EVALUATION OF THE EFFECTS OF RANOLAZINE ON CHRONIC INFLAMMATION IN MALE WISTAR RATS

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LIST OF ABBREVIATIONS

AA	:	Arachidonic acid
ANOVA	:	One-way analysis of variance
PGs	:	Prostaglandins
IL-1	:	Interleukin one
IL-6	:	Interleukin six
IL-1 β	:	Interleukin one beta
CRP	:	C reactive protein
TNF-α	:	Tumor necrosis factor–α
MCP-1	:	Monocyte chemotactic protein one
ROS	:	Reactive Oxygen Species
O^{2-}	:	Superoxide
H_2O_2	:	Hydrogen Peroxide
OH	:	Hydroxyl radicle
ONOO-	:	Peroxy-nitrite
HOCl	:	Hypochlorous Acid
RNS	:	Reactive Nitrogen Species
ICAM	:	Intra cellular cell adhesion molecule
TLR	:	Toll Like Receptor
NF-κB	:	Nuclear factor kappa-light-chain-enhancer of activated B cell
IL	:	Interleukin
NSAIDs	:	Non-steroidal anti-inflammatory drugs

RA	:	Rheumatoid arthritis
CAD	:	coronary artery disease
MI	:	Myocardial infarction
Eg.	:	example
LT-B	:	Leukotriene B
CS	:	Corticosteroids
PAF	:	Platelet activating factor
HETE	:	Hydroxy eicosa tetranoic acid
NO	:	Nitric oxide
CD	:	Cluster differentiation
PDGF	:	Platelet derived growth factor
TGF	:	Transforming growth factor
HT	:	Hydroxy tryptamine
BK	:	Bradykinin
SP	:	Substance p
NK	:	Neurokinin
GM-CSF	:	Granulocyte-macrophage colony stimulating factor
IFNγ	:	Interferon gamma
VCAM	:	vascular cell adhesion molecule
CGF	:	Cell growth factor
CCL4	:	Carbon tetra chloride
IP	:	Intra peritoneal
MCP-1	:	Monocyte Chemo Attractant Protein
GM-CSF	:	Granulocyte Macrophage–colony-stimulating

factor		
PGH	:	prostaglandin endoperoxide
ACEI	:	Angiotensin-converting inhibitors
ARB	:	Angiotensin receptor blocker
PPAR	:	Peroxisome proliferator- Activated receptor
CYP	:	Cytochrome P
SOD	:	Superoxide dismutase
RCT	:	Randomized Controlled Trial
CPCSEA	:	Committee for the purpose of control and supervision of experiments on animals
IAEC	:	Institutional animal ethics committee
CFA	:	Complete Freund's adjuvant
SEM	:	Standard error of mean

ABSTRACT

Introduction & Objective:

Inflammation is the main cause for the most of diseases; it may acute or chronic in nature. Chronic inflammation type of diseases needs to take medications long time, which is totally not curable and for controlling limited medications available with greater adverse reactions. The piperazine derivative of Ranolazine shows effect on acute and sub-acute inflammation. The current experimental study was planned with the primary objective to evaluate the effects of drug Ranolazine on Chronic inflammation in male Wistar rats. The secondary objective was to study the effect on inflammatory markers like TNF-α, IL-1β, IL-6 and CRP.

Materials and Methods:

Chronic inflammation induction by 2 types of models was used:

1. Cotton pellet and Grass pith. (Foreign body induced granuloma)
2. Complete Freund's adjuvant (CFA) induced chronic inflammation. (Rat paw edema)

Test animals were divided as 3 groups (n=6) in each model, drugs given per orally vehicle, aspirin and ranolazine in clinically equivalent dose. In rats cotton pellets and grass piths induction was done by subcutaneously, (Model-1) and CFA injection at right hind limb (Model-2). After induction every 24hours drug was given to animals up to 21 days. The rats were sacrificed to obtain cotton pellets and grass piths. Mean granuloma dry weight of cotton pellets for various groups was measured and percentage inhibition of granuloma dry weight was calculated. Post study period blood samples collected and estimated the serum inflammatory markers. The grass pith sections were stained with haematoxylin and eosin for histopathological studies. CFA induced rats; hind limb paw edema was measured by plythesmometer on 1st day and 21st day of study. Post study period blood samples collected and evaluated the serum inflammatory markers and joints dissected and histopathological studies done.

Results:

Ranolazine shows significant reduction of chronic inflammation in foreign body and CFA induced rats. In foreign body induced model, granuloma dry weight, inflammatory

cytokine levels are statistically reduced. In histopathological studies monocyte and lymphocyte infiltration decreased when compare to disease control group. In CFA induced model, rat paw edema, inflammatory cytokine levels are statistically decreased. In histopathological studies exhibits granuloma formation, lymphocyte and monocyte infiltration decreased when compare to the disease control group.

Conclusion:

The current study exhibited that Ranolazine has anti-inflammatory effect in chronic inflammatory models like foreign body and CFA induced models of inflammation. Ranolazine shows anti-inflammatory effect in previous studies like acute and sub-acute models. However, these findings need to be confirmed in other models of chronic inflammation.

Keywords: Ranolazine, aspirin, chronic inflammation, Complete Freund's adjuvant.

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INTRODUCTION

Inflammation is defined as the local response of the living mammalian tissue to injury due to any agent. It is common clinical condition said to be protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from the original insult.[1] The process of inflammation through brought about by vascular as well as cellular events. The vascular events consist of initial brief vasoconstriction followed by vasodilatation and increased capillary permeability leading to leukocytic infiltration into inflamed tissue.[2]

Inflammation is a response of vascular tissues to infections and damaged tissues that brings cells and molecules of host defense from the circulation to the sites where they are needed, in order to eliminate the offending agents.[1] Inflammation is of two types based on the response to stimuli, acute and chronic.

An acute inflammatory response is manifested as redness, heat, swelling, pain, and the loss of function. Increased vascular permeability, accelerated blood flow and nerve fiber sensitization are associated with swelling, redness, and pain respectively.[2] The cardinal signs brought about by complex actions of various inflammogens like histamine, bradykinin, prostaglandins (PGs), leukotrienes etc. It is of short duration, lasting for several hours or a few days; its main characteristics are the exudation of fluid and plasma proteins (edema) and the emigration of leukocytes, predominantly neutrophils (also called polymorphonuclear leukocytes).[1] The inflammatory response could be suppressed logically, by inhibiting the activity of these endogenous mediators.

The sub-acute inflammation lasts for 1-6weeks or more. The type which is neither acute nor chronic is termed as sub-acute inflammation. Microscopically vascular, exudative as well as proliferative changes of acute and chronic inflammation are present. Exudate chiefly consists of eosinophils, lymphocytes, plasma cells, histiocytes and fibroblasts.[3]

In acute inflammation, if the response fails to clear the stimulus, the reaction can progress to a protracted phase that is called chronic inflammation. Chronic inflammation is of longer duration and is associated with more tissue destruction, the presence of lymphocytes and macrophages, the proliferation of blood vessels, and the deposition of connective tissue.[1]It is usually characterized by substantial destruction and recovery of injured tissues from an inflammatory response.[3]

In rats the acute phase typically lasts 1–3 days. The sub-acute phase may last from 3–4 days to ~1 month. If the sub-acute phase is not resolved within ~1 month, then inflammation is said to be chronic inflammation and can last for several months.[4] The inflammation severity is assessed by pro inflammatory cytokines like tumor necrosis factor-α, IL-1, IL-6, IL-1β, CRP and chemokines like IL-8, MCP-1.[4]

In chronic inflammation plasma cells in the tissue site produces inflammatory cytokines, growth factors, and enzymes which contribute to the progression of tissue damage and secondary repair including fibrosis and granuloma formation.[5] Chronic inflammation frequently begins insidiously as a low grade, smoldering after asymptomatic response. Chronic inflammation is characterized by infiltration with mononuclear cells, tissue destruction, and repair by connective tissue replacements. Uncontrolled inflammation may arise due to numerous diseased states like rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, psoriasis, immune-inflammatory ailments, and neoplastic transformations.[6]

Rheumatoid arthritis (RA) is an autoimmune disorder in which the immune system recognizes the normal component of the body as a foreign antigen, and attacks healthy tissue giving rise to diseases. In inflammatory process of RA, muscle cytokines are produced locally by myofibers, resident inflammatory cells. Proinflammatory cytokines, such as TNF can decrease the storage capacity of adiposities and increase the lypolysis.[7]

Inflammation is involved in the pathogenesis of atherosclerosis which is one of the major causes of coronary artery disease (CAD).[8] Inflammations is involved in the process of post-myocardial infarction (MI) healing process. Ischemic myocardium produces certain important cytokines such as interferon gamma, tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1). These cytokines stimulate the production of IL-6 which triggers the inflammatory response and the platelet aggregation.[7] Inflammatory mediators such as C-reactive protein (CRP) suggest that an inflammatory marker are considered as direct inflammatory promoters.[8]

The inflammatory response could be suppressed logically, by inhibiting the activity of these endogenous mediators. However, the use of antagonists of some of the mediators to suppress inflammation may not be feasible clinically.[6] Drugs like prostaglandin synthesis inhibitors like NSAIDs (eg. Aspirin) are clinically effective and are widely used in the practice.

Ranolazine is a neutral antianginal, anti-ischemic compound and a derivative of piperazine. It inhibits the late sodium current channel in ventricular myocardial cells, which reduces the calcium overload and associated contractile dysfunction.[9] It increases exercise capacity and reduces angina attacks in patients with chronic stable angina.[10] It improves endothelial function by altering the inflammatory mediators. It has also been shown to decrease inflammatory mediators such as IL-1 and TNF-α and increase anti-inflammatory peroxisome proliferator-activated receptor gamma[11].

Animal studies have shown that Ranolazine produces significant inhibition of inflammation in acute and sub-acute models in rats,[12] but as there is no data available on effect of ranolazine on chronic inflammation, the current study was planned "Evaluation of the effects of Ranolazine on chronic inflammation in male Wistar rats".

OBJECTIVES

Primary objective:

- To evaluate the effects of Ranolazine on Chronic Inflammation in male Wistar rats using:

a. Cotton pellet granuloma model

b. Chronic Arthritis (Complete Freund's adjuvant) model

Secondary objectives:

- To determine the effects of Ranolazine on inflammatory cytokines IL-1β, TNF-α, IL-6, CRP level and tissue on chronic inflammation in male Wistar rats.

REVIEW OF LITERATURE

History of inflammation is as old as mankind, since every man born suffers from inflammation one or the other time during his/her life. The term inflammation takes us back a long way in the history of medicine. When living tissue is injured, a series of changes which may last for hours, days or weeks, occur in and around the area of injury and this response itself is inflammation[13]. The word inflammation is derived from latin "inflammare" meaning to burn. [14]

Inflammation because of its frequent external obvious reaction has rich history that is intimately linked to the history of wounds, and infections[15]. The reactive nature of inflammation was first recognized by Scottish surgeon, John Hunter who after his studies of war wounds concluded "inflammation is itself not to be considered as a disease but non-specific response that had a salutary effect on its host.

Inflammation is defined as the local response of living mammalian tissue to injury due to any agent. It is a body defense reaction in order to eliminate or limit the spread of injurious agent[15]. Inflammation is characterized by accumulation of fluid and leukocytes in extravascular tissue and it is closely intertwined with process of repair.[16]

The clinical expression of the process of inflammation was described by a Roman writer Celsus in the first century A. D. named the four cardinal signs of inflammations as, Rubor (redness), Tumour (swelling), Calor (heat), Dolor (pain) and Function laesa (loss of function) by Virchow[1].

TYPES OF INFLAMMATION:

Depending upon the defense capacity of the host and duration of response, mainly three types of inflammation recognized or inflammatory response occurs in three distinct phases, each apparently mediated by different mechanisms[17].

1) ACUTE INFLAMMATION: Characterized by local vasodilation and increased capillary permeability. It is immediate and early response to an injurious agent having three major components[17].

i) Increases in blood flow.

ii) Structural changes that leads to permits plasma protein and leukocytes into circulation.

iii) Accumulation of leukocyte in focus of injury.

2. SUB ACUTE INFLAMMATION: The type which is neither acute nor chronic is termed as sub-acute inflammation. It last for 1 to 6 weeks or more. It is less exposive and last longer as compared to acute inflammation. Microscopically vascular exudative as well as proliferative changes of acute and chronic inflammation are present. Exudate chiefly consists of eosinophils, lymphocytes plasma cells, histocytes and fibroblasts.[3]

3. CHRONIC INFLAMMATION: It is considered to be inflammation of prolonged duration (weeks or months) in which active inflammation, tissue destruction and attempts at healing are preceding simultaneously[17]. Chronic inflammation frequently begins insidiously as a low grade, smoldering after asymptomatic response. The most common chronic inflammation in human beings such as rheumatoid arthritis, atherosclerosis, tuberculosis and chronic lung diseases.[3,17]

Such inflammation arises due to:

1) Persistent infections by certain micro-organisms like tubercular bacilli, fungi etc. These organisms evoked delay hypersensitivity reaction. [17]

2) Prolonged expose to potentially toxic agents exogenous or endogenous. Eg. Plasma lipid components if chronically elevated produced atherosclerosis. [17]

3) Under certain condition immune reactions are set up against the individuals own tissues leading to autoimmune diseases like rheumatoid arthritis and lupus erythematous[17].

Chronic inflammation is characterized by[1, 17]

i) Infiltration with mononuclear cells.

ii) Tissue destruction

iii) Repair by connective tissue replacements.

PROCESS OF INFLAMMATION: It is clearly known that the inflammation involves many mechanisms that are continuously operating during the process. The histo-pathological and biochemical studies of inflammation indicate that it develops in 2 distinct phases.[17]

1) Vascular phase

2) Cellular phase

1. VASCULAR PHASE:

The vascular changes such as vasodilatation and stasis of blood are the central point of the whole process and essence of inflammation. The initial phase of brief vasoconstriction is followed by vasodilatation and increased capillary permeability ultimately leading to leukocytic infiltration into the inflamed tissue[2].

The vascular events in the acute inflammation are due to alteration in vascular calibre, blood flow and permeability. Changes in vascular calibre and flow of blood include[1],

1) Transient vasoconstriction of arterioles.

