Kollias G, Steiner G, Smolen JS, Wagner EF, Schett G. Osteoclasts are essential for TNF-α–mediated joint destruction. The Journal of clinical investigation. 2002 Nov 15;110(10):1419-27.

108. Crotti TN, Smith MD, Weedon H, Ahern MJ, Findlay DM, Kraan M, Tak PP, Haynes DR. Receptor activator NF-κB ligand (RANKL) expression in synovial tissue from patients with rheumatoid arthritis, spondyloarthropathy, osteoarthritis, and from normal patients: semiquantitative and quantitative analysis. Annals of the rheumatic diseases. 2002 Dec 1;61(12):1047-54.

109. Smolen JS, Landewé RB, Bijlsma JW, Burmester GR, Dougados M, Kerschbaumer A, McInnes IB, Sepriano A, Van Vollenhoven RF, De Wit M, Aletaha D. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. Annals of the rheumatic diseases. 2020 Jun 1;79(6):685-99.

110. Sewerin P, Vordenbaeumen S, Hoyer A, Brinks R, Buchbender C, Miese F, Schleich C, Klein S, Schneider M, Ostendorf B. Silent progression in patients with rheumatoid arthritis: is DAS28 remission an insufficient goal in RA? Results from the German Remission-plus cohort. BMC musculoskeletal disorders. 2017 Dec;18:1-9.

111. Kuijper TM, Lamers-Karnebeek FB, Jacobs JW, Hazes JM, Luime JJ. Flare rate in patients with rheumatoid arthritis in low disease activity or remission when tapering or stopping synthetic or biologic DMARD: a systematic review. The Journal of rheumatology. 2015 Nov 1;42(11):2012-22.

112. Dörner T, Strand V, Cornes P, Gonçalves J, Gulácsi L, Kay J, Kvien TK, Smolen J, Tanaka Y, Burmester GR. The changing landscape of biosimilars in rheumatology. Annals of the rheumatic diseases. 2016 Jun 1;75(6):974-82.

113. Smolen, J.S.; Aletaha, D.; McInnes, I.B. Rheumatoid arthritis. Lancet 2016, 388, 2023–2038.

114. Ferraz-Amaro I, Machín S, Carmona L, Gonzalez-Alvaro I, Díaz-González F, EMECAR study group. Pattern of use and safety of non-steroidal anti-inflammatory drugs in rheumatoid arthritis patients. A prospective analysis from clinical practice. Reumatologia Clinica. 2009 Nov 1;5(6):252-8.

115. Crofford LJ. Use of NSAIDs in treating patients with arthritis. Arthritis research & therapy. 2013 Jul;15:1-0.

116. Nissen SE, Yeomans ND, Solomon DH, Lüscher TF, Libby P, Husni ME, Graham DY, Borer JS, Wisniewski LM, Wolski KE, Wang Q. Cardiovascular safety of celecoxib, naproxen, or ibuprofen for arthritis. New England Journal of Medicine. 2016 Dec 29;375:2519-29.

117. Scheiman JM. NSAID-induced gastrointestinal injury. Journal of clinical gastroenterology. 2016 Jan 1;50(1):5-10.

118. Ong CK, Lirk P, Tan CH, Seymour RA. An evidence-based update on nonsteroidal anti-inflammatory drugs. Clinical medicine & research. 2007 Mar 1;5(1):19-34.

119. Strehl C, Bijlsma JW, De Wit M, Boers M, Caeyers N, Cutolo M, Dasgupta B, Dixon WG, Geenen R, Huizinga TW, Kent A. Defining conditions where long-term glucocorticoid treatment has an acceptably low level of harm to facilitate implementation of existing recommendations: viewpoints from an EULAR task force. Annals of the rheumatic diseases. 2016 Jun 1;75(6):952-7.

120. Van der Goes MC, Jacobs JW, Boers M, Andrews T, Blom-Bakkers MA, Buttgereit F, Caeyers N, Cutolo M, Da Silva JA, Guillevin L, Kirwan JR. Monitoring adverse events of low-dose glucocorticoid therapy: EULAR recommendations for clinical trials and daily practice. Annals of the rheumatic diseases. 2010 Nov 1;69(11):1913-9.

121. Ozen G, Pedro S, Holmqvist ME, Avery M, Wolfe F, Michaud K. Risk of diabetes mellitus associated with disease-modifying antirheumatic drugs and statins in rheumatoid arthritis. Annals of the rheumatic diseases. 2017 May 1;76(5):848-54.

122. Ruyssen-Witrand, A.; Fautrel, B.; Saraux, A.; Le Loët, X.; Pham, T. Cardiovascular risk induced by low-dose corticosteroids in rheumatoid arthritis: A systematic literature review. Jt. Bone Spine 2011, 78, 23–30.

123. Liu, D.; Ahmet, A.; Ward, L.; Krishnamoorthy, P.; Mandelcorn, E.D.; Leigh, R.; Brown, J.P.; Cohen, A.; Kim, H. A practical guide tothe monitoring and management of the complications of systemic corticosteroid therapy. Allergy Asthma Clin. Immunol. 2013, 9,30.

124. Wang F, Sun L, Wang S, Davis III JM, Matteson EL, Murad MH, Luo F, Vassallo R. Efficacy and safety of tofacitinib, baricitinib, and upadacitinib for rheumatoid arthritis: a systematic review and meta-analysis. InMayo Clinic Proceedings 2020 Jul 1 (Vol. 95, No. 7, pp. 1404-1419). Elsevier.

125. Rinaudo-Gaujous M, Blasco-Baque V, Miossec P, Gaudin P, Farge P, Roblin X, Thomas T, Paul S, Marotte H. Infliximab induced a dissociated response of severe periodontal biomarkers in rheumatoid arthritis patients. Journal of clinical medicine. 2019 May 26;8(5):751.

126. Zrubka Z, Gulácsi L, Brodszky V, Rencz F, Alten R, Szekanecz Z, Péntek M. Long-term efficacy and cost-effectiveness of infliximab as first-line treatment in rheumatoid arthritis: systematic review and meta-analysis. Expert Review of Pharmacoeconomics & Outcomes Research. 2019 Sep 3;19(5):537-49.

127. Cohen, S.; Tuckwell, K.; Katsumoto, T.R.; Zhao, R.; Galanter, J.; Lee, C.; Rae, J.; Toth, B.; Ramamoorthi, N.; Hackney, J.A.; et al. Fenebrutinib Versus Placebo or Adalimumab in Rheumatoid Arthritis: A Randomized, Double-Blind, Phase II Trial. Arthritis Rheumatol. 2020, 72, 1435–1446.

128. Cagnotto G, Willim M, Nilsson JÅ, Compagno M, Jacobsson LT, Saevarsdottir S, Turesson C. Abatacept in rheumatoid arthritis: survival on drug, clinical outcomes, and their predictors—data from a large national quality register. Arthritis Research & Therapy. 2020 Dec;22(1):1-1.

129. Blair HA, Deeks ED. Abatacept: a review in rheumatoid arthritis. Drugs. 2017 Jul; 77:1221-33.

130. Scott LJ. Tocilizumab: a review in rheumatoid arthritis. Drugs. 2017 Nov; 77:1865-79.

131. Pandolfi F, Franza L, Carusi V, Altamura S, Andriollo G, Nucera E. Interleukin-6 in rheumatoid arthritis. International journal of molecular sciences. 2020 Jul 23;21(15):5238.

132. Ruscitti P, Masedu F, Alvaro S, Airò P, Battafarano N, Cantarini L, Cantatore FP, Carlino G, D'Abrosca V, Frassi M, Frediani B. Anti-interleukin-1 treatment in patients with rheumatoid arthritis and type 2 diabetes (TRACK): A multicentre, open-label, randomised controlled trial. PLoS medicine. 2019 Sep 12;16(9):e1002901.

133. Köhler BM, Günther J, Kaudewitz D, Lorenz HM. Current therapeutic options in the treatment of rheumatoid arthritis. Journal of clinical medicine. 2019 Jun 28;8(7):938.

134. Jevremovic M, Kartaljevic G, Jelusic V, Vodnik T, Pesic M, Filipovic S. Determination of thymosin alpha 1 with enzyme-immunoassay in colorectal cancer patients. Arch Oncol. 1997;5:193-194

135. Gomez-Marquez J, Segade F, Dosil M, Pichel JG, Bustelo XR, Freire M. The expression of prothymosin alpha gene in T lymphocytes and leukemic lymphoid cells is tied to lymphocyte proliferation. J Biol Chem. 1989;264:8451-8454.

136. Conteas CN, Mutchnick MG, Palmer KC, et al. Cellular levels of thymosin immunoreactive peptides are linked to proliferative events: evidence for a nuclear site of action. Proc Natl Acad Sci U S A. 1990;87:3269-3273

137. WELCH RA, LEE HH, SOKOL RJ, MUTCHNICK MG. Amniotic fluid thymosin α1 levels increase during gestation. American journal of reproductive immunology and microbiology. 1988 Jul;17(3):96-7.

138. Grottesi A, Sette M, Palamara AT, Rotilio G, Garaci E, Paci M. The conformation of peptide thymosin α1 in solution and in a membrane-like environment by circular dichroism and NMR spectroscopy. a possible model for its interaction with the lymphocyte membrane. Peptides. 1998 Jan 1;19(10):1731-8.

139. Di Francesco P, Pica F, Gaziano R, Favalli C, Garaci E. In vivo recovery of natural killer cell activity by the association of thymosin Î±1 and cytokines during cocaine administration.

140. Favalli C, Mastino A, Jezzi T, Grelli S, Goldstein AL, Garaci E. Synergistic effect of thymosin α1 and αβ-interferon on NK activity in tumor-bearing mice. International journal of immunopharmacology. 1989 Jan 1;11(5):443-50.

141. Favalli C, Jezzi T, Mastino A, Rinaldi-Garaci C, Riccardi C, Garaci E. Modulation of natural killer activity by thymosin alpha 1 and interferon. Cancer Immunology, Immunotherapy. 1985 Dec 1;20(3):189-92.

142. Mastino A, Favalli C, Grelli S, Innocenti F, Garaci E. Thymosin α 1 potentiates interleukin 2-induced cytotoxic activity in mice. Cellular immunology. 1991 Mar 1;133(1):196-205.

143. Garaci E, Rocchi G, Perroni L, D'Agostini C, Soscia F, Grelli S, Mastino A, Favalli C. Combination treatment with zidovudine, thymosin α 1 and interferon-α in human immunodeficiency virus infectionand interferon-α in human immunodeficiency virus infection. International Journal of Clinical and Laboratory Research. 1994 Jan 1;24(1):23-8.

144. Kawarabayashi N, Seki S, Hatsuse K, Ohkawa T, Koike Y, Aihara T, Habu Y, Nakagawa R, Ami K, Hiraide H, Mochizuki H. Decrease of CD56+ T cells and natural killer cells in cirrhotic livers with hepatitis C may be involved in their susceptibility to hepatocellular carcinoma. Hepatology. 2000 Nov 1;32(5):962-9.

145. Hsia J, Sarin N, Oliver JH, Goldstein AL. Aspirin and thymosin increase interleukin-2 and interferon-γ production by human peripheral blood lymphocytes. Immunopharmacology. 1989 May 1;17(3):167-73.

146. Koziel MJ. Cytokines in viral hepatitis. InSeminars in liver disease 1999 (Vol. 19, No. 02, pp. 157-169). © 1999 by Thieme Medical Publishers, Inc..

147. Foschi FG, Gramenzi A, Castelli E, Cursaro C, Pagani S, Margotti M, D'Errico A, Andreone P, Stefanini GF, Bernardi M. Soluble CD30 serum level in HCV-positive chronic active hepatitis: a surrogate marker of disease activity? Cytokine. 2000 Jun 1;12(6):815-8.

148. Loggi E, Gramenzi A, Margotti M, Cursaro C, Galli S, Vitale G, Grandini E, Scuteri A, Vukotic R, Andreone P, Bernardi M. In vitro effect of thymosin-alpha1 and interferon-alpha on Th1 and Th2 cytokine synthesis in patients with eAg-negative chronic hepatitis B. Journal of viral hepatitis. 2008 Jun;15(6):442-8.

149. Romani L, Bistoni F, Gaziano R, Bozza S, Montagnoli C, Perruccio K, Pitzurra L, Bellocchio S, Velardi A, Rasi G, Di Francesco P. Thymosin α 1 activates dendritic cells for antifungal Th1 resistance through Toll-like receptor signaling. Blood. 2004 Jun 1;103(11):4232-9.

150. Giuliani C, Napolitano G, Mastino A, Di Vincenzo S, D'Agostini C, Grelli S, Bucci I, Singer DS, Kohn LD, Monaco F, Garaci E. Thymosin-α1 regulates MHC class I expression in FRTL-5 cells at transcriptional level. European journal of immunology. 2000 Mar;30(3):778-86.

151. Sinibaldi Vallebona P, Pierimarchi P, Moroni G, Serafino A, Fuggetta MP, Tuthill C, Rasi G. Thymalfasin up-regulates tumor antigen expression in colorectal cancer cells. Tumor Biol. 2002;23:45.

152. Garaci E, Pica F, Serafino A, Balestrieri E, Matteucci C, Moroni G, Sorrentino R, Zonfrillo M, Pierimarchi P, Sinibaldi-Vallebona P. Thymosin α1 and cancer: action on immune effector and tumor target cells. Annals of the New York Academy of Sciences. 2012 Oct 1;1269(1):26-33.

153. Chang CC, Ogino T, Mullins DW, Oliver JL, Yamshchikov GV, Bandoh N, Slingluff CL, Ferrone S. Defective human leukocyte antigen class I-associated antigen presentation caused by a novel β2-microglobulin loss-of-function in melanoma cells. Journal of Biological Chemistry. 2006 Jul 7;281(27):18763-73.

154. Garaci E, Favalli C, Pica F, SINIBALDI VALLEBONA PA, TERESA PALAMARA AN, Matteucci C, Pierimarchi P, Serafino A, Mastino A, Bistoni F, Romani L. Thymosin alpha 1: from bench to bedside. Annals of the New York Academy of Sciences. 2007 Sep;1112(1):225-34.

155. Romani L, Bistoni F, Montagnoli C, Gaziano R, Bozza S, Bonifazi P, Zelante T, Moretti S, Rasi G, Garaci E, Puccetti P. Thymosin α1: an endogenous regulator of inflammation, immunity, and tolerance. Annals of the New York Academy of Sciences. 2007 Sep;1112(1):326-38.

156. Wara DW, Ammann AJ. Thymosin treatment of children with primary immunodeficiency disease. InTransplantation Proceedings 1978 Mar 1 (Vol. 10, No. 1, pp. 203-209).

157. Wara DW, Goldstein AL, Doyle NE, Ammann AJ. Thymosin activity in patients with cellular immunodeficiency. New England Journal of Medicine. 1975 Jan 9;292(2):70-4.

158. Schulof RS, Lloyd MJ, Cleary PA, Palaszynski SR, Mai DA, Cox Jr JW, Alabaster O, Goldstein AL. A randomized trial to evaluate the immunorestorative properties of synthetic thymosin-α1 in patients with lung cancer. Journal of Immunotherapy. 1985 Apr 1;4(2):147-58.

159. Yumin L, Hao C, Xun L, Wence Z, Minyan H, Chiriva-Internati M, Wachtel MS, Frezza EE. A new immunomodulatory therapy for severe sepsis: ulinastatin plus thymosin α 1. Journal of Intensive Care Medicine. 2009 Jan;24(1):47-53.

160. Tuthill CW, King RS. Thymosin apha 1–A peptide immune modulator with a broad range of clinical applications. Clin Exp Pharmacol. 2013;3(4):133.

161. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of immunological methods. 1983 Dec 16;65(1-2):55-63.

162. Sun J, Zhang X, Broderick M, Fein H. Measurement of nitric oxide production in biological systems by using Griess reaction assay. Sensors. 2003 Aug 22;3(8):276-84.

163. Brand DD, Latham KA, Rosloniec EF. Collagen-induced arthritis. Nature protocols. 2007 May 1;2(5):1269-75.

164. Smith DM, Jones MR, Patel S, et al. Comparative anti-inflammatory effects of pharmacologic agents in collagen-induced arthritis in rats. *J Pharmacol Exp Ther*. 2020;372(3):257-267.

165. Gao S, Chen Y, Tan D, Guo Q, Liu Y, Su R. Immunomodulatory and antioxidant activities of polysaccharides from Ganoderma lucidum on RAW 264.7 cells. *Int J Biol Macromol.* 2021;166:1596–1602. doi:10.1016/j.ijbiomac. 2020.11.023

166. Sun H, Zhu X, Zhang W, Lu W. Anti-inflammatory effects of Ficus carica L. extracts on lipopolysaccharide-stimulated RAW 264.7 macrophages. *J Ethnopharmacol*. 2018; 225: 152–158. doi:10.1016/j.jep.2018.06.004.

167. Park H, Kim H, Lee S. Cytotoxicity assessment using RAW 264.7 cell line in an in vitro model of drug development. *Biotechnol Bioproc Eng*. 2020; 25(4): 611–618. doi:10.1007/s12257-019-0241-4.

168. Kim YJ, Kim EH, Ham SK. ROS and NO production assays for RAW 264.7 macrophages in response to oxidative stress: A review of methodologies. *Free Radic Biol Med*. 2019; 132: 48–56. doi:10.1016/j.freeradbiomed.2018.12.011.

169. Hussain A, Ali R, Nafees S, et al. Toxicity profiling of nanoparticles: emerging in vitro models for preclinical testing. *Biomed Pharmacother*. 2021; 139: 111634. doi:10.1016/j.biopha.2021.111634.

170. Egan CG, Lockhart JC, Ferrell WR. Pathophysiology of vascular dysfunction in a rat model of chronic joint inflammation. The Journal of Physiology. 2004 Jun;557(2):635-43.

171. Granado M, Priego T, Martín AI, Villanúa MA, López-Calderón A. Anti-inflammatory effect of the ghrelin agonist growth hormone-releasing peptide-2 (GHRP-2) in arthritic rats. American Journal of Physiology-Endocrinology and Metabolism. 2005 Mar;288(3):E486-92.

172. Saha S, Ghosh S, Sinha M, Mukherjee S. Anti-inflammatory effects of herbal compounds on paw volume in arthritis-induced rats. *J Inflamm Res.* 2019;12:71–78. doi:10.2147/JIR.S183938.

173. Lee SJ, Lee HJ, Kim YJ. Paw swelling and inflammation in arthritis: comparative results across treatment models. *BMC Musculoskelet Disord.* 2020;21(1):85. doi:10.1186/s12891-020-3104-y.

174. Patel H, Joshi R, Sharma N. Dose-dependent reduction of inflammation by novel therapeutic compounds in arthritis models. *Inflammopharmacology*. 2021;29(3):651–663. doi:10.1007/s10787-021-00778-6.

175. Zhang Q, Wang S, Zhu Q. Anti-inflammatory effects of plant-based compounds in rheumatoid arthritis models: focus on paw swelling reduction. *Int J Rheum Dis.* 2018;21(4):789–798. doi:10.1111/1756-185X.13274.

176. Rasool M, Sabina EP, Lavanya B. Anti-inflammatory effect of Spirulina fusiformis on adjuvant-induced arthritis in mice. Biological and Pharmaceutical Bulletin. 2006;29(12):2483-7.

177. Curtis JR, Beukelman T, Onofrei A, Cassell S, Greenberg JD, Kavanaugh A, Reed G, Strand V, Kremer JM. Elevated liver enzyme tests among patients with rheumatoid arthritis or psoriatic arthritis treated with methotrexate and/or leflunomide. Annals of the rheumatic diseases. 2010 Jan 1;69(01):43-7

178. Niino-Nanke Y, Akama H, Hara M, Kashiwazaki S. Alkaline phosphatase (ALP) activity in rheumatoid arthritis (RA): its clinical significance and synthesis of ALP in RA synovium. Ryumachi.[Rheumatism]. 1998 Aug 1;38(4):581-8.

179. Rothwell RS, Davis P. Relationship between serum ferritin, anemia, and disease activity in acute and chronic rheumatoid arthritis. Rheumatology International. 1981 Jun;1:65-7.

180. Hussein R, Aboukhamis I. The association of serum RANKL levels with disease activity and hematological parameters in Syrian patients with rheumatoid arthritis. Biochemistry and Biophysics Reports. 2022 Dec 1;32:101373.

181. Tanaka S, Tanaka Y. RANKL as a therapeutic target of rheumatoid arthritis. Journal of bone and mineral metabolism. 2021 Jan; 39:106-12

182. Monteagudo L, Gravely A, Valen P, Ewart D. The Clinical Characteristics of Patients with Inflammatory Arthritis and a Persistently Low Alkaline Phosphatase Level in a Veteran Affairs Rheumatology Clinic. InARTHRITIS & RHEUMATOLOGY 2019 Oct 1 (Vol. 71). 111 RIVER ST, HOBOKEN 07030-5774, NJ USA: WILEY

183. Skoumal M, Haberhauer G, Kolarz G, Hawa G, Woloszczuk W, Klingler A, Varga F, Klaushofer K. The imbalance between osteoprotegerin and cathepsin K in the serum of patients with longstanding rheumatoid arthritis. Rheumatology international. 2008 May;28:637-41.

184. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ: Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. Cell 1998, 93:165-17.

185. Bucay N, Sarosi I, Dunstan CR, Morony S, Tarpley J, Capparelli C, Scully S, Tan HL, Xu W, Lacey DL, Boyle WJ. Osteoprotegerin-deficient mice develop early onset osteoporosis and arterial calcification. Genes & development. 1998 May 1;12(9):1260-8.

186. Gevers G, Devos P, De Roo M, Dequeker J. Increased levels of osteocalcin (serum bone Gla-protein) in rheumatoid arthritis. British journal of rheumatology. 1986 Aug 1;25(3):260-2.

187. Kishimoto Y, Fukumoto S, Nishihara S, Mizumura H, Hirai K, Teshima R. Gene expression relevant to osteoclastogenesis in the synovium and bone marrow of mature rats with collagen-induced arthritis. Rheumatology. 2004 Dec 1;43(12):1496-503

188. Udden J, Björntorp P, Arner P, Barkeling B, Meurling L, Rössner S. Effects of glucocorticoids on leptin levels and eating behaviour in women. Journal of internal medicine. 2003 Feb;253(2):225-31.

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Recent Developments in the Management of Rheumatoid Arthritis

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Abstract. Rheumatoid arthritis (RA) is a chronic inflammatory condition that affects 1% of adult population. The characteristic symptoms of RA, such as joint pain and stiffness, typically get worse in the morning and are strongly related with severe co-morbidities. The range of pro- and anti-inflammatory cytokines has rapidly increased, and the discovery of new members has shown that they play varying degrees of roles in causing chronic immune cell related inflammatory conditions such as RA. It is crucial to give novel substances to patients who do not respond to currently available strategies, such as glucocorticoids, T-cell targeted therapies, TNF-alpha inhibitors, etc., in order to develop new targeted therapies. This review paper's goal is to give an insight into of the role of newly discovered cytokines- the diseases caused by them and their potential in the therapy in RA. A thorough literature review was done to find articles related to any cytokine the study covered. As per some studies, there is sufficient evidence from RA patients and animal models to proceed with medication discovery, whereas in others conflicting analyses and insufficient knowledge call for more research.

Keywords: inflammatory; inhibitors; strategies.

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic, inflammatory autoimmune disease that primarily affects the aged women more frequently than males. As per a survey, there was geographical variance in the prevalence rate in 2002, which varied from 0.5 to 1 percent of the population [1]. The primary site of RA damage is the synovial joint lining, which can result in progressive disability, early death, and socioeconomic burden. The clinical symptoms of symmetrical joint involvement include arthralgia, edema, redness, and possibly a restriction in range of motion. As the ideal therapeutic window is thought to be the first 12 weeks after the onset of initial symptoms, the first point of diagnosis is considered as the most important for improvement index leading to less radiological progression, decreased joint damage, no functional impairment, and free remission of disease-modifying antirheumatic drugs (DMARD) as well as cost effectiveness [2,3,4].

However, early detection is still challenging because it heavily relies on clinical information obtained from the patient. The causes of delayed DMARD treatment in RA patients mostly depend on the patient and the treating physician, although the contributing factors for late diagnosis range dramatically between countries with various healthcare systems. In poorly controlled cases, there is a chance of developing extra-articular signs including inflammation of the cornea, pulmonary granulomas, pericarditis/pleuritis, small vessel vasculitis, and other nonspecific extra-articular symptoms. Although there is presently no cure for RA, the treatment strategy aims to accelerate diagnosis and attain low disease activity level as soon as possible (LDAS). The Disease Activity Score using 28 joints (DAS-28), Simplified Disease Activity Assessment Index (SDAI), and Clinical Disease Assessment Index (CDAI) are three composite ratings that assess the disease's activity [5]. To completely suppress disease activity, rheumatologists must track disease activity reliably and regularly and adjust the therapy plan as necessary (clinical remission). NSAIDs and corticosteroids, when used universally, have been demonstrated to be effective in

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reducing pain and stiffness, although they may not slow the disease's course. The efficacy of DMARDs has gained immense popularity over the last 20 years as they can effectively alleviate disease activity and significantly minimize the deformity of the joints [6].

The therapeutic intervention includes conventional medicines, biological DMARDs, and recently discovered potential small molecules. In recent years, a small number of DMARDs of biological origin have been developed such as- JAK inhibitors, TNF inhibitors, anti-CD20 antibodies, IL-6 receptor antibodies and RANKL antibodies. This review aims to summarize the most recent findings on the pathophysiology of RA and the possibility for novel pharmacological strategies to enhance RA treatment regimens in the future.