2) Persistent progressive vasodilatation.

3) Slowing of the blood flow and stasis.

1. Transient vasoconstriction of arterioles: Regardless the stimulus, there may be the transient vasoconstriction of the arterioles immediately followed by an injury probably due to antidromic nerve reflex mechanism[1]. This results in reduction of blood flow to the injured areas and lasts for few minutes, if the injury is mild and for several minutes, if the injury is severe as in burns.

2. Persistent progressive vasodilatation: Immediately after transient vasoconstriction of arterioles, the progressive dilatation of the blood vessels, which involves mainly the arterioles and to a less extent affects other components of microcirculation like venules and capillaries. These changes appear within half an hour of injury. Initially vasodilatation is mediated by vaso-active amines but after 30 to 60 minutes it appears to be due to kinins, prostaglandins etc[18]. Though, kinins have been proposed to be the natural mediators of delayed responses[19], there is no convincing evidence for this proposal.

3. Slowing of the blood flow and stasis: The concomitant change in vascular permeability due to released mediators leads to the movement of fluid and protein into the tissues (exudates). The escape of plasma into the tissues causes consequently rise in viscosity of blood leading to increased resistance to flow, in venules and a corresponding rise in hydrostatic pressure in capillary bed[20]. The latter effect assists the expulsion of more plasma, leading to further increase in viscosity and blood stasis.

II) CELLULAR EVENTS IN INFLAMMATION:

The gross cellular event at the site of inflammation is accumulation of leukocytes, to engulf the inflammogens in order to protect rest of the normal tissue. For this purpose, leukocytes, reach the site of injury, by marinization, pavement, sticking, emigration and chemotaxis. Following stagnant red blood cells stick together to form clumps by a process known as sludging. These red blood cell clumps largely occupy central axis of blood stream leaving white blood cell to move peripherally[21]. Neutrophils are said to extrude pseudopods to separate the intracellular junctions between the endothelial cells to emigrate between them to pass through basement membrane. Finally, neutrophils penetrate the peri endothelial sheath and emerge into connective tissue within 3 to 9 minutes. Eosinophils and monocytes migrate through the venular wall in the same manner as neutrophil.[22]

The unidirectional migration of cells toward an attractant, or more simply, locomotion oriented along a chemical gradient is called as chemotaxis. There are separate chemotactic factors for neutrophils monocytes, eosinophils[21, 22]. Bacterial products, components of complement system are reported to be chemotactic agents for neutrophils, and eosinophilic granulocytes. The various factors recognized to influence the process include leukotriene B (LTB4), 15 Hydroxyeicosa tetraenoic acid (15-HETE) platelet activating factor (PAF) and component of complement system (C_5 A) [23]. The inert precursor, serine esterase contained in neutrophil is converted to active enzyme after its exposure to chemotactic factor and inhibition of this active enzyme inhibits chemotaxis[24].

MEDIATORS OF INFLAMMATION:

Locally released chemical substances accountable for the development of inflammatory signs are referred to as "mediators of inflammation". Mediators originate either from plasma or from cells. Mediators perform their biologically activity by initially binding with specific receptors on target cells, enzymatic activation or oxidative damage and they are grouped as[1, 17]

1. Vasoactive amines: Histamine, Serotonin
2. Membrane derived lipid substance

a Eicosanoids: i. Prostaglandins ii. Leukotrienes

b. Platelet activating factor (PAF)

3. Kinins: a. Bradykinin and related kinins, b. Tachykinins
4. Clotting system
5. Complement system

6. Lysosomal proteases

7. Cytokines

8. Biologically derived oxidants: Hydroxyl radical (OH), Hydrogen peroxide(H_2O_2), Hypochloride (Hocl), Super-oxide anion radical (O)

9. Nitric oxide (NO)

10. Adhesion molecules

a) Beta - 2 Integrins or CD 18: include P 150, 95, LFA-1, Mac-1.

b) Ig superfamily or Intracellular adhesion molecule include

ICAM-1 Intracellular adhesion molecule- 1

ICAM-2 Intracellular adhesion molecule- 2

ICAM-3 Intracellular adhesion molecule- 3

VCAM-1-Vascular cell adhesion molecule-1

c) Selectins: P Selectin, L Selectin and E selectin

d) Unclassified: CD-44

11. Growth Factors: Platelet derived growth factor (PDGF), Transforming growth factor (TGF)

1. VASOACTIVE AMINES:

i. Histamine is generally present in all tissues and richest source being the mast cells that are present in connective tissue[17] having role in the inflammatory process by its action on vasculature[25]. Chemically histamine is 5-(2 amino ethyl) imidazole formed by decarboxylation of histidine. It is also found in basophils and platelets.

Irrespective of nature of noxious stimulus, histamine is released from basophils and mast cells in acute inflammation. Histamine release causes increase in blood flow and microvascular permeability[25, 26] leading to edema formation and is also implicated with pain and itching[27]. It dilates small arteries and constricts large arteries pharmacologically proved that involvement of histamine receptor in inflammatory responses indicates that the vasodilation involves both H1 and H2 receptors[28]. Although individual H_1 and H_2 receptor antagonists have been reported to possess the anti-inflammatory activity, the combination of H1 and H2 receptor antagonists is more effective than any one receptor antagonist[29]. H3 receptor doesn't have any role in inflammation.

ii. Serotonin (5-hydroxytryptamine, 5-HT): is a second preformed vasoactive mediator. It is a monoamine formed by decarboxylation of amino acid tryptophan. It is present in

platelets and enterochromaffin cells and in mast cells in rodents[16] and in man it is present in neuroendocrine cells of gastrointestinal tract and respiratory tract and certain nerves. It is usually release with histamine in inflammatory exudate. In rats, it increases capillary permeability like histamine and hence appears to be a mediator of acute inflammation.[30]

2. MEMBRANE LIPID DERIVED SUBSTANCES:

A. Eicosanoids: The exudative product of arachidonic acid called as eicosanoids are increased in inflammation. These include cyclo-oxygenase product (Prostaglandins, thromboxane's and prostacyclin's) and the lipoxygenase products (Leukotrienes and lipoxins).[1, 16]

1. Prostaglandins (PGS):

Prostaglandins are group of pharmacologically active lipids which are widely distributed in mammalian tissue and were described for the first time by Vonruler in 1935. The most widely studied prostaglandins are PGE1, PGE2, PGE2α and PGA1. They are formed enzymatically by oxidation and cyclization of certain 20 carbon polyunsaturated fatty acids and are released at the site of inflammation[31]. Of the various products of cyclooxygenase pathway, PG-E or F are recovered in large quantities from many types of damaged or inflamed tissue, lesions of contact dermatitis[32], UV-ray induce inflammation[33], rat paw injected with carrageenan[34] and mono articular arthritis[35]. Their role is well established in the pathogenesis of rheumatoid arthritis[36] and is also released by immunological injuries[37]. PGs are reported to induce vasodilation, increased vascular permeability, mast cell degranulation and leukocytic accumulation[38]. Although all the major immune inflammatory cell types except lymphocytes are capable of generating prostaglandins by appropriate stimulus, in general but the macrophages are the major sources.[39]

PGE series, particularly PGE-2, is a powerful vasodilator which produces long lasting erythema following intradermal injection, plays a major role in production of edema, erythema and pain during inflammation[40]. Experimental evidences suggest that PGI2 and PGF2α also contribute to inflammatory responses of PGE.[35, 36]

In carrageenan induced biphasic inflammation, Prostaglandins contribute for the latter phase while histamine and 5-HT for the earlier one[18]. It has been suggested that PGEs act as modulators of immune response. Prostaglandins inhibit the release of lymphokines and it is possible that PG production by macrophages act as negative feedback mechanism

to suppress lymphokine release by activated lymphocytes. Further, it inhibits lymphocyte mitogenesis and tumor killer activity of interferon activated mouse macrophages[43]. PGs also appear to be involved in chronic inflammation, since several studies have shown the presence of prostaglandins in the synovial fluid from the joint of chronic arthritic patients.[36]

2. Leukotrienes: These are the products of lipoxygenase pathway of arachidonic acid metabolism. Arachidonic acid is converted by the enzyme 5 lipoxygenase to 5-HPETE. The same enzyme catalyzes conversion of 5-HPETE to leukotriene A4 (LTA4) and its conversion to leukotriene B4 (LTB4) or its conjugation with reduce glutathione to form LTC4. Further, the formation of LTD4 and LTE4 which are products of LTC4 is also catalyzed by same enzyme.[44] Leukotrienes also have a role in allergic and inflammatory diseases. LTB4 is a potent chemotactic factor for neutrophils as compared to eosinophils. In addition to their chemotactic role in acute inflammation, they are also implicated with pathogenesis of chronic inflammation like rheumatoid arthritis[44]. 5 HPEPE and its alcoholic metabolite, 5HETE stimulate the superoxide generation in Guinea pig and human neutrophils by augmenting intercellular calcium level that facilitates the protein-kinase activity[45]. Thus, leukotrienes also appear to play a substantial role in the pathogenesis of inflammatory diseases.

B. PLATELET ACTIVATING FACTOR (PAF): PAF is another phospholipid derived mediator. Its name comes from its initial discovery as a factor derived from antigen stimulated immunoglobulin (IgE) sensitized basophils, which cause platelet aggregation. Chemically, it is an acetyl glycerol ether phosphocholine and is synthesized from membrane phospholipids by activation of phophalipaseA2 (PLA2). PAF causes vasoconstriction, bronchoconstriction; in extremely low concentration induces vasodilation and increase venular permeability with potency 100 to 1000 times greater than histamine. Thus, PAF can elicit most of the cardinal signs of inflammation. It acts directly on targets by its receptors and it also boosts the synthesis of other mediators like eicosanoids. PAF increases vascular permeability leading to platelet accumulation and it is known to induce acute inflammation in experimental animals[46]. The intradermal injection of PAF in humans induce a biphasic inflammatory response characterized by endothelial swelling, perivascular infiltration of mononuclear cells as well as neutrophils

and intravascular accumulation of neutrophils in earlier phase: while appearance of lymphocytes and histocytes dominates in later phase.[47]

In vitro PAF has been shown to induce aggregation of platelets[48], neutrophils and monocytes[49], with subsequent release of secondary inflammatory mediators including lipoxygenase and cyclooxygenase products, oxygen radicals and lysosomal enzymes. These actions of PAF appear to involve different receptor subtypes located in various tissues like platelets, neutrophils & lung membranes.[50] In addition to quoted specific PAF blockers, like kadsurenone, a series of terpenes the most active being ginkogolide B isolated from Chinese tree, Ginkgo biloba are potent and selective inhibitors. Surprisingly certain substances like calcium channel blockers, calcium chelators, cromoglycate, naloxone, atropine, doxepin and tri-azolo benzodiazepines (alprazolam, triazolam) are reported to be antagonists of PAR.[50]

3. KININS: There are two types of kinins which are polypeptide substances,

a) Bradykinin and related kinins (Kallidin and met-lys- bradykinin) derived from plasma and liver, and

b) Tachykinins- the polypeptide substances located in sensory nerve endings.

a) Bradykinin and related kinins: Bradykinin (BK), an appropriate physiological or pathological stimulus activates prekallikreins, the nonapeptide. bradykinin formed in blood by the action of plasma kallikreins on high molecular weight kininogens. It contributes variety of biological actions like inflammation, pain etc. which is mediated by endogenous agents such as prostaglandin, histamine & serotinin[51]. It also stimulates collagen synthesis & cell proliferation in human fibroblast[52]. Once released in peripheral tissues, kinins act on blood vessels & produce both peripheral vasodilatation & increase in capillary permeability[53]. It has been suggested that production of kinins may be prolonged in such a way that these peptides could participate not only in the initial but also intermediate & late phase of the inflammatory process.[54]

These effects of BK appear to be mediated through its various receptor subtypes. Lerner and Mooder (1991) demonstrated that both BK-1 and BK-2 receptors are involved in vasodilatation and fibroblast production while BK1 receptors play a prominent role in angiogenesis[55]. The action of BK is short lived because it is quickly inactivated by enzyme called kiniase[17]. Hence the BK and related kinins appear to play a prominent role in inflammation.

b) Tachykinins: The neuropeptides like substance P(SP), neurokinin A(NKA), neurokinin B (NKB) and neuropeptide K (NPK)located in sensory nerves appear to be involved in inflammation and immune functions and play a role in the pathogenesis of allergic and inflammatory diseases.[56]

Substance P (SP) causes vasodilatation and increased vascular permeability both directly and by stimulating histamine release and eicosanoid production by mast cells and also enhanced neutrophil adhesion and chemotaxis[57]. Release of SP into human skin induces wheal, flare and itch, while NKA is slightly weaker in causing flare without pain or itch.[58] Several tachykinin antagonists including a recently developed nonapeptide antagonist depress the increase in blood flow and plasma extravasation elicited by noxious stimuli in rat skin[59]. These results strongly suggest that tachykinins might also play a role in the pathogenesis of inflammation.