PATHOGENESIS OF RA

Numerous immune modulators (cytokines and effector cells), as well as signaling pathways, are involved in the pathophysiology of RA [7]. The joint damage, which begins at the synovial membrane and covers the majority of the IA structures, is caused by the complex interactions between immune modulators' [7]. The factors causing synovitis are angiogenesis and mononuclear cell infiltration or local activation, or both (including T cells, B cells, plasma cells, dendritic cells, macrophages, and mast cells) [7]. The cytokine network is a complicated area in rheumatoid arthritis (RA), with multiple cytokines demonstrating pleiotropic activities and several different targets. The pro-inflammatory and the anti-inflammatory cytokines can be divided into two types to better understand the network. It is regarded as an important therapeutic objective to control the equilibrium between these two classes.

The two main pro-inflammatory cytokines in RA are IL-1 and TNF. Controlling these cytokines is crucial in the treatment of RA illness. Early clinical trial data demonstrated efficacy but also suggested that not all patients' arthritis was entirely controlled by blocking these cytokines.

We will be able to better understand the aetiology of chronic arthritis as a result of recent discoveries of novel cytokines in arthritis pathology, including as IL-17, IL-18, and RANK ligand (RANKL). These discoveries may also result in the improvement of present treatments. Pleiotropic cytokines IL-4 and IL-10 are well-known as prospective RA control modulators.

ROLES OF CYTOKINES IN RA

For bone homeostasis in RA, such as TNF, IL-1, IL-6 and IL-17, the inflammatory environment plays an orchestrating role [8,9]. Proinflammatory cytokines affect the osteoclasts, osteoblasts and are linked to RA patients' higher disease activity [10]. TNF has a major role in the development of inflammatory RA [8]. Additionally, its antagonistic impact in the differentiation of osteoclast precursor cells and its expansion, while preventing differentiation of the osteoblast. Through the stimulation of NF-B and TNF-induced osteoclast-associated receptor modulation, TNF-driven activation of NFATc1 signaling also promotes osteoclast maturation [11,12,13]. In addition, TNF inhibits osteoblast development by lowering Runx2 and osteocalcin expression in mature osteoblasts [14,15]. Such behavior ultimately promotes loss of bone mass. TNF and IL-1 support differentiation of the osteoclast and thereby causing disease progression. Increases in IL-1 and IL-1 receptors are caused by TNF, and IL-1 then makes it easier for TNF to induce osteoclast differentiation [16]. Similar studies have been reported [16]. IL-1 also reduces pre-osteoclastic and osteoclastic apoptosis, enhancing survival and promoting bone resorption. Alkaline phosphatase and Type I collagen levels fall as a result of IL-1 osteoblast therapy, which also affects calcium levels and the formation of mineralized nodules. As a result, osteoblasts' capacity to build bone is dramatically diminished [17]. IL-1and TNF induce an increase in the levels of IL-6 mRNA in osteoblasts [18].

Inhibiting IL-6 production in TNF transgenic mice resulted in less number of osteoclasts at the joints and decreased joint damage in the murine collagen-induced arthritis (CIA) model [19]. Patients with RA who are treated with TNF or IL-6 inhibition frequently experience a delay in the onset of joint deterioration [20,21]. The development of Th17 cells and subsequent production of IL-17 are both significantly influenced by IL-6 [22]. When synovial fibroblasts and osteoblasts secrete RANKL and prostaglandin E2, the differentiation and function of the osteoclast are improved [23]. IL-17 suppression may increase inflammatory arthritis in murine CIA and antigen-induced arthritis models as well as the rat adjuvant arthritis model [24,25].

Minicircle DNA constructs used in mice to overexpress IL-17A led to an increase in osteoclast precursors and the stimulation of bone resorption indicators [26]. In RA synovium, Th-17 cells are common, and the ratio of these cells to TREG's is significantly higher [27]. IL-17A suppression was demonstrated to have no effect on the percentage of patients who responded to the ACR20 in a clinical RA trial, despite encouraging preclinical evidence on the function of IL-17 in maintaining bone mineral balance in rheumatoid arthritis [28].

The rise in Th17 cell development following IL-6 therapy is due to IL-34 which is a proinflammatory cytokine. TNF and IL-1 can regulate the expression of IL-34 mRNA in human synovial fibroblasts, which increases osteoclastogenesis. TNF therapy reduces FLS, but RA patients had increased amounts of IL-34 in their serum, synovial fluid, and synovial tissues. Increased serum levels of IL-34 are also a sign of bone degradation [29]. IL-34 may potentially show to be a new biological target as well as a biomarker in RA [30]. IL-3, a cytokine released by the Th cells, stimulates the proliferation, development, and survival of pluripotent hematopoietic stem cells [31]. Human osteoclasts require RANKL for differentiation from blood monocytes and bone marrow cells. This is inhibited by IL-3 [32]. TNF-a-induced pathological bone resorption is also prevented by IL-3 in the presence of other proinflammatory cytokines like "IL-1a, TGF-b1, TGF-b3, IL-6, and PGE2", and pre-treatment with IL-3 guards against disease progression [33]. Osteoclastogenesis is elevated despite deficient Treg cells in RA patients and CIA model [34,35,36,37]. Reduced levels of circulating Treg cells are associated with increased bone resorption and osteoclast development in people [38]. Treg cells also significantly suppress osteoclast development, bone resorption, and CIA expansion [37,39]. Both in vitro and in vivo settings, IL-3 is crucial in controlling the development of Treg cells. Numerous studies have demonstrated that IL-3 indirectly increases the proportion of Foxp3+ Treg cells by causing non-Treg cells to produce IL-2. In CIA mice, IL-3 therapy reduces the likelihood of developing arthritis and prevents the loss of "Foxp3+ Treg" cells from lymph node, spleen, and thymus.

TH1 AND TH2 IMMUNE RESPONSE IN RA

Helper T cells are responsible for inflammation in RA. According to the cytokine microenvironment, CD4 T cells split into subpopulations, such as "Th1, Th2, Th17, and T regulatory cells", and play a significant role in the pathogenesis of RA [40]. Th1/Th17/Treg subsets have the potential to exert a significant influence on the intricate web of interactions that regulate the onset and course of RA. More importantly, they have the capacity to show this influence at different stages of the illness and to variable degrees of intensity. Interleukin-2 and/or interferon-gamma are defensive according to recent research [35,36,37,38,39,41]. In light of the contentious findings [42], it is still unclear exactly how Th1 cell responses affect the systemic distribution of Th17/Treg cells in RA.

CURRENT TREATMENTS FOR RA AND THE ISSUES THEY CAUSE

Recent advancements in RA pharmaceutical therapy have allowed many patients to achieve remission improving their quality of life and reducing late consequences from RA. Early intervention in disease management helps in reducing damage along with continuous care. However, a significant issue with traditional RA therapy is their high cost, which is mostly due to disease-modifying anti-rheumatic medications "DMARDs" and "selective synthetic DMARDs". Clinicians should take into account treatment costs when deciding on a course of action [43]. However, a number of problems arise, including the likelihood of an elevated risk of cardiovascular disease and the occurrence of disease flare-ups [44,45]. The discovery and acceptance of biological DMARD-like generic substances has been the subject of another recent discussion. Some studies have indicated that biosimilars are as effective as the originals. They represent a significant alternative to cutting costs, expanding treatment options, and reducing access disparities between wealthy and developing countries to care [46]. Additionally, Smolen et al. [47] noted that therapy failure is a common occurrence in RA patients. Therefore, even after all therapeutic options have been explored, it is still critical to find novel treatments and understand the mechanisms underlying therapy toxicity and failure in many patients who don't experience remission. One of the main issues with treatment is adverse effects and dangers, particularly in individuals with concurrent comorbidities. In addition to the high cost, side effects impede patient adherence to the medications [43].

(a) Non-steroidal anti-inflammatory drugs (NSAIDs)

The majority of patients use NSAIDs for self-medication prior to seeing a doctor and getting a diagnosis. NSAIDs are adjuvant drugs that can be used to manage the symptoms of RA, allow quick analgesia, and reduce inflammation. Continuous use must be avoided because it is linked to a wide range of side effects such as nausea, abdominal pain, liver damage [48,49], increased risk of heart attacks [50], and gastrointestinal problems like ulcers and blending [51]. These effects can vary depending on the kind of "non-steroidal anti-inflammatory drugs (NSAIDs)", and they may be controlled by other pharmaceuticals, such as antacids & inhibitors of proton pumps, or dietary adjustments [52,48].

(b) Glucocorticoids

Glucocorticoids are better as compared to NSAIDs in conjunction with DMARDs and in severe systemic RA, and they are routinely advised. According to Strehl et al. [53], a number of negative consequences might happen. The severity of the symptoms frequently depends on the dosage and duration of treatment and the individual patient [55,56] are documented in the literature. The medications may frequently lead to an increase in the frequency of cardiovascular events, however there are insufficient results in the literature [57]. Careful patient monitoring are required throughout the use of glucocorticoids [58], and patients with comorbidities such as diabetes, hypertension, and dyslipidaemia [53], need to be given special consideration.

(c) TNF-alpha inhibitors

TNF-alpha is created and triggers an immunological response in T-lymphocytes, monocytes, and activated macrophages. Increased TNF-alpha expression promotes bone deterioration and eventually speeds up the disease's course. As a result, various TNF-alpha inhibitors have been developed as medications for the treatment of RA [62]. The first chimeric monoclonal antibody, Infliximab, has a human antibody backbone and a mouse idiotype. By interacting with all forms of TN alpha, this antibody can block the effects of TNF-biological alpha [63]. It has a long-term safety profile and is administered as an intravenous infusion. In individuals receiving infliximab, significant drops in cytokines like “IL-8, IL-6, MCP-1, and IL-1” have been observed. Infliximab exhibits substantial adverse effects, including malignancies, lymphoma, and the reactivation of hepatitis B or T, even after the safety profile [64]. Adalimumab, a totally humanised antibody and another illustration of a TNF-alpha inhibitor that is administered subcutaneously, is one more option. When administered, it has a lesser potential for toxicity and has effects similar to those of MTX. Commonly harmful outcomes include cardiac arrest, cutaneous responses, and latent reactions [65].

(d) T-cell targeted therapies

Numerous T-cells enter the synovium and a small number of them move into the synovium where they increase the expression of proinflammatory cytokines which damage the cartilage and cause bone degradation. Several T-cell-specific therapies are proposed which inhibit this pathway e.g., Abatacept, a T-cell activation modulator [66], it blocks signalling between CD 80 and CD 86. Both injectable and infusion versions are offered. The most common adverse effects include a sore throat, headache, cold, infection, and nausea [67].

(e) Targeted treatments for IL-6

IL-6 stimulates Pannal development and ultimately heightens bone resorption in RA by increasing VEGF expression. The “human-based antibody” tocilizumab targets IL-6 in particular. It has a lower immunogenicity and can be given as intravenously and subcutaneously [68]. Sirukumab, Clazakizumab, Alacizumab, and Sarilumab are other similar examples. They have common side effects such as headaches, hypertension and respiratory tract infections. However, to establish the therapeutic effectiveness of these drugs against RA, additional clinical investigations are also required [69].

(f) Targeted treatments for IL-1

Once-daily injection of Anakinra, which functions as an IL-1 receptor antagonist has been used for treatment. The activity of IL-1a and IL-1b is inhibited by directly inhibiting the action of “IL-1 receptors”. These formulations have the immediate side effects of causing itchy rashes, asthma, gastrointestinal tract infections and respiratory tract infections [70, 71].

CONCLUSION

Customized cytokine-targeted medicines (such anti-TNF, anti-IL-1, and anti-IL-6) have previously been given the go-ahead for use in clinical settings. This type of therapy is now evolving extremely quickly. Furthermore, cytokines are recognized as potentially potent RA indicators, and their functions are anticipated to expand in the coming years. A thorough understanding of the cytokine balance in RA will improve both the diagnostic and therapeutic processes

REFERENCES

[1] A. J. Silman and J. E. Pearson, Arthritis Res **4**, 265–272 (2002).

[2] M. P. V. D. Linden, L. Cessie, S. Raza, K. V. D. Woude, D. Knevel, R. Huizinga, T. W. V. D. H.-V. Mil, and A. H, Arthritis & Rheumatism **62**, 3537–3583 (2010).

[3] C. S. Moura, M. Abrahamowicz, M. E. Beauchamp, D. Lacaille, Y. Wang, G. Boire, P. R. Fortin, L. Bessette, C. Bombardier, J. Widdifield, and J. G. Hanly, 2015.

[4] S. K. Cho, D. Kim, S. Won, J. Lee, C. B. Choi, J. Y. Choe, S. J. Hong, J. B. Jun, T. H. Kim, E. Koh, and H. S. Lee, 2019.

[5] K. Raza, R. Stack, K. Kumar, A. Filer, J. Detert, H. Bastian, G. R. Burmester, P. Sidiropoulos, E. Kteniadaki, A. Repa, and T. Saxne, 2011.