4) CLOTTING SYSTEM: The clotting system consists of series of plasma proteins that play the important role in inflammation. The final step of the cascade is the conversion of fibrinogen to fibrin by the action of thrombin. During this conversion fibrinopeptides are formed, which induced increased vascular permeability and chemotactic activity to leukocytes and neutrophils[60]. Thrombin also has inflammatory properties including causing increased leukocyte adhesion and fibroblast proliferation. The fibrinolytic system contributes to the vascular phenomenon of inflammation in several ways Plasminogen activators released from the endothelium leukocytes and other tissues cleave plasminogen that bind to evolving fibrin clot to generate plasmin that break down fibrin clots and by products released, increase vascular permeability and induce chemotaxis.[61]

5) THE COMPLEMENT SYSTEM: The Complement system is an enzyme cascade consisting of 9 major components; designated as C-1 to C-9. Activation of this cascade can be initiated by substances derived from microorganism, such as yeast cell walls, endotoxins & so on. One of the main events in this pathway is the enzymic splitting of C-3 which gives rise to various peptides, two of which are C-3a & C-3b. C-3a termed as anaphylatoxin & C4a to a small extent stimulates the mast cells, & basophil cells result in release of histamine, which increase vascular permeability causing edema in tissue & can also directly stimulates the smooth muscle of bronchi[62]. Whereas C-3b & C-3bi, when fixed to bacterial cell wall act as an opsonin & favor phagocytosis by neutrophils & macrophages[17]. Enzymic action on later components C-5 releases C-5a & C-5a des Arg,

may also contribute to inflammatory reactions by stimulating the release of mediators like histamine, degradative enzymes, cationic protein & interlukein-I[63], leukotrienes [64]& platelet activating factor[65]. Both C-5a and C5b-9 lytic complex stimulates prostaglandin synthesis & potentiate inflammatory reactions[66, 67]. C-3a also appears to be involved in chronic inflammation like rheumatoid arthritis[68]. C-5a is a powerful chemotactic agent for neutrophils, monocytes, eosinophils & basophils. It also increases the adhesion of leukocytes to endothelium by activating leukocytes & increasing the avidity of surface integrins to their endothelial ligand. C-5a des Arg. which also chemotactic in the presence of serum polypeptide called as co-chemotaxin. Among the complement components, C-3 & C-5 are the most important inflammatory mediators. C-3 & C-5 can be activated by several proteolytic enzymes like plasmin & lysosomes released from neutrophils. Thus, the chemotactic effect of complement & the complement activating effect of neutrophils can set up a self-perpetuating cycle of neutrophil emigration.[17]

6) LYSOSOMAL PROTEASES: The inflammatory cells-neutrophils & monocytes contain lysosomal granules, which when released may contribute to the inflammatory response.[17]

Neutrophils exhibit two main types of granules. The smaller specific granules contain lactoferrin, lysozyme, alkaline phosphatase, the components of NADPH oxidase, the intracytoplasmic pool of integrins & a collagenase. The large granules contain bactericidal factors acid hydrolases & a variety of neutral proteases. These enzymes are secreted by variety of mechanism.[1, 17]

1. Acid proteases degrade protein at acidic Ph. Their most likely action is to degrade bacteria & debris within the phagolysosomes.
2. Neutral proteases are capable of degrading various extracellular components. This enzyme can attach collagen, basement membrane, fibrin, elastin & cartilage resulting in the tissue destruction characteristics of purulent & deforming inflammatory process.

Neutral proteases also cleave C3 & C5 directly, releasing anaphylatoxins & release kinin like peptide from kininogen- monocytes & macrophages. On degranulation also release mediators of inflammation like acid proteases, collagenase, elastase & plasminogen activator. The lysosomal proteases play a role in chronic as well as acute inflammation[1, 17].

7. CYTOKINES: These are products of lymphocytes and macrophages that are activated by antigens. The cytokines that are of particular importance to allergic inflammatory conditions as described earlier are summarized below[69]:

A. Macrophage activating factors:

i. Migration inhibitory factor.
ii. Granulocyte-macrophage colony stimulating factor (GM-CSF)
iii. Interferon-gamma.
iv. Macrophage colony stimulating factor (M-CSF)
v. Tumor necrosis factors (TNFS)

B. Factors that affect lymphocyte function:

i. IL- 1 - Lymphocyte activating factor.
ii. IL- 2 T cell growth factor.
iii. IL- 4- CGF
iv. IL- 5-CGF
v. IL- 6-CGF

C. Factors that affect cells other than leukocytes:

i. Interleukin - 3
ii. Interleukin-9
iii. Interleukin 10
iv. Lymphotoxin
v. Osteoclast activating factor
vi. Collagen producing factor.
vii. Histamine releasing factor (IL-8)

These cytokines have a definite role in immune response and autoimmune or inflammatory disorders. Cytokines that may contribute to the local accumulation and activation of eosinophils include IL-5, GM-CSF and IL-3. These cytokines are also activating factors for neutrophils and macrophages. IF gamma and IL-4 are other neutrophil activating factors. The other cytokines that may have important roles in allergic inflammation include tumor necrosis factor and IL- 6 and IL-8.[69]

Tumor necrosis factors were discovered on the basis of their capacity to induce the destruction of tumors. TNF represents two closely related proteins (TNF-α) and (TNF-β) that are primarily derived from mononuclear phagocytes and lymphocytes respectively. Pro-inflammatory activities of TNF include activation of eosinophils and neutrophils leading to their adherence to endothelial cells, chemotaxis and degranulation.[70]

Interluekin-1 is primarily produced by cells of the mononuclear phagocytic lineage, but IL-1 production also has been shown to occur in endothelial cells, synovial cells, osteoclasts and other cells[69]. IL-1 is an essential factor in the proliferation and differentiation of certain stem cells in the thymus that results in emergence of mature T cells. Another consequence of activation of helper T cells is in the synthesis and release of variety of cytokines (notably IL-4, IL-5, IL-6) that control both cellular and humoral arms of immune response.[71] IL-3 and IL- 4 serve as growth factors for mast cells and as co-mitogens for hematopoietic cells. While IL-6 shares several activities with IL- 1.[72]
Interleukin-8, a macrophage derived neutrophil chemotactic factor is equipotent to LTB4.[73] Hence cytokines play a dominant role in immune response and autoimmune inflammatory disorders.

8. BIOLOGICALLY DERIVED OXIDANTS: These are chemical species with one or more unpaired electrons in their outer orbital & they are O_2, H_2O_2, OH & Hypochlorite (HOCL). Although, their production is essential for normal metabolism but they are theoretically destructive unless tightly controlled. Their major sources are the respiratory burst produced by neutrophils, sensitized monocytes, macrophages & eosinophils in response to particulate & non particulate stimuli (Eg: bacteria aggregated IgG, complement & interleukin-1)[74]. Free radicals like OH having a potentiating effect on prostaglandin synthesis[75]. Free radical not only like O_2 & H_2O_2 but transition metals also stimulate the lipid peroxidation by increasing the rate of decomposition of hydroperoxide into peroxy or alkoxy radicals result in producing long lived secondary radicals & toxic aldehydes of varying reactivity evoke a broad spectrum of inflammatory effect along with changes in protein composition lead to critical effects on cells[76]. Reactive oxygen species act not only as an inflammatory mediator but also, they are having modulating effect on other inflammatory process like lipid peroxidation results in enzymatic leakage from cells & cellular compartments into area where their activity is damaging. In addition, they also act synergistically to serum protease like alpha-1 proteases[77]. This free radical also effects

on chronic inflammation like rheumatoid inflammation. Trials of free radical scavengers, including superoxide dismutase, catalase & iron chelators have been most effect in suppressing many diverse animal models of acute & chronic inflammation.

9. NITRIC OXIDE: (NO) This is a relatively new mediator of inflammation. Nitric oxide is a soluble free radical gas that is produce not only by endothelial cells but also by macrophages and specific neurons in the brain.[17] In addition to vascular smooth muscle relaxation, NO plays important role in inflammation[78]. The NO produced by macrophages, acting as a free radical reacts with superoxide anion to form oxidant nitrogen dioxide and reactive hydroxyl radical. Uncontrolled NO production by macrophages lead to peripheral' vasodilation leading to increase vascular permeability and shock and has implicated in variety of inflammatory diseases.

10. ADHESION MOLECULES: The immune system is constantly being challenged by amyraid of microbial pathogens. Neutrophils are the front lines of defense and are rapidly mobilized and recruited to sites of tissue injury or infection[1]. A key step in inflammatory responses is the ability of circulating neutrophils which are in non-adhesive state, to be adhesive and attach to the vascular endothelium at the appropriate place and time[1]. This adhesive interaction is highly selective and transient in nature & has been referred to as an adhesion cascade- a precisely orchestrated cascade of events which ensures a rapid self-limiting response to isolate and destroy invading microbes while minimizing damage to healthy tissue. According to an earlier report to date, three families of adhesion molecules have emerged as key players in neutrophil endothelial cell interactions.[17]

1. CD18 or Beta- 2 integrins which includes, P 150, 95, LFA-1, Macrophages-1

2. The intracellular adhesion molecules (ICAMS) which include.

ICAM-1, ICAM-2, ICAM-3 and VCAM-1

3. The selectins which includes: L-Selectin, E-Selectin and P-Selectin.

4. Unclassified which include: CD44, CD-18 or Beta - 2 Integrins.

1.The CD18 integrin family includes three structurally related alpha beta hetero dimers- LFA-1, MAC-1 and P 150, 95. To date there are at least 12 alpha subunits and seven beta

subunits in the integrin family & LFA-1, MAC-1 and P150, 95, have distinct alpha subunits. Most of other integrin members are extracellular matrix receptors. The CD-18 integrins involved in cell-cell interactions of leukocytes & their functions are magnesium or calcium dependent.[1, 17] The CD18 are having expression to leukocytes while LFA-1 is having expression to all lineages of w. b. cells like lymphocytes, monocytes and granulocytes. MAC-1 and P150, 95 are restricted primary to myeloid cells.
CD-18 integrins play a contributing role in most leukocyte adhesion to endothelial related function[1].

2. Intracellular Adhesion Molecules: All three ICAMs are also structurally related members of the immunoglobulin (Ig) Superfamily & were all originally defined functionally as ligands for LFA-1.[1, 17] The ICAM 1 and 3 are having 5 Ig like domain and ICAM-2 having 2-Ig like domains. ICAM-1 is expressed basally only at low levels on vascular endothelial cells, lymphocytes and at moderate levels on monocytes.

However, ICAM-1 expression can be induced to high level by stimulation with inflammatory cytokines like IL-1, TNF and γ- interferon. Induced or greatly increase expression of ICAM-1 has been reported on endothelial cells, keratinocytes, synovial cells, epithelial cells, fibroblast, hepatocytes and myocytes.[1] ICAM-2 expression is restricted to endothelial cells and mononuclear leukocytes. ICAM-2 is not expressed by inflammatory cytokines. ICAM-3 is even more restricted in expression & it is expressed only on monocyte, lymphocyte and granulocyte. Interestingly ICAM- 3 is the only ICAM molecule highly expressed by neutrophils. ICAM-1 is involved in neutrophil endothelial cell interactions, trans-endothelial migration and adhesion dependent respiratory burst.[1, 17] The functional role of ICAM-2 and 3 is not clear in inflammatory response[1]. VCAM-1: expression is very low on unstimulated endothelial cells but is induced following stimulation with IL-1, TNF or to a lesser extent, IL-4.[1, 17]

3.Selectins: The three members of selectin family shared a common structural motif: an N-terminal C-type lectin domain, an epidermal growth factor and variable number of short consensus repeats.[1, 17]
a) L-Selectin: It is restricted in expression to white blood cells like lymphocytes, monocytes and granulocytes. L-selectin has been demonstrated to mediate, in part, the adherence of neutrophil to cytokine stimulated endothelial cells.[1, 17]

b) E-Selectin: It is restricted in expression to cytokine stimulated endothelium & the characterization of this molecule is involved in neutrophil & monocyte adhesion to stimulated endothelial cells. E- selectin expression is prominent on endothelial cells in acute inflammatory lesions and correlates with large influx of neutrophils. E-selectin can also be expressed on endothelium at some chronically inflamed sites of skin and synovial joints.[1, 17]

c) P-Selectin: It is restricted in cell surface expression to activated platelets and endothelial cells. In unstimulated cells it is stored in the a-granules of platelets. The cytoplasmic domain of P-selectin mediates transport of P-selectin to these intracellular compartments. It is implicated in the interactions of leukocytes with both platelets and endothelial cells. Cell activation with histamine or thrombin results in rapid recruitment of P-selectin to cell surface & thus, P-selectin may bridge the early events of hemostasis and inflammation.[79]

4) Unclassified- CD44: A polymorphic membrane protein widely expressed on lymphoid and myeloid cells, epithelial cells, and fibroblasts is involved in adhesion of tissue leukocytes to several matrix components including hyaluronate and fibronectin. It has been proposed that CD44 is involved in lymphocyte adhesion to vascular endothelial cells and it expressed on many synovial cells in patient with trauma.[80]

11. GROWTH FACTORS: They consist of platelet derived growth factor and transforming growth factor which may be chemotactic to leukocytes, mesenchymal cells in addition they have other activities resembling those of cytokines.[1, 17]

SCREENING METHODS FOR ANTI-INFLAMMATORY ACTIVITY

Anti-inflammatory activity of a compound is determined by observing specific suppression of inflammatory signs in laboratory animals. To mimic the clinical condition, animal models of inflammation could be grouped into 3 categories viz[81]: acute, subacute and chronic.