[6] F. Ometto, C. Botsios, B. Raffeiner, P. Sfriso, L. Bernardi, S. Todesco, A. Doria, and L. Punzi, Autoimmunity reviews **9**, 161–165 (2010).

[7] J. S. Smolen and G. Steiner, 2003.

[8] E. M. Gravallese and N. C. Walsh, Nature Reviews Rheumatology **7**, 626–626 (2011).

[9] T. N. Crotti, M. D. Smith, H. Weedon, M. J. Ahern, D. M. Findlay, M. Kraan, P. P. Tak, and D. R. Haynes, 2002.

[10] A. Yarilina, K. Xu, J. Chen, and L. B. Ivashkiv, Proceedings of the National Academy of Sciences **108**, 1573–1581 (2011).

[11] N. Kim, M. Takami, J. Rho, R. Josien, and Y. Choi, Journal of Experimental Medicine **195**, 201–210 (2002).

[12] S. Herman, R. B. Müller, G. Krönke, J. Zwerina, K. Redlich, A. J. Hueber, H. Gelse, E. Neumann, U. Müller- Ladner, and G. Schett, Arthritis & Rheumatism: Official Journal of the American College of Rheumatology **58**, 3041–50 (2008).

[13] J. H. Shim, Z. Stavre, and E. M. Gravallese, 2018.

[14] L. Gilbert, X. He, P. Farmer, J. Rubin, H. Drissi, A. J. V. Wijnen, J. B. Lian, G. S. Stein, and M. S. Nanes, Journal of Biological Chemistry **277**, 2695–701 (2002).

[15] S. Wei, H. Kitaura, P. Zhou, F. P. Ross, and S. L. Teitelbaum, 2005.

[16] P. Stashenko, F. E. Dewhirst, M. L. Rooney, L. A. Desjardins, and J. D. Heeley, Journal of Bone and Mineral Research **2**, 559–65 (1987).

[17] Y. O. Ishimi, C. H. Miyaura, C. H. Jin, T. A. Akatsu, E. T. Abe, Y. U. Nakamura, A. K. Yamaguchi, S. H. Yoshiki, T. A. Matsuda, and T. O. Hirano, The Journal of Immunology **145**, 3297–303 (1990).

[18] N. Takagi, M. Mihara, Y. Moriya, N. Nishimoto, K. Yoshizaki, T. Kishimoto, Y. Takeda, and Y. Ohsugi, 1998.

[19] J. S. Smolen, J. C. Avila, and D. Aletaha, 2012.

[20] R. Maini, E. W. S. Clair, F. Breedveld, D. Furst, J. Kalden, M. Weisman, J. Smolen, P. Emery, G. Harriman, M. Feldmann, and P. Lipsky, 1999.

[21] E. Bettelli, Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner, and V. K. Kuchroo, Nature **441**, 235–235 (2006).

[22] F. Zhang, H. Tanaka, T. Kawato, S. Kitami, K. Nakai, M. Motohashi, N. Suzuki, C. L. Wang, K. Ochiai, K. Isokawa, and M. Maeno, Biochimie **93**, 296–305 (2011).

[23] K. A. Bush, K. M. Farmer, J. S. Walker, and B. W. Kirkham, Arthritis & Rheumatism **46**, 802–807 (2002).

[24] E. Lubberts, M. I. Koenders, B. Oppers-Walgreen, L. V. D. Bersselaar, C. J. C.-D. Roo, L. A. Joosten, V. Den, and W. B. Berg, Arthritis & Rheumatism: Official Journal of the American College of Rheumatology **50**, 650–659 (2004).

[25] I. E. Adamopoulos, E. Suzuki, C. C. Chao, D. Gorman, S. Adda, E. Maverakis, K. Zarbalis, R. Geissler, A. Asio, W. M. Blumenschein, and T. Mcclanahan, 2015.

[26] M. Noack and P. Miossec, Autoimmunity reviews **13**, 668–77 (2014).

[27] M. C. Genovese, P. Durez, H. B. Richards, J. Supronik, E. Dokoupilova, V. Mazurov, J. A. Aelion, S. H. Lee, C. E. Codding, H. Kellner, and T. Ikawa, 2013.

[28] S. J. Hwang, B. Choi, S. S. Kang, J. H. Chang, Y. G. Kim, Y. H. Chung, D. H. Sohn, M. W. So, C. K. Lee, W. H. Robinson, and E. J. Chang, 2012.

[29] M. Chemel, L. Goff, B. Brion, R. Cozic, C. Berreur, M. Amiaud, J. Bougras, G. Touchais, S. Blanchard, F. Heymann, M. F. Berthelot, and J. M, 2012.

[30] F. Zhang, R. Ding, P. Li, C. Ma, D. Song, X. Wang, T. Ma, and L. Bi, Int J Clin Exp Med **8**, 7809–7815

(2015).
[31] J. W. Schrader, *Interleukin-3*, edited by T. C. H. A. W. Thomson, , and M. T. Lotze (Academic Press, London, 2003), pp. 201–225.
[32] N. Gupta, A. P. Barhanpurkar, G. B. Tomar, R. K. Srivastava, S. Kour, S. T. Pote, G. C. Mishra, and M. R. Wani, The Journal of Immunology **185**, 2261–72 (2010).
[33] S. D. Yogesha, S. M. Khapli, R. K. Srivastava, L. S. Mangashetti, S. T. Pote, G. C. Mishra, and M. R. Wani, The Journal of Immunology **182**, 361–70 (2009).
[34] M. R. Ehrenstein, J. G. Evans, A. Singh, S. Moore, G. Warnes, D. A. Isenberg, and C. Mauri, Journal of Experimental Medicine 277–85 (0200).
[35] H. Kelchtermans, D. Klerck, B. Mitera, T. V. Balen, M. Bullens, D. Billiau, A. Leclercq, G. Matthys, and P, Arthritis Res Ther **7**, 402–402 (2005).
[36] S. Ochi, M. Shinohara, K. Sato, H. J. Gober, T. Koga, T. Kodama, T. Takai, N. Miyasaka, and H. Takayanagi, Proceedings of the National Academy of Sciences **3**, 11394–11403 (2007).
[37] H. Kelchtermans, L. Geboes, T. Mitera, D. Huskens, G. Leclercq, and P. Matthys, 2009.
[38] M. M. Zaiss, B. Frey, A. Hess, J. Zwerina, J. Luther, F. Nimmerjahn, K. Engelke, G. Kollias, T. Hünig, F. Schett, and J. P. David, The Journal of Immunology **184**, 7238–7284 (2010).
[39] M. A. Burchill, J. Yang, C. Vogtenhuber, B. R. Blazar, and M. A. Farrar, 2007.
[40] M. L. Toh and P. Miossec, 2007.
[41] R. Setoguchi, S. Hori, T. Takahashi, and S. Sakaguchi, Journal of Experimental Medicine **201**, 723–758 (2005).
[42] E. F. Rosloniec, K. Latham, and Y. B. Guedez, Arthritis Research & Therapy **4**, 333–333 (2002).
[43] J. S. Smolen, R. B. M. Landewé, J. W. J. Bijlsma, G. R. Burmester, M. Dougados, A. Kerschbaumer, I. B. Mcinnes, A. Sepriano, R. F. V. Vollenhoven, and M. Dewit, Ann. Rheum. Dis **2020**, 685–699.
[44] P. Sewerin, S. Vordenbaeumen, A. Hoyer, R. Brinks, C. Buchbender, F. Miese, C. Schleich, S. Klein, M. Schneider, and B. Ostendorf, BMC Musculoskelet. Disord **18**, 163–163 (2017).
[45] T. M. Kuijper, F. B. G. Lamers-Karnebeek, J. W. G. Jacobs, J. M. W. Hazes, and J. J. Luime, J. Rheumatol **42**, 2012–2022 (2015).
[46] D. Thomas, V. Strand, P. Cornes, J. Gonçalves, L. Gulácsi, J. Kay, T. K. Kvien, J. Smolen, Y. Tanaka, and G. R. Burmester, Ann. Rheum. Dis **75**, 974–982 (2016).
[47] J. S. Smolen, D. Aletaha, and I. B. Mcinnes, Lancet **388**, 2023–2038 (2016).
[48] I. Ferraz-Amaro, S. Machín, L. Carmona, I. González-Alvaro, and F. Díaz-González, Reumatol. Clin **5**, 252–258 (2009).
[49] L. J. Crofford, Arthritis Res. Ther **2**, 15–15 (2013).
[50] S. E. Nissen, N. D. Yeomans, D. H. Solomon, T. F. Lüscher, P. Libby, M. E. Husni, D. Y. Graham, J. S. Borer, L. M. Wisniewski, and K. E. Wolski, N. Engl. J. Med **375**, 2519–2529 (2016).
[51] J. M. Scheiman, J. Clin. Gastroenterol **50**, 5–10 (2016).
[52] C. K. S. Ong, P. Lirk, C. H. Tan, and R. A. Seymour, Clin. Med. Res **5**, 19–34 (2007).
[53] C. Strehl, J. W. J. Bijlsma, M. D. Wit, M. Boers, N. Caeyers, M. Cutolo, B. Dasgupta, W. G. Dixon, R. Gee- nen, and T. W. J. Huizinga, Ann. Rheum. Dis **75**, 952–957 (2016).
[54] R. S. Weinstein, N. Engl. J. Med **365**, 62–70 (2011).
[55] M. C. V. D. Goes, J. W. G. Jacobs, M. Boers, T. Andrews, M. A. M. Blom-Bakkers, F. Buttgereit, N. Caeyers, L. Cutolo, J. A. P. D. Silva, and L. Guillevin, Ann. Rheum. Dis **69**, 1913–1919 (2010).
[56] G. Ozen, S. Pedro, M. E. Holmqvist, M. Avery, F. Wolfe, and K. Michaud, Ann. Rheum. Dis **76**, 848–854 (2017).
[57] A. Ruyssen-Witrand, B. Fautrel, A. Saraux, X. L. Loët, and T. Pham, Jt. Bone Spine **78**, 23–30 (2011).
[58] D. Liu, A. Ahmet, L. Ward, P. Krishnamoorthy, E. D. Mandelcorn, R. Leigh, J. P. Brown, A. Cohen, and H. Kim, Allergy Asthma Clin. Immunol 30–30 (2013).
[59] R. Conway, C. Low, R. J. Coughlan, M. J. Donnell, and J. J. Carey, Arthritis Rheumatol **66**, 803–812 (2014).
[60] R. Conway, C. Low, R. J. Coughlan, M. J. Donnell, and J. J. Carey, Semin. Arthritis Rheum **45**, 156–162 (2015).
[61] J. Braun and R. Rau, Curr. Opin. Rheumatol **21**, 216–223 (2009).
[62] F. Wang, L. Sun, S. Wang, J. M. Davis, E. L. Matteson, M. H. Murad, F. Luo, and R. Vassallo, Mayo Clin. Proc **2020**, 1404–1419.
[63] M. Rinaudo-Gaujous, V. Blasco-Baque, P. Miossec, P. Gaudin, P. Farge, X. Roblin, T. Thomas, S. Paul,

and H. Marotte, J. Clin. Med **8**, 751–751 (2019).
[64] Z. Zrubka, L. Gulácsi, V. Brodszky, F. Rencz, R. Alten, Z. Szekanecz, and M. Péntek, Expert Rev. Pharmacoecon. Outcomes Res **19**, 537–549 (2019).
[65] S. Cohen, K. Tuckwell, T. R. Katsumoto, R. Zhao, J. Galanter, C. Lee, J. Rae, B. Toth, N. Ramamoorthi, and J. A. Hackney, Phase II Trial. Arthritis Rheumatol **72**, 1435–1446 (2020).
[66] G. Cagnotto, M. Willim, J. Å. Nilsson, M. Compagno, L. T. H. Jacobsson, S. Saevarsdottir, and C. Turesson, Arthritis Res. Ther **22**, 15–15 (2020).
[67] H. A. Blair and E. D. Deeks, 2017.
[68] L. J. Scott and Tocilizumab, 2017.
[69] F. Pandolfi, L. Franza, V. Carusi, S. Altamura, G. Andriollo, and E. Nucera, Int. J. Mol. Sci **2020**, 5238–5238.
[70] P. Ruscitti, F. Masedu, S. Alvaro, P. Airò, N. Battafarano, L. Cantarini, F. P. Cantatore, G. Carlino, V. Abrosca, and M. Frassi, PLoS Med 1002901–1002901 (2019).
[71] B. M. Köhler, J. Günther, D. Kaudewitz, and H. M. Lorenz, J. Clin. Med **8**, 938–938 (2019).