A) ACUTE INFLAMMTION MODELS:

i) Paw edema

ii) Mouse ear edema

iii) Ultraviolet light induced erythema.

iv) Chemical peritonitis (ascites)

v) Chemical pleurisy (Pleural effusion)

B) SUB ACUTE INFLAMMATION MODELS:

i) Granuloma pouch

ii) Cotton pellet granuloma

iii) CCL4 induced granuloma

C) CHRONIC INFLAMMATION MODELS

i) Adjuvant induced arthritis.

ii) Crystal induced synovitis

A) ACUTE INFLAMMATION MODELS:

i. Paw edema: Edema represents the early phase of inflammation. Carrageenan induced paw edema is the simplest and most widely used model for studying the anti-inflammatory activity of new compounds[81]. Similarly paw edema induced by Egg white[82], Kaolin, dilute formalin, brewer's yeast, dextran. histamine, mustard, serotonin, creatinine proteolytic enzyme like trypsin, and glass powders are also used for producing localized swelling. These agents can be injected in 0.1mL volume of suitable concentration in sterile saline in to the sub plantar tissue of the rat hind foot & paw volume can be measured immediately & then at pre-determined intervals by the plethysmometric methods as described by Singh and Ghosh (1968)[84]. The difference between subsequent reading & 0-hour reading is actual edema at a given time.

ii. Mouse ear edema: According to the methods of brown and Robson (1964)[85], the ear edema is produced in mice by the application of xylol to one of the ears while, the other ear serves as control. The difference in weight between control and inflamed ear is taken as the measure of the edema. The percentage inhibition can be calculated using formula:

$$\% \text{ Inhibition} = 100 \times \frac{Wc - Wt}{Wc}$$

Wt= gain in ear weight (edema volume) of treated group

Wc= gain in ear weight (edema volume) in control group.

iii. Ultraviolet light induced erythema in skin: This test is used to measure anti-inflammatory activity against erythema caused by radiation.[84]

A small area on the back of a depilated, albino guinea pig is exposed to ultraviolet radiation from a lamp for 20 sec. The animal is given saline or a test substance orally 30 minutes before radiation and the degree of erythema (0-4) is estimated 120 minutes later. Animals given saline produce erythema of degree 3 or 4. The effective dose to inhibit erythema is defined as the dose of the test compound which in a group of animals reduces the standard erythema to a second degree response.[84] It is the only assay that utilizes a species other than rat as the test animal and measure the vascular component of Inflammation.[82]

iv. Chemical peritonitis (ascites): Rats weighing almost 300gm are treated with test substance and are given 1ml of 1.5% formalin solution IP. Four to eight hours later, the rats are sacrificed to measure the ascitic fluid. Anti-inflammatory agents significantly diminish the ascitic fluid volume.[82]

v. Chemical Pleurisy (Pleural effusion): In order to test anti-inflammatory activity, rats are lightly anaesthetized with ether. Six hours after injecting and aqueous solution of Evans blue into the pleural cavity, the animals are killed with ether. The pleural cavity is opened, and the fluid collected is measured. Pleural effusion is significantly reduced by anti-inflammatory drugs.[82]

B) SUBACUTE INFLAMMATION MODELS:

i) Granuloma pouch: In this method, Wistar rats, weighing 150 - 200g are injected subcutaneously with 25ml air, and then 0.5ml of a sterile 1% solution of croton oil in cotton seed oil into the same spaces[86]. On the second day after formation of the pouch, the air is removed by vacuum. On the third day, the pouch is compressed manually to prevent the formation of adhesions and on the fourth day, the pouch is opened and the exudative fluid is aspirated to measure its volume. Anti-inflammatory drugs significantly suppress the exudate formation.[82]

ii) Cotton pellet granuloma: The cotton pellet granuloma inhibition assay was introduced by Meijer in 1950 and has been modified subsequently by several investigators[87, 88]. It is one of the most widely used assays for measuring the activity of anti-inflammatory drugs

acting on proliferative component of sub-acute and chronic inflammatory process. The general procedure consists of implantation of sterile cotton pellets weighing 10mg in-to the sub-cutaneous space of groin or axilla of the albino rats. The duration of implantation varies from one day to fourteen days. New connective tissue synthesis as reflected by the appearance of collagen which is not observed before 4th day and this is consistent with the earlier reports.[89]

On the last day the rats are killed with anesthetic ether and the pellets are dissected out. After removal of fat and extraneous tissue, the pellets are dried overnight at 60°C and are weighed. Anti-inflammatory drugs significantly suppress the dry weight of granulation tissue.

iii) Carbon tetrachloride induced granuloma: It has been described earlier[90] but rarely used model. In rats 24 hours after sub- cutaneous injection of CCL4 granulomas appeared. These reactions were inhibited by orally administered anti-inflammatory drugs like streptokinase, plasminogen or trypsin. Suggesting that the effect of these substances is due to their enzymatic activity.[91]

C) CHRONIC INFLAMMATION MODEL:

i. Adjuvant induced arthritis: Adjuvant induced arthritis is the only experimental model which has been often considered to parallel the human arthritis more closely than any other laboratory model. The arthritic syndrome is induced by the intradermal injection of 0.05ml of a Freund's adjuvant[82] is a mixture of dead mycobacterium in liquid paraffin or vegetable oil into the plantar surface of hind foot. The parameters noted in the control and treated groups throughout the period of 14 days include:

i) paw thickness, ii) the severity of secondary lesions and iii) changes in body weight.

Most of the anti-inflammatory agents including steroids, immuno-suppressants, gold and d-penicillamine are effective in this method.[92]

ii. Crystal induced synovitis: Intra-articular injection of micro- crystalline sodium urate into normal human knees and dogs was used to test the anti-inflammatory effect of new drugs. Inflammatory index in treated & control rats derived from objective measurements of knee joint swelling, tenderness, skin temperature the volume of fluid aspirated from the joint helps to assess efficacy of a test drug.[93]

EXISITING NON-STERIODAL ANTI-INFLAMMATORY DRUGS

The ancient herbal remedies, used for the treatment of pain and fever when subjected for chemical analysis revealed the presence of salicylic acid and lead to synthesis of aspirin & later many more compounds. The salicylates, particularly aspirin, make its appearance in 1899 and are one of the most widely used drugs. In one chemical form or another, these drugs which are commonly grouped as nonsteroidal anti-inflammatory drugs (NSAIDs), have been used for hundreds of years to treat a wide variety of inflammatory diseases[94]. Hence, in 1949 introduced corticosteroids as anti-inflammatory agents and Wilhelm showed remarkable clinical efficacy of phenylbutazone as an anti-inflammatory drug in 1942, though its toxicity preludes if it used in long term therapy[95]. Later the introduction of indomethacin in 1963 as a new anti-arthritic agent by Winter et al was followed by other series of NSAIDS.

These NSAIDS can be grouped on the basis of their chemical structure as[96,97:]

1) Salicylates and their congeners:

eg. Aspirin and selected compounds

2) Aniline and para-amino derivatives.

eg. Phenacetin, acetanilide, acetaminophenon etc.

3) Pyrazolon derivatives.

eg. phenylbutazone and oxyphenbutazone etc.

4) Acetic acid derivatives

eg. Indomethacin sulindac and etodolac etc.

5) Phenylacetic acid derivatives.

eg. Alcotenac, Diclofenac and Tolmetin

6) Anthranilic acid derivative.

eg. Mefenamic acid, meclofenamate sodium etc.

7) propionic acid derivatives.

eg. Ibuprofen, Naproxen, fenoprofen, ketoprofen etc.

8) Oxicams.

eg. Piroxicam, meloxicam tinoxicam etc.

9) 8-Aminoquinolones.

eg. Chloroquine and amidoquine etc.

10) Benzoxazocine derivatives.

eg. Nefopam.

11) Pyrole derivatives.

eg. Ketoralac

12) Gold compounds.

eg. Gold sodium thiomalate, gold sodium thiosulphate and aurofin etc.

13) Miscellaneous

a) Chelators – penicillamine

b) Antihistamines

e) Azathioprine

d) Methotrexate

e) Cyclophosphamide

MECHANISM OF ACTION OF NSAIDS: Non-steroidal anti-inflammatory agent's posses wide variety of pharmacological actions. They reduce inflammatory edema, erythema and pain. In 1971 vane and co-workers made the landmark observation that aspirin and other NSAIDs inhibited the biosynthesis of prostaglandin which is major step in the inflammatory process[96]. In addition to the inhibition of biosynthesis of prostaglandins other possible modes of action of NSAIDS have been reviewed.

They are:

1) Irreversibly acetylate ser 530 of cox-1 there by blocking access of arachidonic acid to active site.[94]

2) By unknown means aspirin inhibit cox-2 leads to generation of 15-HETE which is potent inhibitor of neutrophil O2 generation.

3) It alters the uptake of precursor arachidonic and its insertion into the membranes of cultured human monocytes and macrophages.

4) It also inhibits anion transport across a variety of cells membrane.

5) NSAIDS inhibit synthesis of cartilage-proteo-glycan and bone metabolism (both in vitro and vivo) by mechanisms does not depend upon inhibition of PGH synthase.

6) Recent work has shown that aspirin like drugs affect stimulus response coupling in the most abundant cells of acute inflammation neutrophils.

7) NSAIDS inhibit cell-cell aggregation of human neutrophils induced by chemo-attractants.

8) More lipophilic form of NASIDS interferes with cell functions. (Prevent with superoxide anion generation by a cell free membrane rich preparation from neutrophils) [97].

9) It inhibits platelet aggregation and completely inhibits thromboxane biosynthesis via its effect on PGH synthase.

10) NSAIDs uncouple chemo-attractant receptors from their cellular transduction mechanism.

11) High concentration NSAIDS inhibits cell migration.

12) Uncoupling of oxidative phosphorylation by isolated mitochondria.[98]

13) Inhibit of biosynthesis of muco-polysaccharides.[99]

14) Sulfa-hydril disulphide stabilization.[100]

15) Inhibition of chemotaxis.[102]

16) Hyper polarization of neuronal membrane.[101]

17) Displacement of endogenous anti-inflammatory peptide from plasma protein.

18) Antagonist effect on mediators other than PGs.[102]

eg. Histamine and bradykinin.

19) NSAIDS may inhibit expression or activity of certain of these cell adhesion molecules.[17]

Aspirin and aspirin like drugs showed the unwanted effects, the most common is to induce gastric irritation which may lead to ulceration and the result is loss of blood platelet function appears to be disturbed because aspirin like drugs prevents the formation of thromboxane A2 by platelets, a potent platelet aggregating agents and thus worsen gastrointestinal hemorrhage.

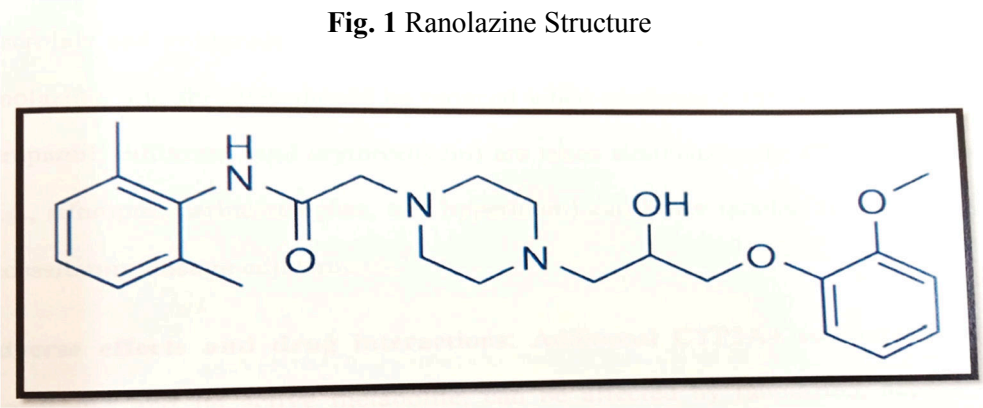
Since a number of NSAIDS are known to inhibit the de-naturation of proteins; the clotting time may be prolonged with aspirin like drugs. Other adverse effects of aspirin and like drugs include prolonged gestation and labor, renal papillary necrosis, intolerance in the form of bronco-constrictions etc.

Because of these adverse effects the search for safes drugs and different safer effective combination continue. The combination of aspirin and paracetamol is reported to be more effective as but safer than aspirin alone. The drugs used in clinical practice like Ca++ channel blockers, adrenergic agonists and antagonists have also been reported to possess anti-inflammatory activity, but their efficacy in the treatment of inflammation in clinical practice is yet to be established.

NEED OF RANOLAZINE AS TEST DRUG IN PRESENT STUDY:

Ranolazine are second-line anti-anginal agent for the treatment of chronic angina. Ranolazine - It is being be used with a number of different drugs, including Beta blockers, Calcium channel blockers, ACEI, ARBs, lipid-lowering drugs and anti-platelet drugs in the treatment of angina pectoris.[103]

Fig. 1 Ranolazine Structure



MECHANISM OF ACTION: Ranolazine works by inhibiting late sodium current that allows calcium to enter through the sodium-calcium exchanger. Ranolazine lowers diastolic tension, cardiac contractility, and workload by decreasing intracellular calcium concentration.[103]

Pleotropic effects:

1. Anti-inflammatory effects: Few studies showed Ranolazine inhibits the inflammatory markers such as IL1β and TNF-α, and increases PPAR-γ level indicating it has anti-inflammatory effects.

2. Anti-Oxidant effects: Furthermore, antioxidant proteins Cu/Zn SOD and Mn- SOD significantly increased after Ranolazine addition in cultured astrocytes.[104]

Pharmacokinetics: Ranolazine available as extended release tablet and administered without regard to meals at 500mg to 1gram twice daily. Higher doses are poorly tolerated. The drug's oral bioavailability is about 75%. P-glycoprotein inhibitors (eg. Digoxin etc.) increase ranolazine absorption and exposure to both ranolazine and the competing drug. Ranolazine's terminal $t_{1/2}$ of is approximately 7 hours; with repeated dosing, a steady-state plasma concentration is reached in 3 days. Ranolazine is metabolized primarily by CYP3A4 and to a lesser extent by CYP2D6. Both the unchanged drug and its metabolites are eliminated in the urine (5 percent). With strong CYP3A4 inhibitors (e.g. macrolide and

imidazole antibiotics, HIV protease inhibitors) should not be used with ranolazine, and dosages should be reduced. CYP3A4 inducers (e.g. rifampin, carbamazepine, and hypericum) can lower ranolazine plasma levels, necessitating dosage adjustments.[103]

Drug interactions: Ranolazine can affect plasma levels of CYP3A4 substrates, such as simvastatin and its active metabolite. It requires ranolazine dose reduction may be required. For narrow therapeutic range drugs (e.g, cyclosporine, tacrolimus, sirolimus) used together with ranolazine also need dose adjustment. Ranolazine with co administration of tri-cyclic antidepressants and antipsychotics, it can increase the CYP2D6 levels.[103]

Adverse effects: Dizziness, headache, nausea, and constipation are the most common side effects. Some CNS effects resemble those of class I anti-arrhythmic (eg. dizziness, fuzzy vision, and disorientation). Although QT prolongations must be noted, there have been no reports of torsade's de pointes arrhythmias or associated occurrences[103].

Therapeutic use: Ranolazine is used in the treatment of chronic angina. Dose required for its effect was 500 to 1000mg twice a day. It is available as in dosage form as extended release.