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Biochemical And Hematological Impacts Of Thymosin Alpha 1 On Bone Tissue In Animal Models

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Abstract

This study aimed to examine the effects of Thymosin Alpha 1 (Tα1) on the degree of arthritis and bone remodeling in animal models. The chronic autoimmune illness known as rheumatoid arthritis (RA) is marked by inflammation and joint damage, frequently with changes in bone metabolism. Tα1, an immune-modulatory peptide found naturally in the body, has demonstrated potential in reducing inflammatory reactions and accelerating tissue healing. The effects of Tα1 on arthritis progression and related bone alterations were evaluated in this study using a collagen-induced arthritis (CIA) model in wistar rats. Following treatment with Tα1 at three different doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), the animals were observed for signs and symptoms of arthritis, joint swelling. As indicated by a decrease in joint inflammation, our findings show that Tα1 therapy reduced the severity of arthritis. In arthritic joints, Tα1 therapy increased anti-inflammatory responses and inhibited the production of pro-inflammatory cytokines. Moreover, the injection of Tα1 resulted in an increase in osteoblast activity and a decrease in osteoclast-mediated bone resorption, hence enhancing bone quality and integrity. Tα1 may be a useful therapeutic agent for reducing the severity of arthritis and maintaining bone health in patients with RA, according to these data. Tα1's therapeutic potential in clinical settings will only be revealed through additional research into the molecular mechanisms behind its effects on arthritis and bone remodeling.

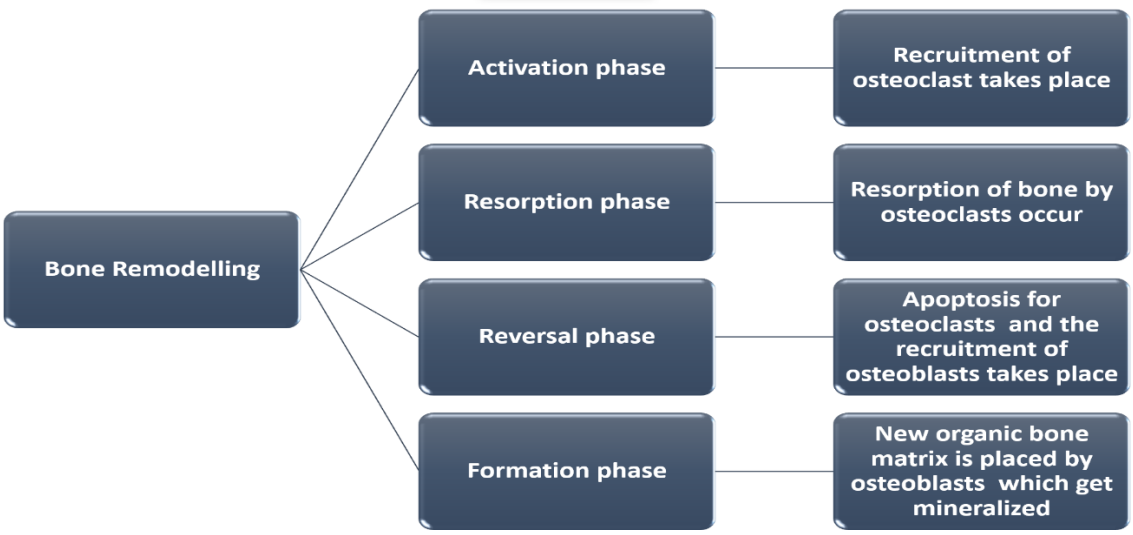
Keywords: CIA rats, rheumatoid arthritis, bone remodeling, inflammation, thymosin alpha-1

Introduction:

Muscles, bones, joints, tendons, ligaments, and other body parts can all be significantly impacted by musculoskeletal disorders, which include about 150 known illnesses and conditions. These ailments range from short-term ailments like sprains to long-term, incapacitating conditions like rheumatoid arthritis and osteoarthritis. In addition to causing discomfort and impeding mobility,

musculoskeletal issues can also impair a person's capacity for employment, social obligations, and mental health. Since 1990, lower back pain in particular has continuously been recognized as one of the major causes of disability worldwide, indicating the substantial impact these disorders have on societies all over the world. It is estimated that musculoskeletal disorders cause discomfort to 20% to 33% of the world's population (1). The age and diagnosis factors have a significant impact on this percentage. According to recent data from the USA, the prevalence of musculoskeletal illnesses in adults is on par with chronic respiratory and cardiovascular diseases (2). According to an analysis of data from the "World Health Organization's Study of Global Ageing and Adult Health (SAGE)", people with economically disadvantaged backgrounds are more likely to have arthritis than people in middle-class or wealthy countries (3).

Bone performs vital roles and is made up of cellular, vascular, and calcium compound structures: (a). It functions as a store for phosphate, magnesium, potassium, and bicarbonate and is essential for preserving calcium homeostasis. (b) Bone serves as a lever for muscle activity and gives soft tissues structural support. (c) It functions as our body's main location for hematopoiesis, the process that creates new blood cells (4). Bone needs to continually grow, repair, and break down (resorb) in order to stay healthy. All of these modifications to bones are collectively referred to as "remodeling". Remodeling is a result of osteoblast and osteoclast activity. There are four steps involved in bone remodeling (5):



A number of cytokines and hormones are essential for controlling bone remodeling. Bone loss, a defining feature of numerous skeletal illnesses such as osteoporosis and rheumatoid arthritis (RA), arises when the rate of new bone formation is not keeping up with the rate of bone destruction (6). Among the many systemic autoimmune diseases, RA stands out for its high frequency and enduring synovial inflammation, which deteriorates cartilage and joints. Research points to immune system dysfunctions, especially those involving pro-inflammatory reactions, as a factor in arthritis's chronicity. Th cells, in particular, are essential for both triggering and maintaining inflammation. Synthetic peptide thymosin alpha 1 reduces inflammation and increases the generation of different T cell subsets and natural killer cells, which in turn modifies immunological responses (7). Clinical research has investigated its potential impact on autoimmune illnesses including psoriatic arthritis, as well as diseases like cancer, severe sepsis, and hepatitis B and C (8). Researchers discovered that Ta1 may alter immune function and had conflicting effects on joint pain in individuals who had survived breast cancer (9). Significantly, patients with chronic inflammatory autoimmune illnesses have been found to have lower serum levels of Thymosin alpha 1, indicating the possible therapeutic value of this protein (10).

Experimental Model:

The Wistar strain albino male rats used in the experiment were 6-8 weeks old and were obtained from the Invivo Biosciences animal house facility located in Bengaluru, Karnataka 560091. They were allowed to acclimate to the animal house environment, maintained on a 12-hour light-dark cycle. Food and water were provided ad libitum. Eight rats per experimental group were divided into three categories: CIA control rats, normal control rats, and arthritic rats treated with 0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg of Thymosin alpha-1. The animal ethics committee at the Invivo Biosciences animal house facility in Bengaluru, Karnataka, approved all experimental methods in advance.

CIA induction and dosage schedule:

The experimental design of the study included five groups of animal models, each subjected to different treatments to assess the role of Thymosin-α 1 in bone health. Group I served as the control group and received a vehicle treatment administered intraperitoneally (I.P.). Group II, the arthritis group, was induced with arthritis through the administration of Type II collagen on day 0 and lipopolysaccharide (LPS) on day 3. Group III, designated as the arthritis + Thymosin-α 1 (1 mg/kg) group, received the same arthritis-inducing agents as Group II, but with the addition of Thymosin-α 1 at a dose of 1 mg/kg administered I.P. on the 1st, 3rd, and 5th days. Group IV, the arthritis + Thymosin-α 1 (0.5 mg/kg) group, followed a similar protocol, receiving Thymosin-α 1 at a reduced dose of 0.5 mg/kg on the same days. Lastly, Group V, the arthritis + Thymosin-α 1 (0.25 mg/kg) group, also received the arthritis-inducing agents along with Thymosin-α 1 at a dose of 0.25 mg/kg I.P. on the 1st, 3rd, and 5th days. This setup allowed for the evaluation of the effects of different doses of Thymosin-α 1 on arthritis-induced bone changes.

After the dosage was finished, blood was drawn using the retro-orbital method, which allowed the serum to be separated. The laboratory study included the measurement of hematological indicators like RBC count, Differential count, and platelet counts in addition to the assessment of serum biochemistry markers like Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), and ferritin.

Statistical analysis:

SEM was computed by averaging all of the data. Dunnett's multiple comparison tests were used to compare all of the treatment groups' parameters with those of the negative control group using a one-way ANOVA. A value of less than 0.05 was deemed statistically significant.

Result:

The rats, induced with arthritis, underwent a treatment regimen involving the administration of test substances on days 1, 3, and 5, with consistent timing of application. Throughout the entire study duration, meticulous daily monitoring was implemented to assess the overall health of all animals and to identify any discernible clinical changes. Swellings in the hind paw region were consistently observed in both the groups receiving treatment and the induced group. Upon the culmination of the 15-day study period, specifically on the 15th day, a thorough examination was conducted for all animals. This comprehensive assessment included an array of biochemical markers and haematological parameters. This approach ensured a comprehensive evaluation of the physiological responses and outcomes associated with the administered treatments and the induced arthritic conditions. An automated blood analyser was used to analyze the blood sample

for hematological parameters, and kit-based techniques were employed to complete all biochemical assays on the RoboniK semi-automated analyser.

Biochemical Parameters:

Serum ALT and AST:

On the 15th day of experimentation, serum ALT levels were examined, showing consistent levels across all groups, regardless of arthritis presence. The arthritic group exhibited ALT levels of 94.1±0.58 IU/ml, while normal rats showed levels of 94.1±0.80 IU/ml. Administration of different doses of Thymosin alpha-1 (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg) did not yield significant changes in ALT levels in arthritic rats. Specifically, ALT levels were 94.8±0.77 IU/ml at 0.25 mg/kg, 95.9±0.81 IU/ml at 0.5 mg/kg, and 95.4±0.84 IU/ml at 1 mg/kg. None of the treatment groups exhibited notable differences compared to the arthritis control group (refer to Figure- 1 and 2). Similarly, serum AST levels remained consistent across all groups, with no significant changes observed following Thymosin alpha-1 administration, underscoring its hepatic safety profile.

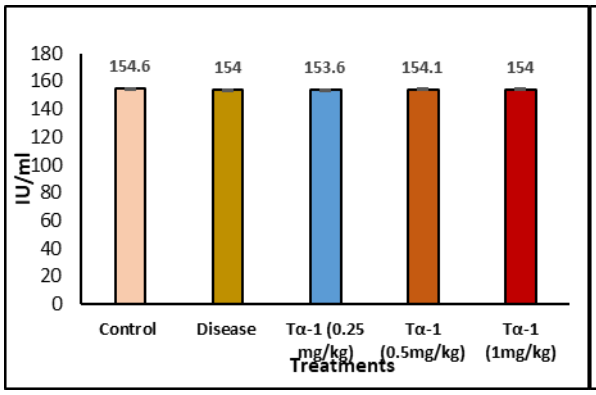


Fig. 1: Graphical representation of Serum ALT in all the five groups

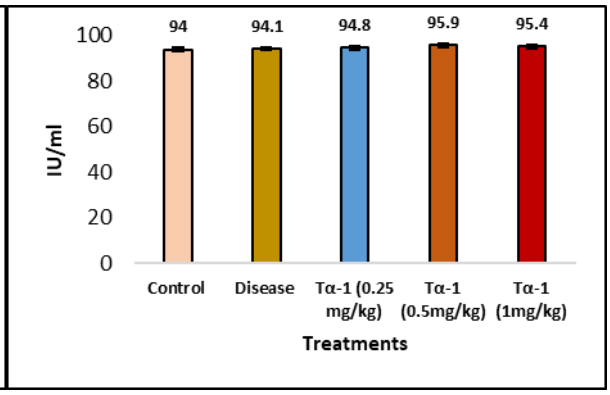


Fig. 2: Graphical representation of Serum levels AST levels in all the five groups

Serum Alkaline Phosphatase:

A significant rise in the serum levels of Alkaline Phosphatase was observed as arthritis progressed in arthritic control rats, with measurements on the 15th day reaching 211.4±0.95 U/L. Arthritic control rats exhibited a noteworthy difference in serum ALP levels compared to normal control rats, recording values at 112.6±0.41 U/L on the 15th day. Following the administration of Thymosin alpha-1 at varying doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a marked decrease in serum levels of ALP in arthritic rats was evident on the 15th day. Specifically, the ALP levels were 197.5±3.75 U/L at the dosage of 0.25 mg/kg, 144.5±0.38 U/L at the dosage of 0.5 mg/kg, and 124.8±1.0 U/L at the dosage of 1 mg/kg. Recent investigations have further unveiled a noteworthy decrease in ALP levels within the low, mid, and high dose groups, as opposed to the arthritis control group **(Figure-3).** This observed reduction in ALP levels suggests a potential influence of the administered doses on the regulatory mechanisms of ALP, emphasizing the need for a more nuanced understanding of the relationship between Thymosin alpha-1 treatment and ALP dynamics in the context of RA. Further exploration of this phenomenon could contribute valuable insights into the complex interplay between Thymosin alpha-1 and the biochemical markers associated with RA pathology.

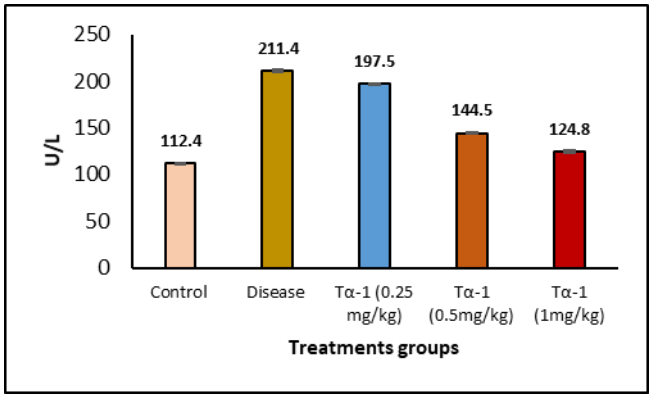


Fig. 3: Graphical representation of reduction in serum ALP levels post treatment with Thymosin alpha-1 comparing to disease group

Serum Ferritin Levels:

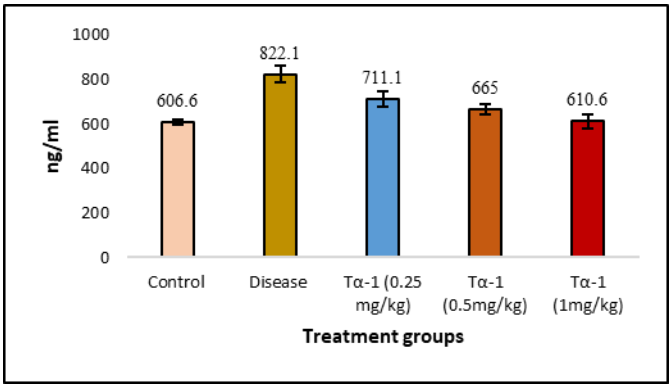


Fig. 4: Graphical representation of significant reduction in the levels of serum ferritin

A marked increase in serum Ferritin levels was evident as arthritis progressed in arthritic control rats, peaking at 822.1±36.14 ng/ml on the 15th day. Notably, arthritic control rats exhibited a substantial disparity in serum Ferritin levels compared to their non-arthritic counterparts, registering values of 606±12.9 ng/ml on the same day. Upon the administration of Thymosin alpha-1 at varying doses (0.25 mg/kg, 0.5 mg/kg, and 1 mg/kg), a discernible reduction in serum Ferritin levels in arthritic rats became observable by the 15th day. Specifically, Ferritin levels recorded were 711.1±35.84 ng/ml at the 0.25 mg/kg dosage, 665±25.19 ng/ml at the 0.5 mg/kg dosage, and 610.1±33.65 ng/ml at the 1 mg/kg dosage. This observed decline suggests a potential therapeutic impact of Thymosin alpha-1 on the modulation of serum Ferritin levels in the context of arthritis progression. The findings emphasize the importance of investigating the influence of Thymosin alpha-1 on Ferritin as a crucial facet in comprehending its potential role in the management of arthritis. A substantial reduction in ferritin levels was evident across all treatment groups when compared to the arthritis control group **(Figure-4).**

Haematological Parameters:

Hematological parameters, including red blood cell (RBC) count, platelet count, neutrophil count, lymphocyte count, eosinophil count, and monocyte count, were assessed on the 15th day of the experiment. Notably, arthritic rats exhibited a significant decrease in RBC count compared to the control group, with a marked reduction observed following administration of Thymosin alpha-1 at varying doses (Figure-5). Platelet counts remained consistent across all groups, unaffected by

Thymosin alpha-1 treatment (Figure-6). Conversely, neutrophil count increased substantially in the arthritic group but decreased notably with Thymosin alpha-1 treatment (Figure-7). Lymphocyte count displayed a significant increase in the arthritic group, with reductions noted after Thymosin alpha-1 administration (Figure-8). Eosinophil and monocyte counts showed no significant changes with Thymosin alpha-1 treatment compared to the arthritic control group (Figure 9 and 10). These findings underscore the potential of Thymosin alpha-1 in mitigating arthritis progression.

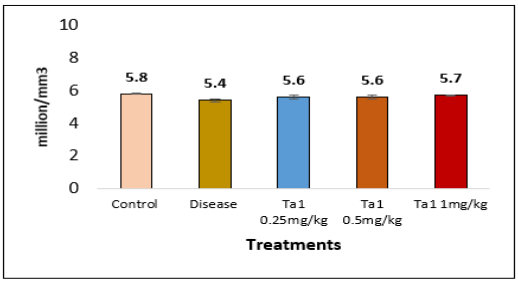


Fig.5: RBC count in all the groups

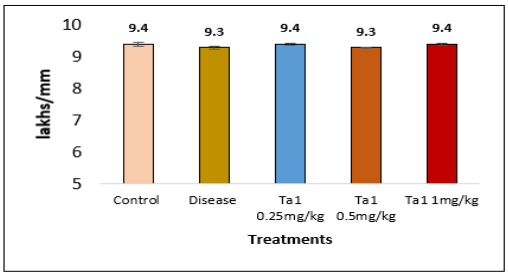


Fig.6: Platelets count in all the groups

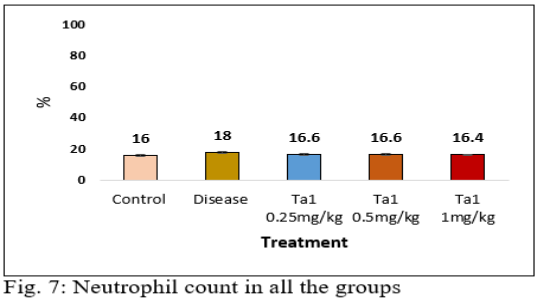


Fig. 7: Neutrophil count in all the groups

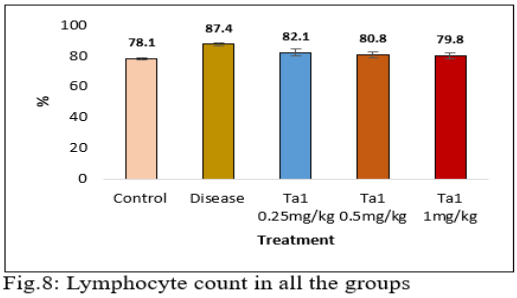


Fig.8: Lymphocyte count in all the groups

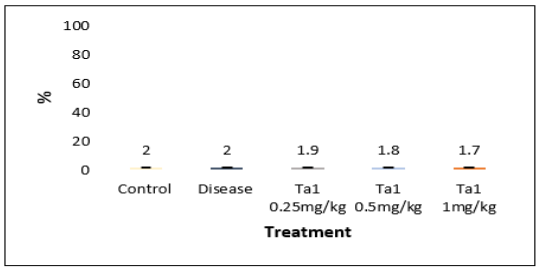


Fig.9: Eosinophil count in all the groups

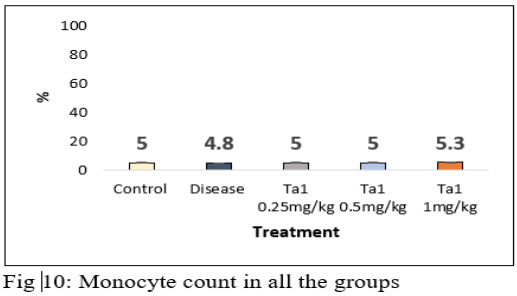


Fig |10: Monocyte count in all the groups

Discussion:

Lately, there has been much discussion about the investigation and adoption of biosimilars generic drugs that mimic biological DMARDs. There is evidence to support their efficacy, providing a noteworthy substitute to lower expenses, increase therapy alternatives, and lessen access discrepancies between developed and underdeveloped countries (11). The tendency of RA patients to have therapy failure even after trying every possible treatment option highlights the need for new medicines and a better understanding of the mechanisms underlying therapy toxicity and failure in non-remissive instances. Adverse effects and high costs present substantial obstacles to patients taking their drugs as prescribed (12). Originally derived from thymus tissue, thymosin alpha 1 is a synthetic peptide made of 28 amino acids that is presently synthesized (13). Research indicates that it may impact immune function and have different impacts on joint discomfort (9). The study findings indicate that following Thymosin alpha-1 treatment, significant improvements were noted in a number of measures, such as paw swelling, both biochemical and hematological parameters.

In 2010, Curtis JR. et al. conducted a research study and discovered that irregular ALT/AST levels emerged in 14-35% of patients starting DMARD therapy for RA or PsA. The risks were higher,

especially for those with PsA and those taking a combination of MTX (at least 10 mg/day) and LEF. These discoveries can guide the monitoring process for potential liver issuesin these patient groups (14). In earlier studies, many treatments approaches were associated with an increase in liver markers. In the current study we have not seen any significant difference in the levels of AST/ALT in all the groups. Thymosin alpha-1 did not exhibit any adverse effects on the liver markers ALT, as observed in those previous studies.

Increased levels of serum ALP are associated with inflammation in RA. In 1998, Niino-Nanke Y. et.al. conducted a study determining that out of 123 patients 37.4% of individuals with rheumatoid arthritis (RA) exhibited elevated serum alkaline phosphatase (ALP) values, measuring at 245.2 +/- 91.2 IU/L. These values were notably higher than those observed in osteoarthritis (OA) patients, averaging 192.3 +/- 45.2 IU/L ($P < 0.01$, RA vs. OA).The research findings in RA patients further confirmed a positive correlation between the increase in serum ALP levels and the activity of the disease (15). However, in the present study with the administration of Thymosin alpha-1 the levels of ALP were reduced in all the 3 groups at dosage (0.25mg/kg, 0.5mg/kg and 1mg/kg).

R. S. Rothwell. et al. also confirmed in their research study that in the context of acute rheumatoid disease, serum ferritin serves as an acute-phase reactant, reflecting the intensity of disease activity. Within the cohort of 15 patients, observations reveal notable declines in serumferritin levels that correspond with a decrease in disease activity, as evaluated through the Ritchie index and ESR (16). The similarity between our study and Rothwell et al.'s research strengthens the idea that serum ferritin is a reliable sign of how active rheumatoid disease is. This agreement not only supports the trustworthiness of using serum ferritin as an indicator but also shows that this link holds true in various research studies. This shared evidence adds what we know about how important serum ferritin is in understanding RA disease better, helping us grasp its clinical significance more fully. In current study also a substantial reduction in ferritin levels was evident across all treatment groups when compared to the arthritis control group. Overall, the present study data showed improvement in the paw, biochemical parameters and hematological parameters. Thymosin alpha-1 has demonstrated promising efficacy in reducing the pathophysiological manifestations of rheumatoid arthritis (RA), suggesting its potential as a superior therapeutic candidate for future RA management strategies. Further detailed analysis of mechanistic studies may offer a deeper understanding of its mode of action and enhance its therapeutic utility in RA treatment.

References:

1. VOS, Theo, et al. Global, regional, and national incidence, prevalence, and years lived with disability for 28 diseases and injuries for 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. The Lancet, 2017, 390.10100: 1211-1259.
2. Bone and Joint Initiative USA. 2016.
3. BRENNAN-OLSEN, Sharon L., et al. Prevalence of arthritis according to age, sex and socioeconomic status in six low- and middle-income countries: analysis of data from the World Health Organization study on global AGEing and adult health (SAGE) Wave 1. BMC musculoskeletal disorders, 2017, 18.1: 271
4. Frost, H. M. Dynamics of bone remodeling. Frost, H. M., ed. Bone Biodynamics. Boston: Little, Brown; 1964: 315-333.
5. Dempster DW. The impact of bone turnover and bone-active agents on bone quality: focus on the hip. Osteoporosis international. 2002;13(5):349-52

6. Parfitt AM. Surface specific bone remodeling in health and disease. Clinical disorders of bone and mineral metabolism. 1989: 7-14.
7. Weyand CM, Goronzy JJ. Pathogenesis of rheumatoid arthritis. Medical Clinics. 1997;81(1):29-55.
8. Goldstein AL, Goldstein AL. From lab to bedside: emerging clinical applications of thymosin α1. Expert opinion on biological therapy. 2009;9(5):593-608.
9. Zhang Q, Tang D, Zhao H. Immunological therapies can relieve aromatase inhibitor-related joint symptoms in breast cancer survivors. American journal of clinical oncology. 2010;33(6):557-60.
10. Pica F, Chimenti MS, Gaziano R, Buè C, Casalinuovo IA, Triggianese P, Conigliaro P, Di Carlo D, Cordero V, Adorno G, Volpi A. Serum thymosin α 1 levels in patients with chronic inflammatory autoimmune diseases. Clinical & Experimental Immunology. 2016;186(1):39-45.
11. Dörner T, Strand V, Cornes P, Gonçalves J, Gulácsi L, Kay J, Kvien TK, Smolen J, Tanaka Y, Burmester GR. The changing landscape of biosimilars in rheumatology. Annals of the rheumatic diseases. 2016;75(6):974-82.
12. Smolen JS, Landewé RB, Bijlsma JW, Burmester GR, Dougados M, Kerschbaumer A, McInnes IB, Sepriano A, Van Vollenhoven RF, De Wit M, Aletaha D. EULAR recommendations for the management of rheumatoid arthritis with synthetic and biological disease-modifying antirheumatic drugs: 2019 update. Annals of the rheumatic diseases. 2020;79(6):685-99.
13. Jevremovic M, Kartaljevic G, Jelusic V, Vodnik T, Pesic M, Filipovic S. Determination of thymosin alpha 1 with enzyme-immunoassay in colorectal cancer patients. Arch Oncol. 1997; 5:193-194
14. Curtis JR, Beukelman T, Onofrei A, Cassell S, Greenberg JD, Kavanaugh A, Reed G, Strand V, Kremer JM. Elevated liver enzyme tests among patients with rheumatoid arthritis or psoriatic arthritis treated with methotrexate and/or leflunomide. Annals of the rheumatic diseases. 2010;69(01):43-7
15. Niino-Nanke Y, Akama H, Hara M, Kashiwazaki S. Alkaline phosphatase (ALP) activity in rheumatoid arthritis (RA): its clinical significance and synthesis of ALP in RA synovium. Ryumachi. [Rheumatism]. 1998;38(4):581-8.
16. Rothwell RS, Davis P. Relationship between serum ferritin, anemia, and disease activity in acute and chronic rheumatoid arthritis. Rheumatology International. 1981:65-7