Selection of Ranolazine as the study drug: Ranolazine is prescribed for the treatment of chronic angina in conjunction with other anti-anginal medications such as beta blockers, calcium channel blockers, and long acting nitrates, among other things. Ranolazine has been shown to be safe in patients with cardiovascular disease and has also been shown to result in a reduction in inflammation.[105]

Ranolazine has also been proven to have anti-inflammatory qualities, as demonstrated by the studies listed below. This is according to the findings of a study entitled 'Vagal stimulation Facilitates Improving Effects of Ranolazine on Cardiac Function in Rats with Chronic Ischemic Heart Failure,' which suggested that a combination of vagal stimulation and ranolazine improved cardiac function by attenuating the exaggerated levels of NE/BNP-45 and cytokines in the rats with chronic ischemic heart failure (IL-1beta, IL-6, and TNF-alpha). [106]

According to the findings of another study titled 'Ranolazine improves endothelial function in patients with stable coronary artery disease,' ranolazine improved endothelial function and C-reactive protein levels in patients with stable CAD, suggesting that ranolazine may have a novel mechanism of action in this setting.[107]

Because of this, ranolazine advantages may extend far beyond its anti-angina capabilities. Along with these things ranolazine also controls the anti-inflammatory property. As a result, ranolazine may be a viable treatment for inflammation.

A study done on rats showed significant inhibition of paw edema in acute inflammation. The anti-inflammatory effect on acute inflammation can be explained on the basis of its effect on one of the inflammatory mediators [108]. It showed a significant decrease in granuloma dry weight in sub-acute inflammation. This can be attributed to a decrease in levels of inflammatory mediators levels such as TNFα, CRP and IL6 which, in turn, could have decreased the cellular infiltration to the site of inflammation [108].This decrease in cytokines level (like TNFα) could be because of decrease in fibroblasts which is known to increase the level of this cytokine.[108]

One more study at Spain by Martin A et.al: Ranolazine reveals that it decreases pro-inflammatory mediators IL-1β and TNF-α release, and increased anti-inflammatory PPAR-γ as well as the antioxidant enzymes Cu/Zn-SOD and Mn-SOD expressions in primary culture of astrocytes. Its increased astrocytes viability and proliferation and reduced LDH release, caspase 3 activity and apoptosis activity via Smac/Diablo protein.[109]

Another study conducted in Alabama on Chronic inflammation in Rheumatoid arthritis; shows that: Inflammation will induce pathology to skeletal muscle and associated impairment of physical function. Effects of chronic inflammation on skeletal muscle mass by Pro-inflammatory cytokines [i.e. tumor necrosis factor (TNF), interleukin-1β (IL-1β) and interleukin-6(IL-6)] are thought to be the key mediators of inflammation responsible for stimulating proteasome-dependent proteolysis and inhibiting anabolic and/ or anti-catabolic signals that lead to low skeletal muscle mass in chronic inflammatory conditions, such as Rheumatoid Arthritis.[110]

Studies have shown that chronic inflammation plays a significant role in the onset of many diseases like rheumatoid arthritis, Diabetes, Coronary artery disease etc. Drugs with anti-inflammatory actions help in prevention of these diseases. Ranolazine an anti-anginal drug has showed anti-inflammatory effect in acute and sub-acute models of inflammation in rats.[12] Anti-inflammatory effect of Ranolazine can be confirmed by further study on chronic inflammation. Thus, to evaluated the chronic inflammatory effect of Ranolazine, the current study was planned.

METERIALS & METHODS

The complete course of the experiment was conducted using healthy adult male Wistar rats weighing about 200±50 grams. Animals were obtained from central animal house of J. N. Medical College. They were housed under standard laboratory conditions and acclimatized to 12-h light/dark cycle for 5 days prior to the day of experimentation. They had free access to food (standard chow pellet) and water *ad libitum.*

All drugs required for this experiment was obtained from standard pharmaceutical companies and all required laboratory equipment and reagents were obtained from standard laboratory equipment and reagent suppliers. The study was approved by the IAEC (Institutional Animal Ethics Committee) (Annexure-I). The study was conducted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi.

Animals were randomly assigned to various groups. Before starting the experiments, coding and masking was done to all the animals used in the experiment by the guide. The study was conducted using two chronic inflammatory models, the first model used was cotton pellet granuloma model and 2nd was CFA induced Chronic inflammation in male Wistar rats.

CHRONIC INFLAMMATORY MODELS

1. Cotton pellet (foreign body)-induced granuloma model[112]

Chronic inflammation was induced in all 3 groups of rats after a washout period of 48h. In overnight starved (with water *ad libitum*) rats, after clipping the hair in axilla and groin, two sterile cotton pellets weighing 10 mg in right and left axilla and 2 sterile grass pith in right and left groin were implanted subcutaneously through a small incision under anesthesia (Thiopentonesodium 30mg/kg). Wounds were sutured and animals were caged individually after recovery from anesthesia. Aseptic precaution was maintained throughout the procedure. Rats in the disease control group were induced with chronic inflammation and they were administered with normal saline orally for 21 days. Rats in the standard drug group were treated with aspirin 200mg/kg, orally for 21 days and rats in the test drug group were treated with ranolazine 180mg/kg orally for 21 days. (Table-1)

On 22nd day, the rats were sacrificed with an overdose of anesthesia (Thiopentone sodium 120mg/kg) and animals were sacrificed. Later, cotton pellets were removed from the both axilla of rats. The cotton pellets freed from extraneous tissue and was dried overnight at 60°C to note their dry weight. Net granuloma weight was calculated by subtracting initial weights of the cotton pellet (10 mg) from the weights noted. Mean granuloma dry weight of cotton pellet in all three groups was calculated and expressed as mg/100 grams of body weight[112, 113].

The percentage inhibition of granuloma dry weight was calculated using the formula[84]: Percentage inhibition granuloma dry weight = 1- dry weight of granuloma in treated group/dry weight of granuloma in control group × 100

The grass piths obtained from right and left groin were preserved in 10% formalin[112, 84] and were processed in the pathology laboratory, Jeevan diagnostics, Belagavi, and sections were stained with H&E, and the granulation tissue in each group was studied microscopically.

Before sacrificing the animal, blood samples were collected through cardiac puncture and checked for the chronic inflammatory parameters like IL-1β, IL-6, Fibrinogen levels and CRP levels.[114]

Table:1 Experimental groups in cotton pellet granuloma model

Group no	**Groups n=6**	**Drug-Route of Administration**	**Dose(mg/kg) per orally**	**Days of drug given**
I	Disease control group	Normal saline-per oral	Equal vol (as standard dose)	21 days
II	Standard drug group	Aspirin-per oral	200mg/kg[12]	21 days
III	Test drug group	Ranolazine-per oral	180mg/kg[12]	21 days

Figure 2: Cotton pellet covered with granulation tissue (R_1-Rat1, R_2-Rat2, and R3-Rat3)

Figure 3: Grass pith covered with granulation tissue (R_1-Rat1, R_2-Rat2, and R3-Rat3)

Figure 4: Cotton pellet covered with granulation tissue in rat.

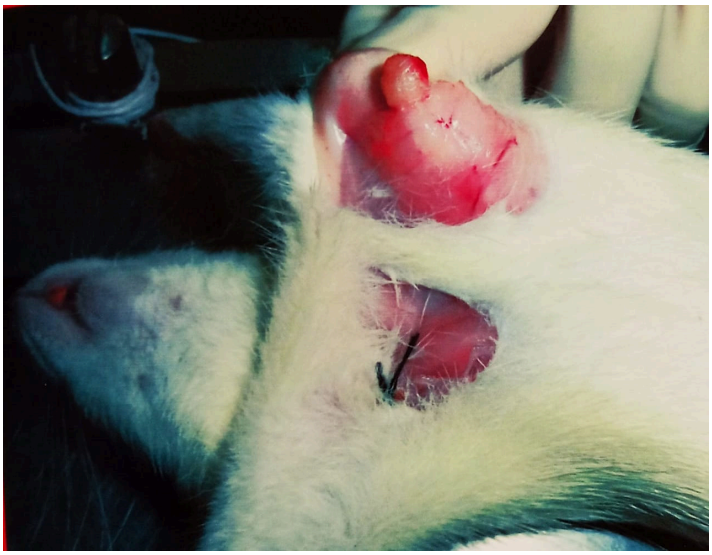
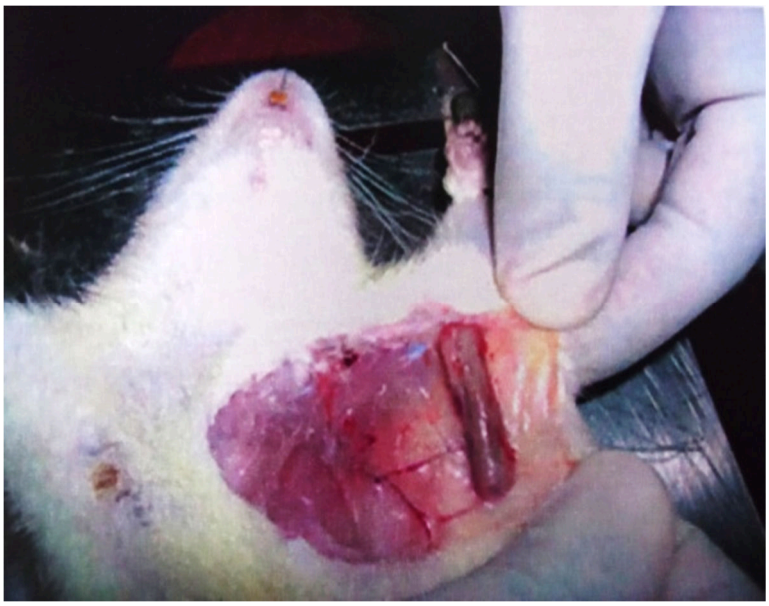


Figure 5: Grass pith covered with granulation tissue in rat.



2. Arthritis Model using Complete Freund's adjuvant[19, 112].

The chronic inflammatory arthritis was induced in all 3 groups of rats (male Wistar rats) using the Complete Freund's adjuvant (CFA), by subcutaneously injection of 0.1 ml into the plantar surface of the right hind paw. Rats in the disease control group were induced with chronic inflammation and they were administered with normal saline orally for 21 days. Rats in the standard drug group were treated with aspirin 200mg/kg, orally for 21 days and rats in the test drug group were treated with Ranolazine 180mg/kg orally for 21 days.

Adjuvant arthritis characterized by chronic proliferative and inflammatory reactions in synovial membranes, producing pain, disability and eventually destruction of joints. Aseptic precaution was maintained throughout the procedure. The treatment was started after CFA induction and was repeated every 24h, regularly for 21 days[114].(Table-2)

On 22nd day, the rats were sacrificed with an overdose of anesthesia using Thiopentone sodium 120mg/kg[19]. After the animals were sacrificed, tissue sections of ankle joints of CFA injected hind paws of rats was collected and checked for collagen content[19, 84]. The blood sample from all the animals of 3 groups was taken after 21 days of treatment to check the levels of investigating parameters i.e. TNF-α, IL-6, IL-1β, CRP and compared with all groups[114].

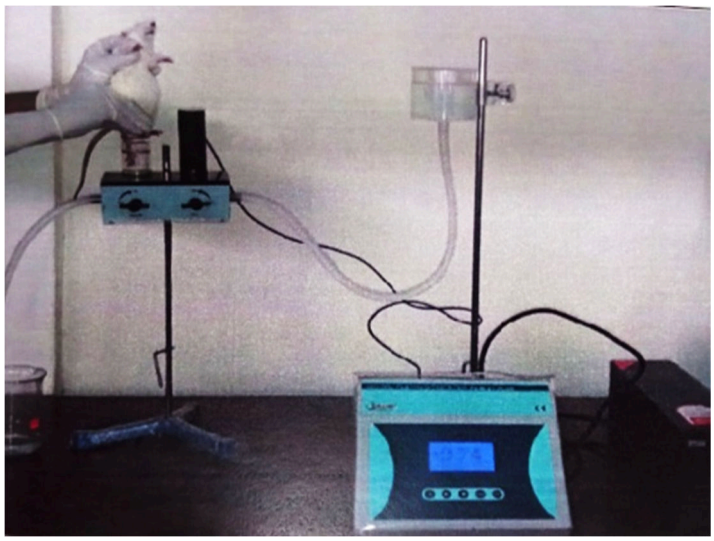
The blood obtained before sacrificing the rats was centrifuged and the serum thus obtained was used to estimate the levels of inflammatory markers using ELISA kits.

Table: 2- Experimental groups in Complete Freund's adjuvant model

Group no	**Group n=6**	**Drug-Route of Administration**	**Dose(mg/kg) per orally**	**Days of drug given**
I	Disease control group	CFA induction- Normal saline-per oral	Equal vol (as standard dose)	21 days
II	Standard drug group	Aspirin-per oral	200mg/kg	21 days
III	Test drug group	Ranolazine-per oral	180mg/kg	21 days

Figure 6: CFA injection in rat paw

Figure 7: Plethysmographic measuring the rat paw edema



Drugs used and their dosages:

1. Aspirin – was obtained as a tablet from Reckitt Benckiser India Limited. The clinical dose of 2222mg was converted to rat equivalent dose that was 200mg/kg of rat. It was administered orally, as a suspension of 1% gum acacia.
2. Ranolazine – was obtained as a tablet form Torrent Pharmaceuticals Ltd. The clinical dose of 1000mg was converted to rat equivalent dose that was 180mg/kg of rat. It was administered orally.
3. Complete Freund's adjuvant – was obtained from (Sigma Aldrich, St. Louis, USA) Rajendra Traders, Dharwad.

Statistical analysis:

The data for all the groups was expressed as Mean±SEM. Data was analyzed by one-way ANOVA (Analysis of variance) followed by Dunnett's test to compare with control group using Graph pad prism software and $p \leq 0.05$ was considered as statistically significant.

Comparison between aspirin and Ranolazine treatment group was done by one-way ANOVA followed by Bonferroni's test using graph pad prism software and $p \leq 0.05$ was considered to be statistically significant between aspirin and Ranolazine treatment groups.

Paw edema volume levels data was compared by Tukeys multiple posthoc test using by graph pad prism software and $p \leq 0.05$ was considered to be statistically significant between aspirin and Ranolazine treatment groups.

RESULTS

In the present study, Ranolazine in its therapeutic equivalent dose was investigated for its possible anti-inflammatory activity using chronic inflammatory models in male Wistar rats.