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The Effects of Thymosin Alpha-1 on Macrophages: A Cytological and Anti-Inflammatory Study

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Abstract

Thymosin alpha 1 (Tα-1) is a naturally occurring substance synthesized by the thymus tissue, known to activate various immune system cells. It has been reported to increase the production of Natural Killer cells, CD4 and CD8 cells, and cytokines such as IL-2, IL-3, and IFNγ. Furthermore, it plays a vital role in regulating immunity, inflammation, and tolerance. Its effect on the immune system is exerted via its modulatory action on innate immune system cells, thereby functioning as an endogenous regulator for both inflammatory and adaptive immune systems. In this study, we sought to evaluate the cytological and anti-inflammatory effects of Tα-1 on RAW 264.7 cells. The cytological effects of Tα-1 were assessed using the MTT assay, with the IC50 value against RAW 264.7 cells determined to be 368.105 ug/ml. The morphological observations made at various concentrations of Tα-1 showed increased cytotoxicity and decreased cell density of RAW 264.7 cells with increasing test concentration. Furthermore, the anti-inflammatory effects of Tα-1 were evaluated by analyzing the nitric oxide (NO) production in RAW 264.7 cells. Treatment with the test items at concentrations ranging from 7.813 to 31.25 ug/ml showed a dose-dependent reduction in NO production compared with the control group. These findings suggest that Tα-1 may have the potential as an anti-inflammatory agent in the treatment of various diseases associated with inflammation.

Keywords: *Thymosin alpha-1; Immune system; Interleukins; Natural killer cells; inflammation.*

1. Introduction

Thymosin alpha-1 (Tα-1), a 28 amino acid glycoprotein, working as an important modulator of immunological responses and released from the thymus. While the thymus has the highest concentrations of T-1, it has also been detected in the kidney, brain, spleen, lungs, and other tissues. T-1 is a physiological regulator that enhances immunological cell activity and is used to manage an array of disorders in which the immune response is impaired or inefficient. Its immunoregulatory actions include increasing T-cell generation, increasing natural killer cell activity, and decreasing inflammatory response. Additionally, T-1 has been used for an array of diseases caused by immunological dysfunctions, including hepatitis B and HCV, many cancers, severe sepsis, and as an adjuvant to enhance vaccinations [1].

Tα-1's cellular method of action has been found to have both immune-modulating and direct-acting properties. It engages with cytoplasmic Toll-Like Receptors and, in organic solvents, can fold into a coiled helix, enabling it to penetrate the cell membrane on its own [2]. Tα-1's immunomodulatory effects are achieved via its impact on innate immune system cells, such as dendritic cells and macrophages, and also cells of the adaptive system, like T-cells and B-cells. Tα-1 has been demonstrated to promote the release of cytokines such as IL-2, IL-4, IL-6, and IFN, all of which play

important roles in the homeostasis of immune responses. In conclusion, Tα-1 works as an important protein hormone that regulates the immune system's functions. Its ability to stimulate immune cell activity and reduce inflammation has made it a promising therapeutic agent for treating a range of diseases. Further research is necessary to uncover Tα-1's full range of mechanisms of action, which may lead to the development of new treatment options for immunological disorders. Tα-1 has been observed to positively impact stem cell and immune cell production. Specifically, it has been shown to stimulate the production of mature T lymphocytes, particularly CD4 cells, by encouraging the proliferation of stem cells. Tα-1 has also been used to promote thymopoiesis in human CD34 stem cells, which has led to increased production of interleukin-7, an essential cytokine for thymocyte development [3].

In addition, Tα-1 has been found to boost the physiological characteristics of Natural Killer (NK) cells in in-vivo system and also in HIV-positive individuals. This is significant, as NK activity tends to decrease in individuals with hepatitis C infections [4-8]. Furthermore, Tα-1 can promote the production of Th1 cytokines like IFN, IL-2, and IL-3, as well as the expression of IL-2 receptors, leading to a Th1 immunological response which is also linked with strong antiviral activity [9]. Conversely, a Th2 immunological response which is linked with the continuation of infections, and the production of Th2 cytokines may serve as a viral defense mechanism. Tα-1 has been found to significantly increase the production of IL-2 hepatitis-C patients, and this impact was more pronounced than that of IFN treatment alone [10-13]. When used in conjunction with IFN therapy, Tα-1 has shown promise in enhancing T-cell proliferation essential for long-lasting removal of the hepatitis-C virus, while simultaneously suppressing IFN-linked Th2 responses [14-16].

Understanding the effects of Tα-1 on macrophages is essential for developing new treatments for diseases characterized by inflammation, such as autoimmune diseases and cancer. Here, we explore the cytological and anti-inflammatory impacts of Tα-1 on macrophages. We hypothesize that Tα-1 will enhance the anti-inflammatory response of macrophages and provide new insights into the potential therapeutic applications of Tα-1 [17-18].

2. Materials And Methods

Cytotoxic assay Methodology:

RAW 264.7 were bought from the NCCS Pune, India. Cells were grown in log stage in “Dulbecco's modified eagle medium (DMEM)” supplemented with 10% (v/v) thermal attenuated “fetal bovine serum (FBS)”, “100 U/mL penicillin”, and “100 ug/mL streptomycin”. The “MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)” test was implemented to assess the cytotoxicity of the substance against the RAW 264.7 cell line [19]. The grown cell from the cultured plates were seeded in 96-well microtiter plate (1x107 cells/well), cultivated for two days at 37°C in a 5% CO2 incubator, and allowed to grow to 70-80% confluence. After being subjected to various quantities of materials, the media was replenished, and the cells were subsequently cultivated for 24 hours. After 24 hours, the morphological changes of untreated (controlled) and treated cells were analysed and photographed using a computerized inverted microscope at 20X magnification.

$$Cell\ viability\ (\%) = \left(\frac{Absorbance\ of\ sample}{Absorbance\ of\ control}\right) X100$$

Anti-inflammatory assay:

RAW 264.7 cells were housed in a humid chamber with 95% air at 37°C and 5% CO2. 1x100,000 cells/mL were pre-incubated for 1 hour with different testing item concentrations before being stimulated for 24 hours at 37°C with 1 ug/mL LPS in the medium. Monitoring the degree of NO generation by measuring the nitrite content in the culture media. To do this, the medium was combined with the Griess reagent system. After 10 minutes of incubation, the absorption spectrum was recorded at 540 nm. The nitrite level was determined using a sodium nitrite calibration curve as a benchmark [20-22].

3. Results and Discussion

Cytotoxic assay:

The MTT test was used to determine the sample's cytotoxicity. After treating with various concentrations of Tα-1, it was discovered that the drug concentrations ranging from 15.62 ug/mL to 31.25 ug/mL were those with the highest cell viability. After the results, it was found that the IC50 value of Tα-1 against RAW 264.7 cells were found to be 368.10 ug/ml.

Table 1. Cytotoxic activities of thymosin alpha-1 RAW 264.7 cells

Concentrations (ug/mL)	Absorbance			Average	Cell Viability (%)	Inhibition (%)
	I	II	III			
Control	0.42	0.42	0.42	0.42	100	0
15.62	0.41	0.41	0.40	0.41	97.71	2.28
31.25	0.40	0.39	0.40	0.39	94.25	5.74
62.5	0.36	0.37	0.38	0.37	88.50	11.49
125	0.35	0.33	0.34	0.34	81.02	18.97
250	0.27	0.27	0.29	0.28	66.14	33.85

Morphological observations of different concentrations were also observed and recorded in the supplementary file. The observations show that increasing cytotoxicity and decreasing cell density of RAW 264.7 cells with an increase in test concentration treatment. Morphological changes are shown in the pictures given below in figure 1.

Anti-inflammatory assay:

This cell line is a commonly used model for studying macrophage biology and immune function. The evaluation of the anti-inflammatory effects of the Tα-1 was done by assessing the nitric oxide (NO) release in RAW 264.7 cell line. The evaluation of the anti-inflammatory effects of Tα-1 on NO production in RAW 264.7 cells was a key aspect of our study. Various concentrations of sample given below in the table-2 was use to evaluate the production of nitric oxide. It was found that the test items at concentrations ranging from 7.81 to 31.25 ug/mL showed a dose-dependent reduction of NO production compared with the control.

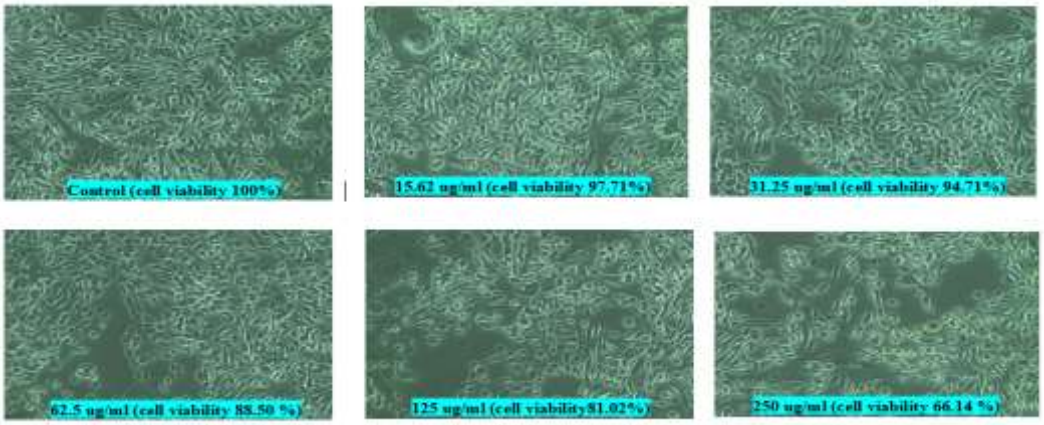

Figure 1. Dose-dependent cytotoxicity effect of Tα-1 and morphological changes in the RAW 264.7 cells

Table 2. Nitric oxide release after the treatment of LPS and different concentrations of Tα-1 in RAW 264.7 cells

Concentrations (ug/mL)	Absorbance			Average	Nitric Oxide (uM/l)
	I	II	III		
Untreated	0.08	0.08	0.07	0.07	7.76
LPS Induced	0.25	0.26	0.26	0.26	40.04
LPS + 7.81	0.24	0.23	0.23	0.23	35.48
LPS + 15.62	0.22	0.21	0.21	0.21	31.91
LPS + 31.25	0.16	0.15	0.15	0.15	21.80

NO is a key mediator of inflammation and is produced by macrophages in response to various stimuli. Our results indicate that Tα-1 treatment leads to a dose-dependent reduction in NO production in RAW 264.7 cells (table 1, figure 2). This finding suggests that Tα-1 has significant anti-inflammatory effects in macrophages and may be an effective therapeutic agent for the treatment of inflammatory diseases. Tα-1's ability to lower NO production might be attributed to its capacity to modulate the functioning of inductive nitric oxide synthase (iNOS), the enzyme accountable for NO production in immune cells. Recent research has demonstrated that Tα-1 can suppress iNOS expression as well as activity, resulting in a reduction in NO generation.

The RAW 264.7 cell line is a widely used model for studying macrophage biology and immune function. In our study, we investigated the effects of Tα-1 on RAW 264.7 cells and observed significant changes in their cytological and anti-inflammatory properties.

Firstly, we found that Tα-1 treatment increased the viability of RAW 264.7 cells. This indicates that Tα-1 may protect macrophages, promoting their survival and preventing cell death. This effect may be due to Tα-1's ability to enhance the expression of anti-apoptotic genes or through the modulation of cellular metabolism. Secondly, we observed that Tα-1 treatment reduced the creation of pro-inflammatory cytokines like TNF-α and IL-6 and increases proinflammatory cytokines synthesis and secretion like IL-10.