1. Cotton pellet-induced granuloma model (Foreign body induced granuloma model)

1. a. Effect of Ranolazine on mean granuloma dry weight of cotton pellet

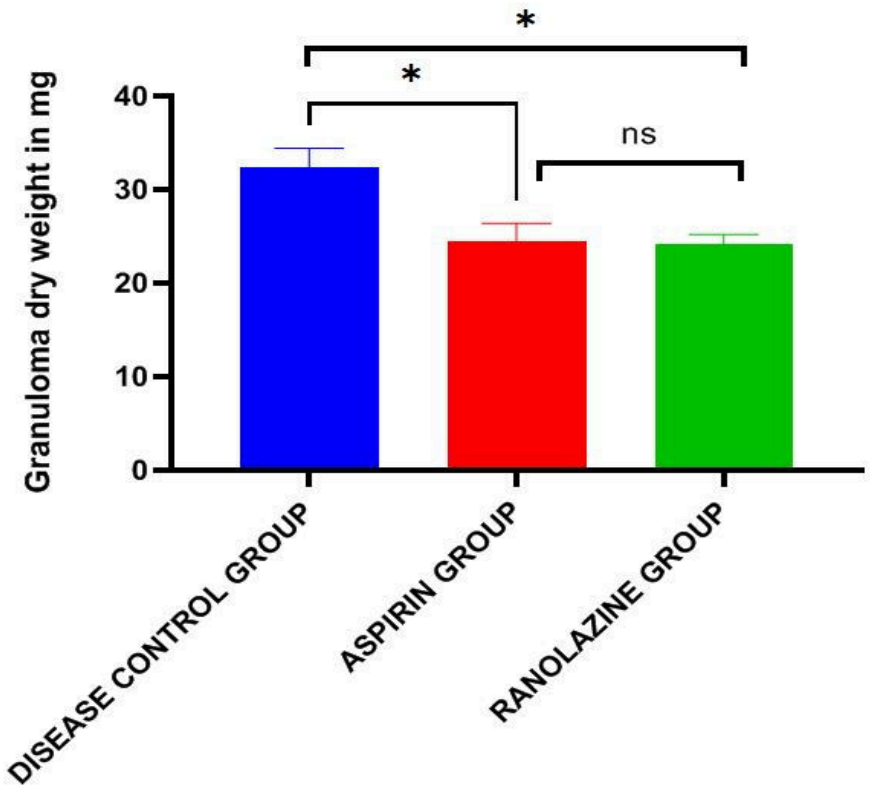
The cotton pellet recovered from the rats of all 3 groups were measured to record its dry weight of granuloma at the end of the study. The mean dry weight of 21 days old granuloma were 32.30±2.10, 24.56±1.862, 24.13±1.04 in disease control group, aspirin treated group and ranolazine treated group respectively. The dry weight of granuloma in Ranolazine treated group was significantly reduced ($p \leq 0.017$) as compared to disease control group. However, there is no significant difference ($p \leq 087$). in the weight of granuloma between ranolazine treated and aspirin treated group. Aspirin treated group showed significant reduction ($p \leq 0.015$) in granuloma dry weight as compared to disease control group. The percentages of inhibition in granuloma dry weight were 25.04% and 24.02% in Ranolazine treated and aspirin treated group respectively. (Table-3, graph-1)

Table-3: Effect of Ranolazine on mean granuloma dry weight of cotton pellet

Sl. No	Groups n=6	Mean granuloma dry weight mg/100gm body weight (Mean±SEM)	Percentage granuloma inhibition
1	Disease control	32.30±2.10	--
2	Aspirin	24.49±1.86*	24.02%
3	Ranolazine	24.13±1.04*	25.04%

ANOVA: Post hoc analysis by Dunnet's test: *p<0.01. SEM-Standard Error of mean.

Graph-1: Effect of Ranolazine on mean granuloma dry weight of cotton pellet



Post hoc analysis by Dunnet's test: Disease control group vs aspirin group *p≤0.05, Disease control group vs Ranolazine group *p≤0.05, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group

1. b. Effect of Ranolazine on Inflammatory markers

Blood samples were collected from the rats at the end of the study and serum inflammatory markers were measured. The effect of Ranolazine was studied on inflammatory markers like CRP, IL-6, IL-1β and TNF-α. The levels of CRP (ng/ml) in disease control group, aspirin treated group and ranolazine treated group were 26.93±0.902, 13.33±0.751, 13.02±1.518 respectively. At the end of study, serum CRP level was significantly increased in disease control group. According to post hoc analysis by Dunnet's test, Ranolazine treated group($p \leq 0.0001$) and aspirin treated group($p \leq 0.0001$) showed statistically significant reduction in serum CRP level as compared to disease control group. However, Boneferonii's test analysis showed no significant difference ($p \leq 0.8$) in serum CRP level between Ranolazine and Aspirin treated groups. (Table-4, Graph-2).

At the end of 21 day, the serum level of IL-6 (pg/ml) was measured in all 3 groups. The levels of IL-6 in disease control group, aspirin treated group and ranolazine treated group were 134.6±9.970, 50.48±2.698 and 41.38±5.688 respectively. At the end of study, serum IL-6 level was significantly increased in disease control group. According to the post hoc analysis by Dunnet's test Ranolazine treated group($p \leq 0.0001$) and aspirin treated group($p \leq 0.0001$) showed statistically significant reduction and in serum IL-6 level respectively as compared to disease control group. However, Boneferonii's test analysis showed no significant difference ($p \leq 0.3$) in serum IL-6 level between Ranolazine and aspirin treated groups. (Table-4, Graph-3)

Similarly, IL-1β levels (pg/ml) were measured at the end of the study and rats in disease control group, aspirin treated group and ranolazine treated group showed 0.605±0.0563, 0.2400±0.0284 and 0.3467±0.0699 respectively. There was a significant increase in serum IL-1β level in disease control group due to foreign body induced inflammatory reaction. According to the post hoc analysis by Dunnet's test, Ranolazine treated group ($p \leq 0.0005$) and aspirin treated group ($p \leq 0.008$) showed statistically significant reduction in IL-1β level as compared to disease control group. However, Boneferonii's test analysis showed no significant difference ($p \leq 0.1$) in serum IL-1β level between Ranolazine and aspirin treated group. (Table-4, Graph-4).

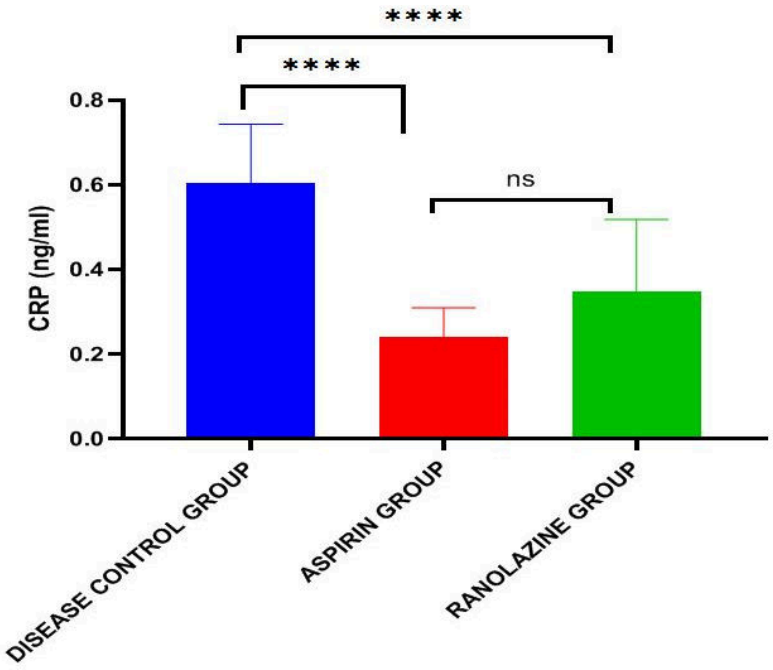
Also, the levels of TNF-α (pg/ml) were measured at the end of the study. The serum level of TNF-α in disease control group, aspirin treated group and ranolazine treated group were 21.12±2.608, 13.24±1.924 and 11.75±0.978 respectively. There was a significant increase in serum TNF-α level in disease control group. According to the post hoc analysis by Dunnet's test, Ranolazine treated group ($p \leq 0.02$) and aspirin treated group ($p \leq 0.007$) showed statistically significant reduction in TNF-α level as compared to disease control group. However, Boneferonii's test analysis showed no significant difference ($p \leq 0.5$) in serum TNF-α level between Ranolazine and aspirin treated group. (Table-4, Graph-5).

Table-4: Effect of Ranolazine on serum inflammatory markers

Sl.No.	Groups n=6	Serum levels (Mean±SEM)			
		CRP (ng/ml)	IL-6 (pg/ml)	IL-1β(pg/ml)	TNF-α (pg/ml)
1	Disease Control	26.93±0.902	134.6±9.970	0.605±0.0563	21.12±2.608
2	Aspirin	13.33±0.751****	50.48±2.698****	0.2400±0.0284***	13.24±1.924*
3	Ranolazine	13.02±1.518****	41.38±5.688****	0.3467±0.0699**	11.75±0.978**

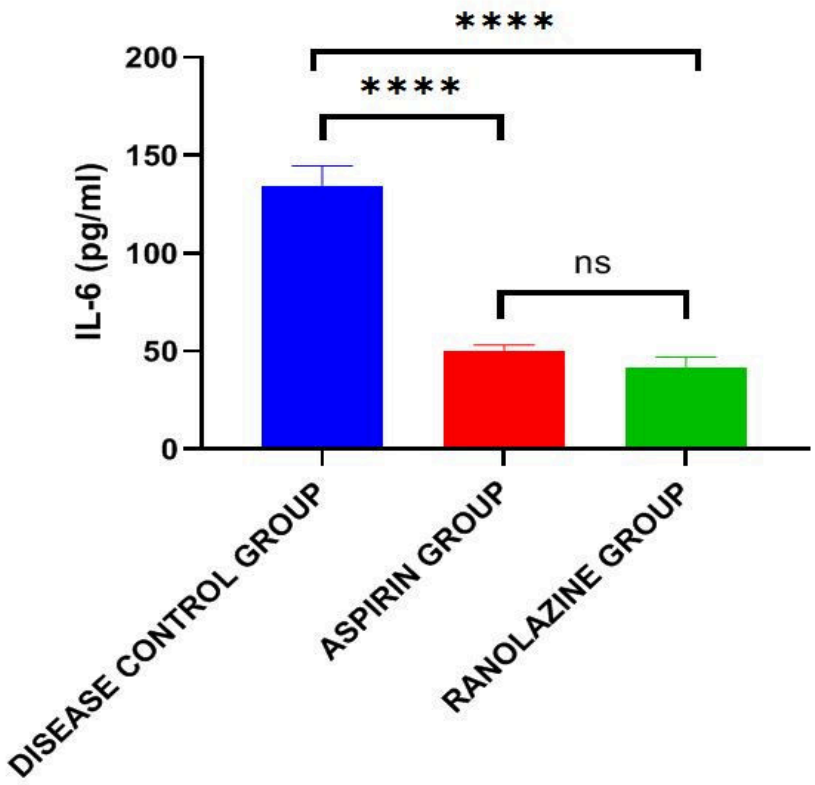
Statistical analysis using ANOVA, Post hoc analysis by Dunnett's test with P value *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, ****$p \leq 0.0001$, n=6 in each group, SEM- Standard error of mean.

Graph-2: Effect of Ranolazine on serum CRP levels in cotton pellet model



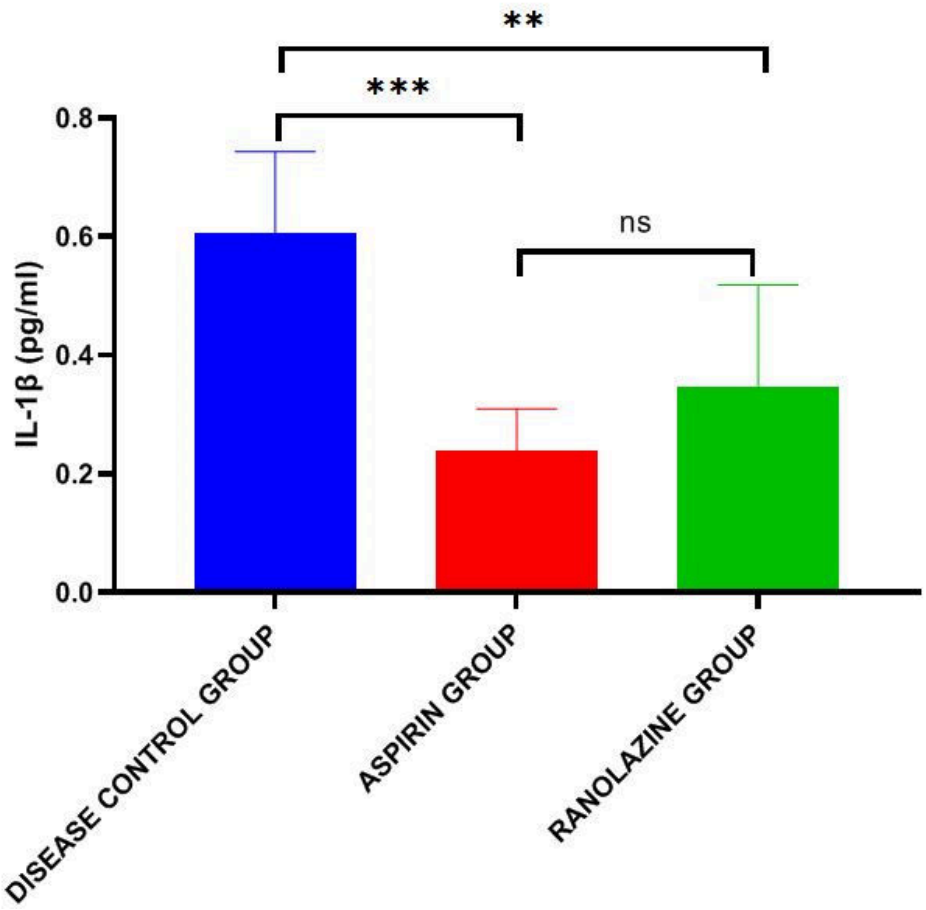
Post hoc analysis by Dunnet's test: Disease control group vs aspirin group ****p≤0.0001, Disease control group vs Ranolazine group ****p≤0.0001, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, CRP=C reactive protein

Graph-3: Effect of Ranolazine on serum IL-6 levels in cotton pellet model



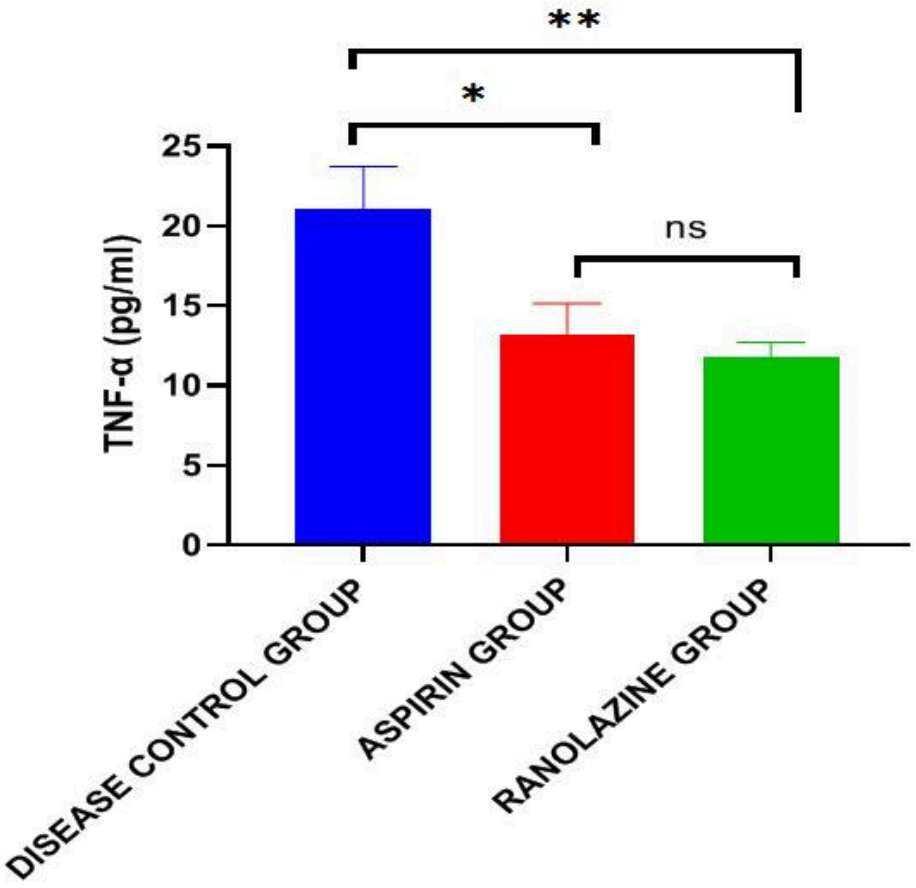
Post hoc analysis by Dunnet's test: Disease control group vs aspirin group ****p≤0.0001, Disease control group vs Ranolazine group ****p≤0.0001, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, IL-6=Interleukin 6

Graph-4: Effect of Ranolazine on serum IL-1β levels in cotton pellet model



Post hoc analysis by Dunnet's test: Disease control group vs aspirin group ***p≤0.0005, Disease control group vs Ranolazine group **p≤0.005, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, IL-1β=Interleukin 1 beta

Graph-5: Effect of Ranolazine treatment on serum TNF-α levels in cotton pellet model



Post hoc analysis by Dunnet's test: Disease control group vs aspirin group *p≤0.05, Disease control group vs Ranolazine group **p≤0.005, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, TNF-α = Tissue Necrosis Factor alpha

1. c. Histopathological images of Grass pith granuloma

The effect of ranolazine on inflammation was further evaluated by histopathological studies. The sections of grass pith when stained with H&E showed dense area of inflammation, abundant granulation tissue, dense band of fibrous tissue in disease control animals, while histopathological report in aspirin and ranolazine treated rats showed sparse inflammation and thin band of fibrous tissue. The result indicates that ranolazine reduces the inflammation as standard drug in chronic inflammatory model.

Figure-8: Histopathological images of grass pith granulation in Disease Control group

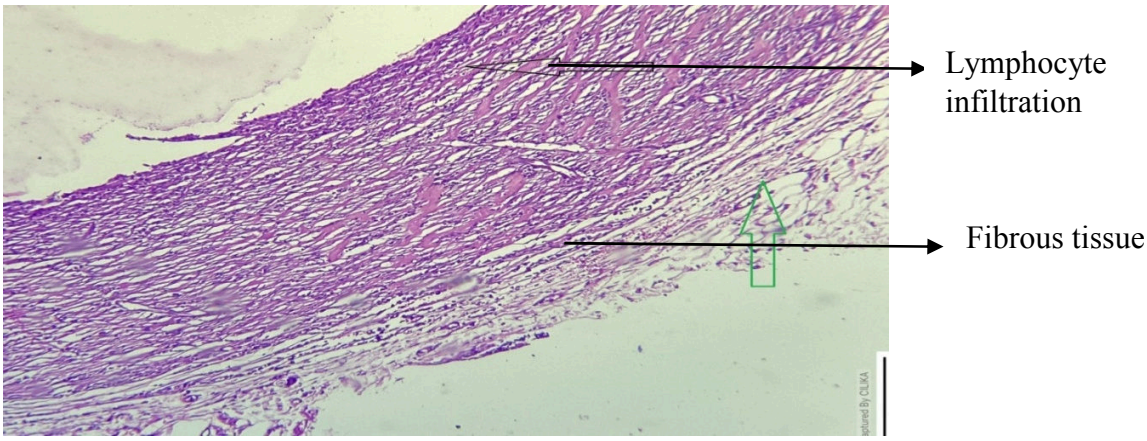


Figure-9: Histopathological images of grass pith granulation in Aspirin treated group

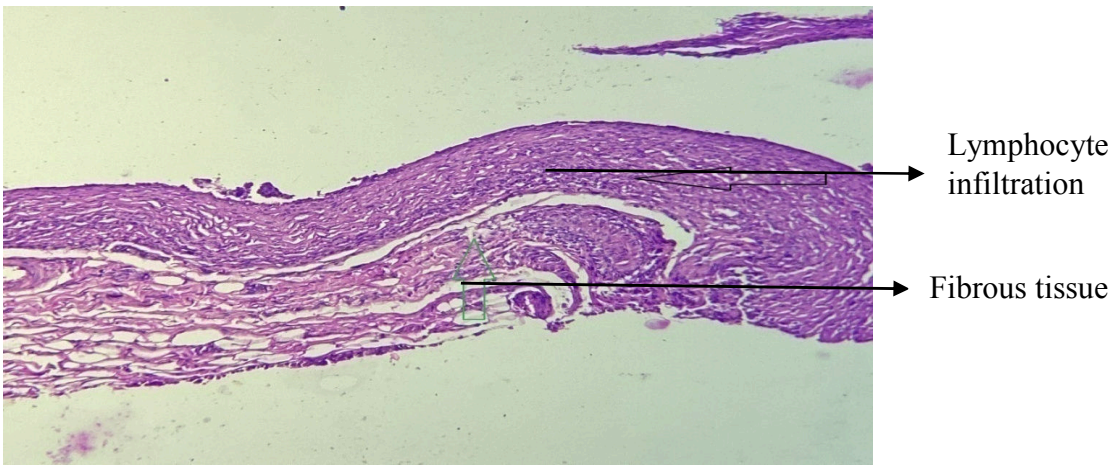
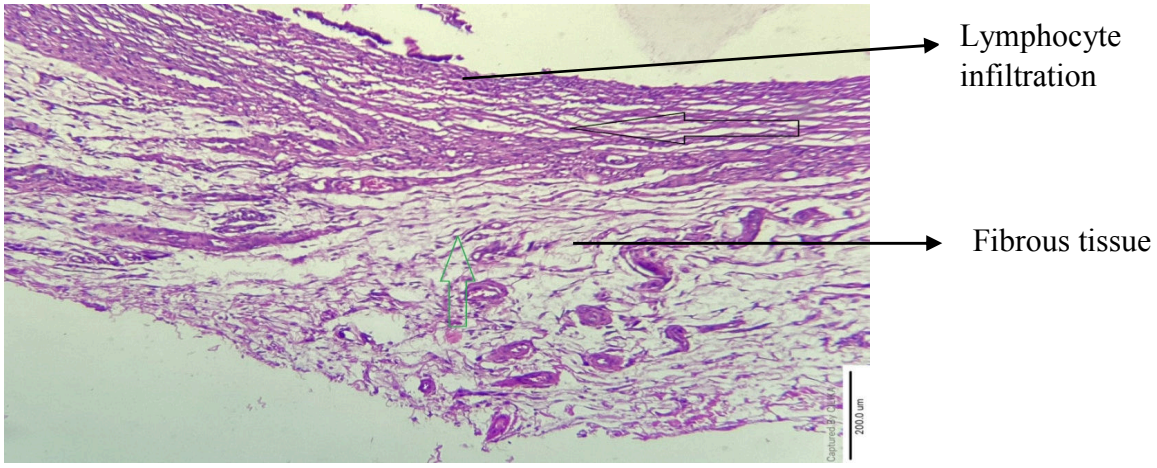


Figure-10: Histopathological images of grass pith granulation in Ranolazine treated group



→ Fibrous tissue →Lymphocyte infiltration

Note: Abundant granulation tissue, inflammatory cells and fibrous tissue in disease control rats. Markedly reduced granulation tissue, fibrous tissue and inflammatory cells in Aspirin and Ranolazine treated rats.

2. Arthritis Model using Complete Freund's adjuvant (CFA)

In this study, Ranolazine in therapeutic equivalent dose was investigated for its possible anti-inflammatory activity on chronic model of inflammation in male Wistar rats.

2. a. Effect of Ranolazine on CFA induced paw edema

The paw edema was measured by water displacement using a digital plethysmometer on day 1 and day 21. The mean paw edema volume ml on day 1 in disease control group, aspirin treated group and Ranolazine treated group were 2.27±0.19, 2.18±0.08 and 2.14±0.13 respectively. And on day 21 the mean paw edema volume in disease control group, aspirin treated group and Ranolazine treated group were 2.45±0.30, 2.00±0.15 and 2.02±0.32 respectively.

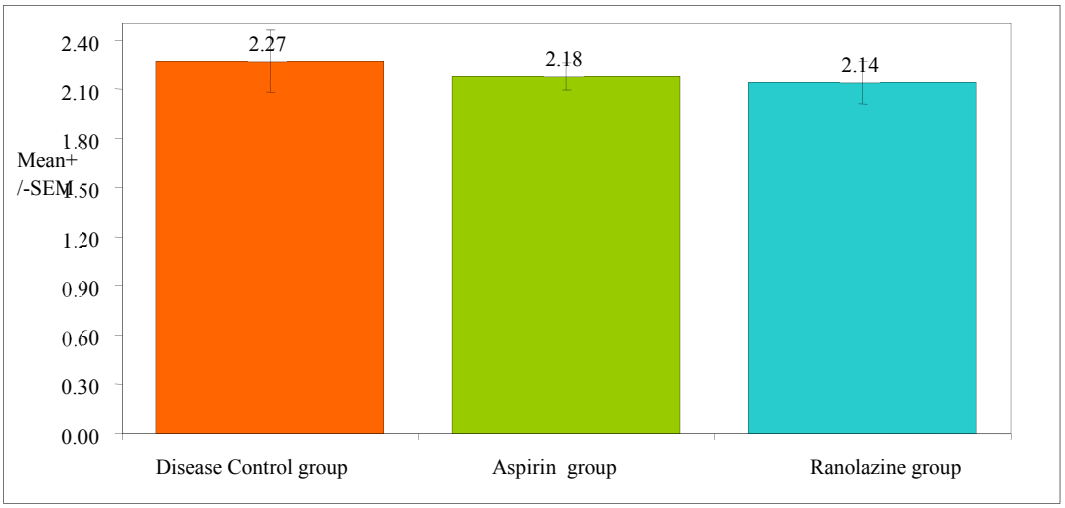
According to Tuekys multiple posthoc procedures test, on day 1 there was no significant difference in the paw edema volume in disease control group, aspirin and Ranolazine treated group. (Table-5 ,Graph-6). But on day 21 rats in aspirin ($p \leq 0.02$) and Ranolazine treated groups ($p \leq 0.03$) showed statistically significant reduction in paw edema volume. (Table-5, Graph-7).

Table-5: Effect of Ranolazine on CFA's induced paw edema.

Day after CFA injection	Paw edema in ml (Mean±SEM)		
	Disease control	Aspirin treated group	Ranolazine treated group
Day 1	2.27±0.19	2.18±0.08	2.14±0.13
Day 21	2.45±0.30	2.00±0.15*	2.02±0.32*
p value	ns	0.024*	0.034*

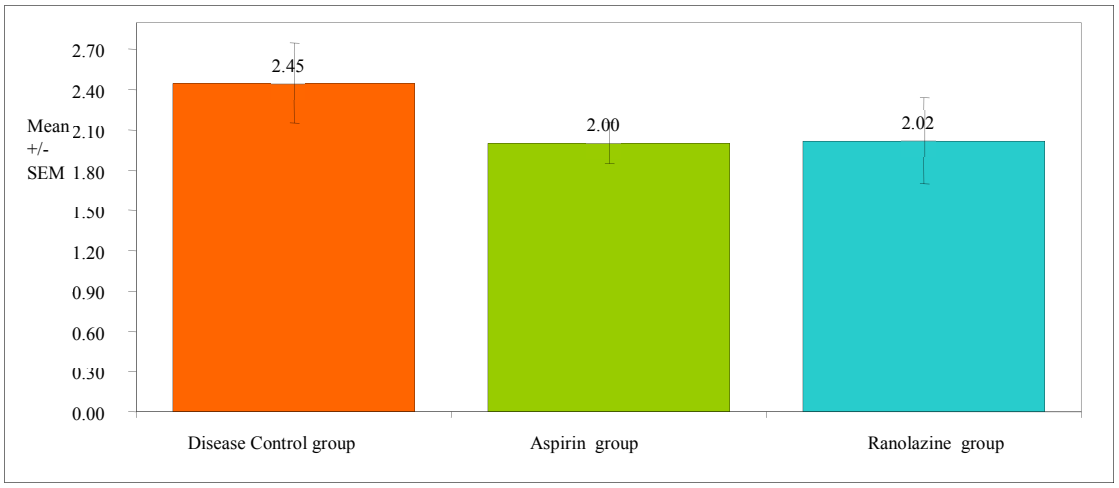
Tukeys multiple posthoc: *$p \leq 0.05$. ns- non significant, SEM=Standard Error of Mean, n=6 in each group

Graph-6: Effect of Ranolazine on CFA induced paw edema on day 1



Tukeys multiple posthoc analysis: p≤0.05. SEM=Standard Error of Mean, n=6 in each group.