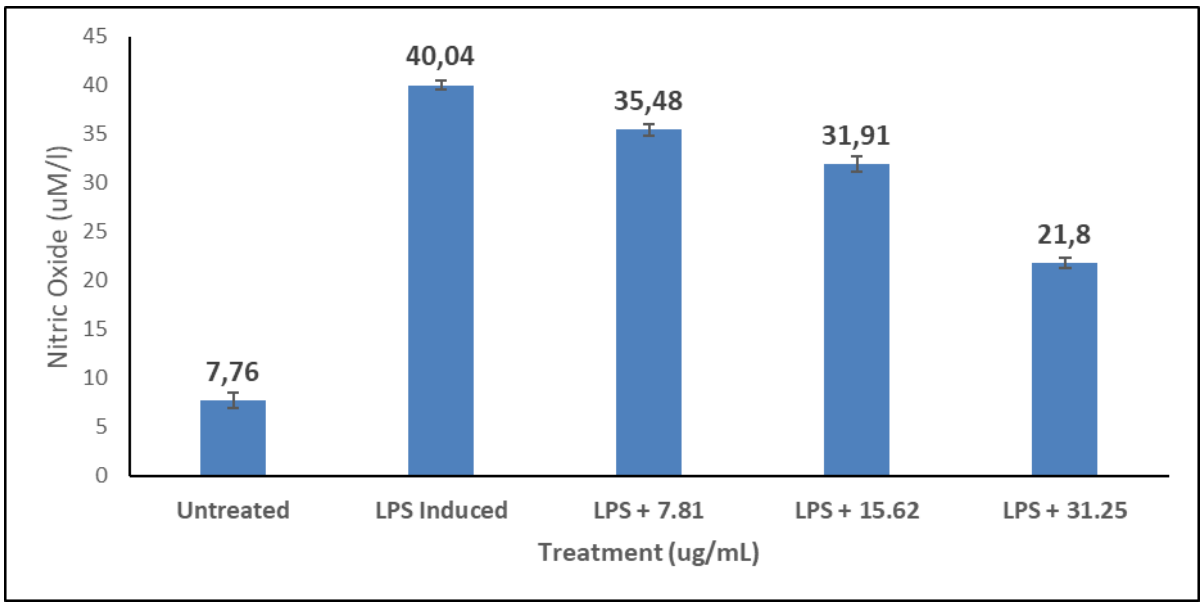

Figure 2. Nitric oxide release after the treatment of LPS and different concentrations of Tα-1 in RAW 264.7 cells

This is consistent with previous studies demonstrating Tα-1's anti-inflammatory effects in various cell types, including macrophages. Tα-1 may achieve this by modulating the activity of transcription factors such as NF-κB, which are known to be involved in the regulation of pro-inflammatory gene expression. The ability of Tα-1 to enhance IL-10 production suggests that it might be an efficient medicinal means in the management of inflammatory diseases. Overall, our results suggest that Tα-1 has significant cytological and anti-inflammatory effects on RAW 264.7 cells, highlighting its potential as a remedial agent in the management of inflammatory diseases. However, further research is needed to investigate the underlying mechanisms of Tα-1's effects on macrophages and to evaluate its efficacy in treating specific diseases.

Tα-1 is a naturally occurring biological modulator that has been shown to enhance immune cell activity and increase the production of cytokines such as IL-2, IL-3, and IFNγ (10-13). Additionally, Tα-1 is a critical regulator of immunity, inflammation, and tolerance. Its immunomodulatory effects are achieved through its action on the innate immune system cells, making it a vital endogenous regulator for both the inflammatory and adaptive immune systems.1 This study aimed to investigate the cytotoxicity and anti-inflammatory effects of Tα-1. In particular, we sought to determine the impact of increasing Tα-1 concentration on nitric oxide production in the anti-inflammatory assay. Our findings indicate that increasing the concentration of Tα-1 reduces the production of nitric oxide, highlighting its anti-inflammatory effects. Overall, our results contribute to the growing body of evidence demonstrating the beneficial effects of Tα-1 on the immune system and its potential as a therapeutic agent in the management of inflammatory diseases. Further research is needed to elucidate the mechanisms underlying its anti-inflammatory effects and to evaluate its efficacy in treating specific diseases.

4. Conclusion

In conclusion, our study has provided evidence that Tα-1 has significant cytological and anti-inflammatory effects on macrophages. Our findings suggest that Tα-1 enhances the anti-inflammatory response of macrophages, reducing inflammation and potentially mitigating the severity of diseases characterized by inflammation, such as autoimmune diseases and cancer. Overall, the evaluation of Tα-1's effects on NO synthesis and secretion provides important insights into its anti-inflammatory properties and potential as a therapeutic agent in managing inflammatory diseases. Further investigation

is ought to investigate the underlying mechanisms of Tα-1's effects on macrophages and to evaluate its efficacy in treating specific diseases.

Our study showed that Tα-1 can modulate macrophage activity and improve their function, highlighting its potential as a therapeutic agent. Further research is needed to investigate the mechanisms underlying Tα-1's effects on macrophages and to evaluate its efficacy in treating specific diseases. Nonetheless, our study provides important insights into the immunomodulatory effects of Tα-1 and its promise as a medicinal agent in the controlling agents of inflammatory diseases.

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Conflicts of Interest

The authors declare no conflict of interest.

References:

1. Wei, Y.; Zhang, Y.; Li, P.; Yan, C.; Wang, L. Thymosin α-1 in cancer therapy: Immunoregulation and potential applications. *Int. Immunopharmacol.* 2023, 117: 109744. https://doi.org/10.1016/ j.intimp. 2023.109744
2. Brusa, I.; Sondo, E.; Falchi, F.; Pedemonte, N.; Roberti, M.; Cavalli, A. Proteostasis regulators in cystic fibrosis: current development and future perspectives. *J. Med. Chem.* 2022, *65*(7), 5212-5243. https://doi. org/10.1021/acs.jmedchem.1c01897
3. Tao, N.; Xu, X.; Ying, Y.; Hu, S.; Sun, Q.; Lv, G. et al., Thymosin α1 and Its Role in Viral Infectious Diseases: The Mechanism and Clinical Application. *Molecules*, 2023, *28*(8), 3539. https://doi.org/ 10.3390/molecules28083539
4. Meoli, A.; Eickmeier, O.; Pisi, G.; Fainardi, V.; Zielen, S.; Esposito, S.. Impact of CFTR modulators on the impaired function of phagocytes in cystic fibrosis lung disease. *Int. J. Mol. Sci.* 2023, *23*(20), 12421. https://doi.org/10.3390/ijms232012421
5. Wu, L.; Luo, P.P.; Tian, Y.H.; Chen, L.Y.; Zhang, Y.L. Clinical efficacy of thymosin alpha 1 combined with multi-modality chemotherapy and its effects on immune function of patients with pulmonary tuberculosis complicated with diabetes. *Pak. J. Med. Sci.* 2022, *38*(1), 179. https://doi.org/ 10.12669% 2Fpjms.38.1.4419
6. Wang, S.; Wei, W.; Yong, H.; Zhang, Z.; Zhang, X.; Zhang, X. et al., Synergistic anti-cancer and attenuation effects of thymosin on chemotherapeutic drug vinorelbine in tumor-bearing zebrafish. *Biomed. Pharmacother.* 2023, *162*, 114633. https://doi.org/10.1016/j.biopha.2023.114633
7. Dominari, A.; Hathaway Iii, D.; Pandav, K.; Matos, W.; Biswas, S.; Reddy, G. et al., Thymosin alpha 1: a comprehensive review of the literature. *World J. Virol.* 2020, *9*(5), 67. https://doi.org/10. 5501% 2Fwjv. v9.i5.67
8. Tao, N.; Xu, X.; Ying, Y.; Hu, S.; Sun, Q.; Lv, G.; Gao, J. Thymosin α1 and Its Role in Viral Infectious Diseases: The Mechanism and Clinical Application. *Molecules*, 2023, *28*(8), 3539. https://doi.org/ 10.3390/molecules28083539
9. Linye, H.; Zijing, X.; Wei, P.; Chao, H.; Chuan, L.; Tianfu, W. Thymosin alpha-1 therapy improves postoperative survival after curative resection for solitary hepatitis B virus-related hepatocellular carcinoma: A propensity score matching analysis. *Medicine* 2021, *100*, e25749. https://doi.org/ 10.1097/ md.0000000000025749
10. Chen, Y.; Zhou, L.; Wang, J.; Gu, T.; Li, S. Clinical effect of Xuebijing combined with thymosinα1 on patients with severe pneumonia complicated with sepsis and its effect on serum inflammatory factors. *Cell. Mol. Biol.* 2022, *67*, 228–235. https://doi.org/10.14715/cmb/2021.67.6.30
11. Chen, J.-F.; Chen, S.-R.; Lei, Z.-Y.; Cao, H.-J.; Zhang, S.-Q.; Weng, W.-Z. et al. Safety and efficacy of Thymosin α1 in the treatment of hepatitis B virus-related acute-on-chronic liver failure: A randomized controlled trial. *Hepatol. Int.* 2022, *16*, 775–788. https://doi.org/10.1007/s12072-022-10335-6
12. Zhang, Y.-H.; Wang, W.-Y.; Pang, X.-C.; Wang, Z.; Wang, C.-Z.; Zhou, H. et al., Thymosin-α1 binds with ACE and downregulates the expression of ACE2 in human respiratory epithelia. *Front. Biosci.* 2022, *27*, 48. https://doi.org/10.31083/j.fbl2702048
13. Chen, M.; Jiang, Y.; Cai, X.; Lu, X.; Chao, H. Combination of Gemcitabine and Thymosin alpha 1 exhibit a better anti-tumor effect on nasal natural killer/T-cell lymphoma. *Int. Immunopharmacol.* 2021, *98*, 107829. https://doi.org/10.1016/j.intimp.2021.107829
14. Huang, C.; Fei, L.; Xu, W.; Li, W.; Xie, X.; Li, Q.; Chen, L. Efficacy Evaluation of Thymosin Alpha 1 in Non-severe Patients With COVID-19: A Retrospective Cohort Study Based on Propensity Score Matching. *Front. Med.* 2021, *8*, 664776. https://doi.org/10.3389/fmed.2021.664776

15. Matteucci, C.; Minutolo, A.; Balestrieri, E.; Petrone, V.; Fanelli, M.; Malagnino, V. et al. Thymosin Alpha 1 Mitigates Cytokine Storm in Blood Cells From Coronavirus Disease 2019 Patients. *Open Forum Infect. Dis.* 2020, *8*, ofaa588. https://doi.org/10.1093/ofid/ofaa588
16. Binder, U.; Skerra, A. PASylated Thymosin α1: A Long-Acting Immunostimulatory Peptide for Applications in Oncology and Virology. *Int. J. Mol. Sci.* 2021, *22*, 124. https://doi.org/10.3390/ ijms 22010124
17. Kl.j Lan, H.; Hao, Y.; Lv, Y.; Li, G.; Mo, Y.; Zheng, C.; Yuanfa, L. Synergistic effect of a combination of granulocyte macrophage colony-stimulating factor and thymosin α1 on Lewis lung cancer transplanted tumor in mice. *Trop. J. Pharm. Res.* 2020, *19*, 759–764. https://doi.org/10.4314/tjpr.v19i4.12
18. Fang, H.; Zhang, X.; Shen, L.; Si, X.; Ren, Y.; Dai, H. et al., RimJ is responsible for N(alpha)-acetylation of thymosin alpha1 in Escherichia coli. *Appl. Microbiol. Biotechnol*. 2009, *84*, 99–104. https://doi.org/ 10. 1007/s00253-009-1994-8
19. Hare, D.N.; Murdza, T.; Collins, S.; Schulz, K.; Mukherjee, S.; de Antueno, R. et al., Differential Cellular Sensing of Fusion from within and Fusion from without during Virus Infection. *Viruses* 2023, *15*, 301. https://doi.org/10.3390/v15020301
20. Mandaliti, W.; Nepravishta, R.; Pica, F.; Vallebona, P.S.; Garaci, E.; Paci, M. Thymosin α1 Interacts with Hyaluronic Acid Electrostatically by Its Terminal Sequence LKEKK. *Molecules* 2017, 22, 1843. https://doi.org/10.3390/molecules22111843
21. Wu, L.; Luo, P.-P.; Tian, Y.-H.; Chen, L.-Y.; Zhang, Y.-L. Clinical efficacy of thymosin alpha 1 combined with multi-modality chemotherapy and its effects on immune function of patients with pulmonary tuberculosis complicated with diabetes. *Pak. J. Med. Sci.* 2021, *38*, 179–184. https://doi.org/ 10.12669/ pjms.38.1.4419
22. Linye, H.; Zijing, X.; Wei, P.; Chao, H.; Chuan, L.; Tianfu, W. Thymosin alpha-1 therapy improves postoperative survival after curative resection for solitary hepatitis B virus-related hepatocellular carcinoma: A propensity score matching analysis. *Medicine* 2021, *100*, e25749. https://doi.org/10.1097/ md.0000000000025749

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