Graph-7: Effect of Ranolazine on CFA induced paw edema volume when compared with aspirin on day 21



Tuekys multiple posthoc analysis: P≤0.05. SEM=Standard Error of Mean, n=6 in each group.

2. b. Effect of Ranolazine on Inflammatory markers

The effect of Ranolazine was studied on inflammatory markers like CRP, IL-6, IL-1β and TNF-α. at the end of the study. The levels of CRP (ng/ml) in disease control group, aspirin treated group and Ranolazine treated group were 28.95±4.382, 17.82±1.320, 18.13±0.252 respectively. At the end of study, serum CRP level was significantly increased in disease control group. According to the post hoc analysis by Dunnet's test Ranolazine treated group ($p \leq 0.02$) and aspirin treated group ($p \leq 0.01$) showed statistically significant reduction in serum CRP level as compared to disease control group. However, Boneferroni's test analysis showed no significant difference ($p \leq 0.5$) in serum CRP level between Ranolazine and aspirin treated groups. (Table-6, Graph-8).

At the end of 21 day, the serum level of IL-6 (pg/ml) was measured in all 3 groups. The levels of IL-6 in disease control group, aspirin treated group and Ranolazine treated group were 58.65±2.874, 39.00±2.920 and 38.55±0.5309 respectively. At the end of study, serum IL-6 level was significantly increased in disease control group. According to the post hoc analysis by Dunnet's test Ranolazine treated group ($p \leq 0.0001$) and aspirin treated group ($p \leq 0.0001$) showed statistically significant reduction in serum IL-6 level respectively as compared to disease control group. However, Boneferroni's test analysis showed no significant difference ($p \leq 0.5$) in serum IL-6 level between Ranolazine and aspirin treated groups. (Table-6, Graph-9)

Similarly, IL-1β levels (pg/ml) were measured at the end of the study and rats in disease control group, aspirin treated group and ranolazine treated group showed 47.37±7.806, 24.02±3.193 and 22.10±2.134 respectively. There was a significant increase in serum IL-1β level in disease control group. According to the post hoc analysis by Dunnet's test Ranolazine treated group ($p \leq 0.005$) and aspirin treated group ($p \leq 0.009$) showed statistically significant reduction in IL-1β level as compared to disease control group. However, Boneferonii's test analysis showed no significant difference ($p \leq 0.6$) in serum IL-1β level between Ranolazine and aspirin treated groups. (Table-6, Graph-10).

Also, the levels of TNF-α (pg/ml) were measured at the end of the study. The serum level of TNF-α in disease control group, aspirin treated group and ranolazine treated group were 20.15±3.930, 11.68±0.472 and 11.22±0.391 respectively. There was a significant increase in serum TNF-α level in disease control group. According to the post hoc analysis by Dunnet's test Ranolazine treated group ($p \leq 0.03$) and aspirin treated group ($p \leq 0.02$)

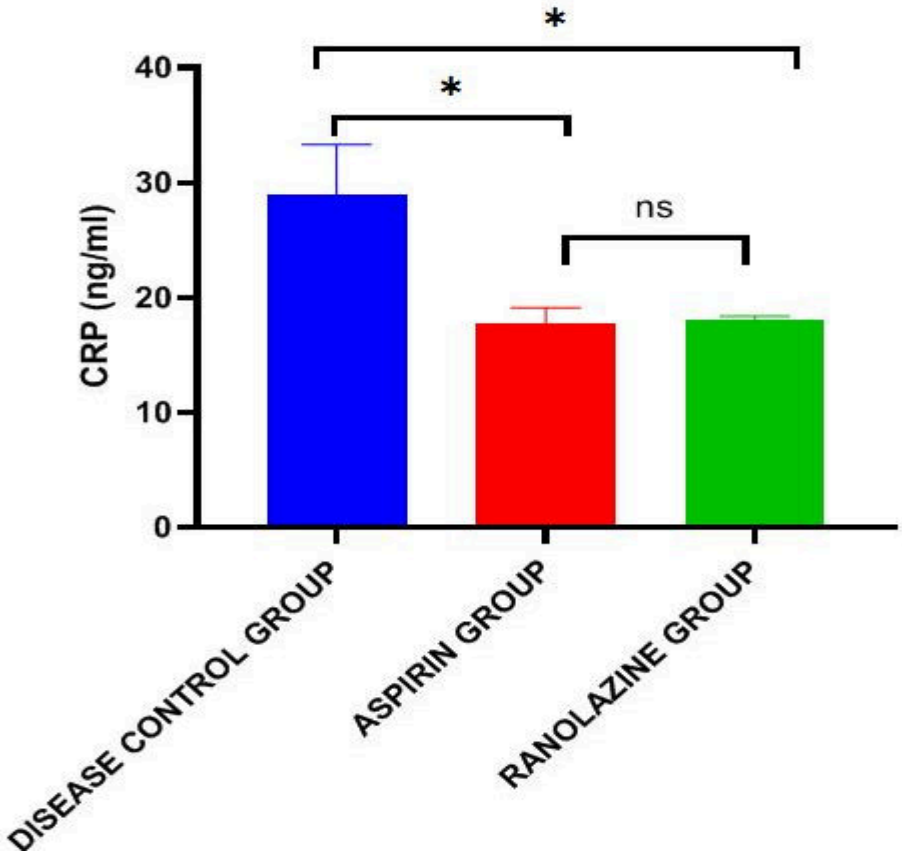
showed statistically significant reduction in TNF-α level as compared to disease control group. However, Boneferonii's test analysis showed no significant difference (p≤0. 7) in serum TNF-α level between Ranolazine and aspirin treated groups. (Table-6, Graph-11).

Table-6: **Effect of Ranolazine on serum inflammatory markers**

Sl.No.	**Groups**	**Serum levels (Mean±SEM)**			
		CRP (ng/ml)	**IL-6 (pg/ml)**	**IL-1β(pg/ml)**	**TNF-α (pg/ml)**
1	Disease Control	28.95±4.382	58.65±2.874	47.37±7.806	20.15±3.930
2	Aspirin	17.82±1.320*	39.00±2.920****	24.02±3.193**	11.68±0.472*
3	Ranolazine	18.13±0.252*	38.55±0.530****	22.10±2.134**	11.22±0.391*

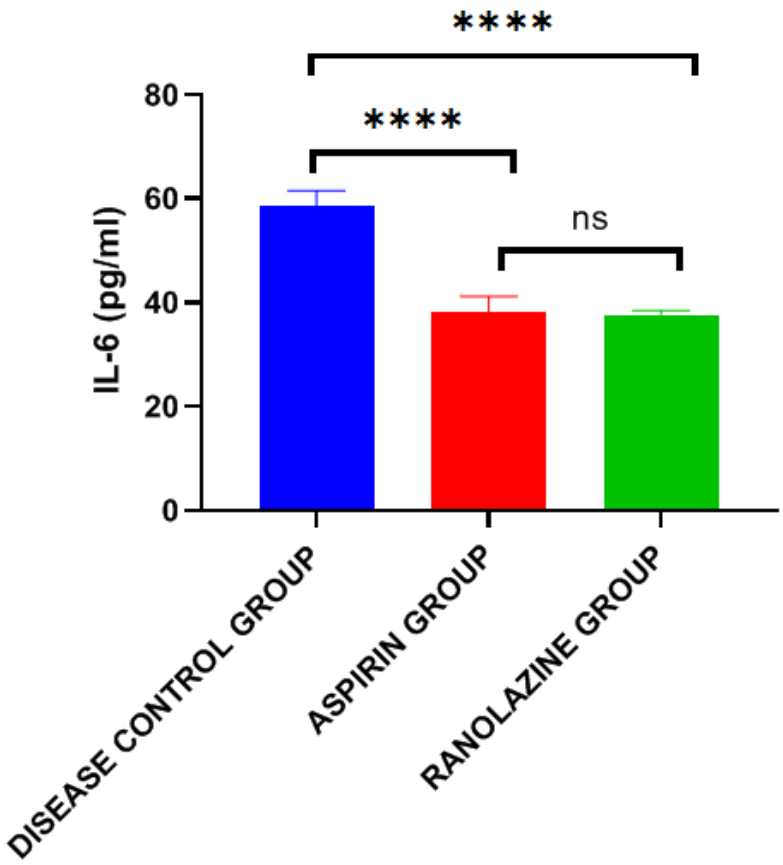
ANOVA: *p≤0.05, **p≤0.005, ****p≤0.0001, SEM=Standard Error of Mean, n=6 in each group

Graph-8: **Effect of Ranolazine on serum CRP levels in CFA induced chronic inflammation**



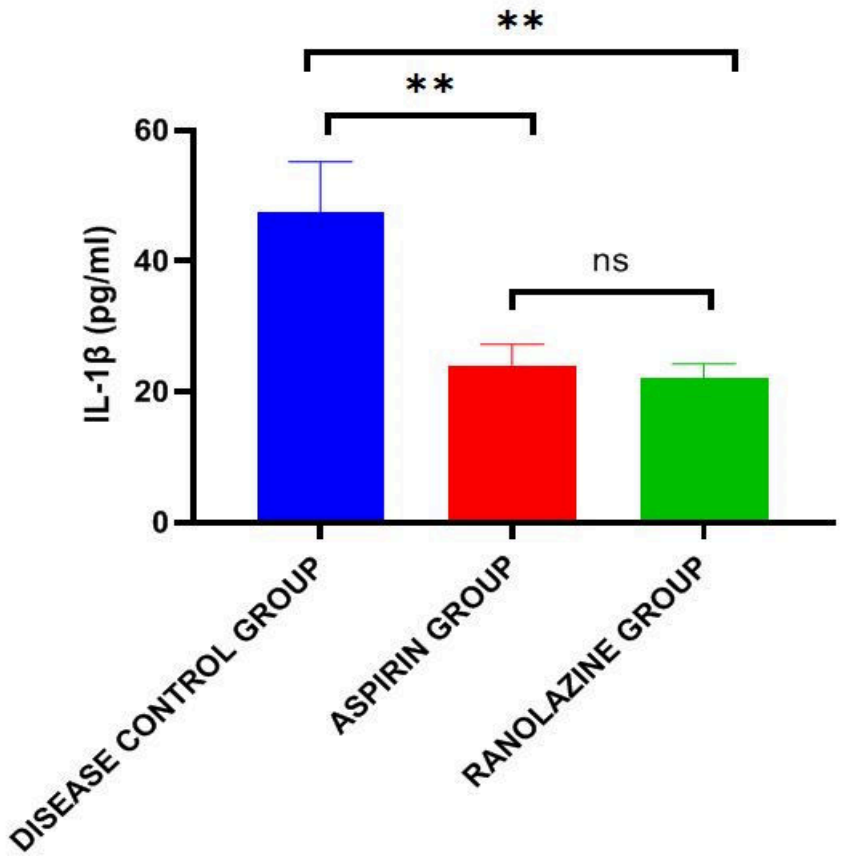
Post hoc analysis by Dunnet's test: Disease control group vs aspirin group *p≤0.05, Disease control group vs Ranolazine group *p≤0.05, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, CRP=C reactive protein

Graph-9: **Effect of Ranolazine on serum IL-6 levels in CFA induced chronic inflammation**



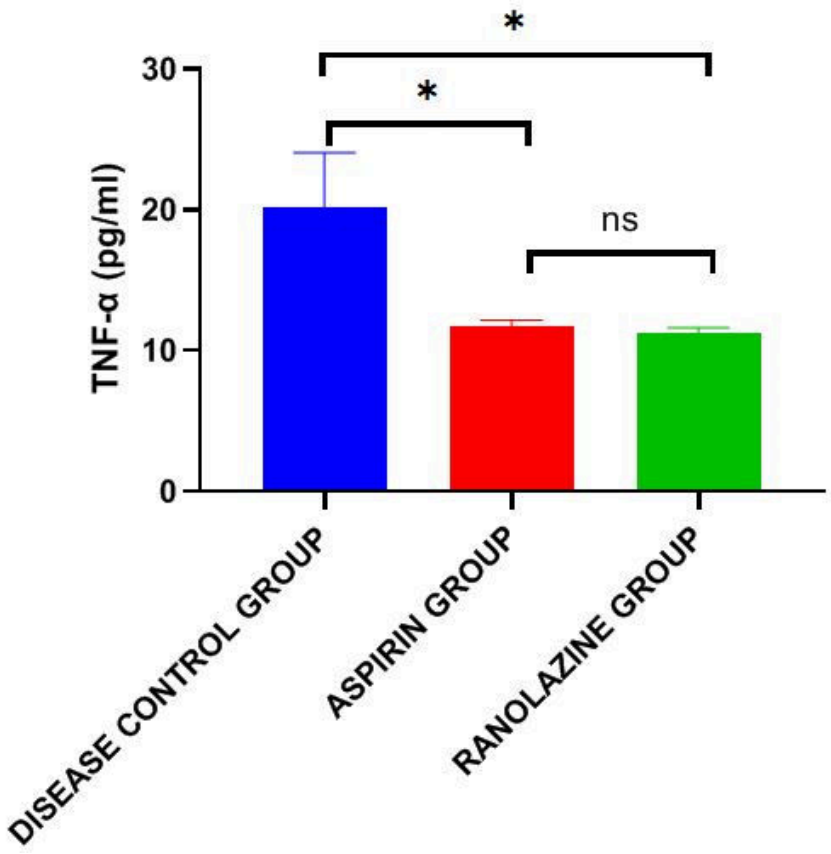
Post hoc analysis by Dunnet's test: Disease control group vs aspirin group ****p≤0.0001, Disease control group vs Ranolazine group ****p≤0.0001, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, IL-6= Interleukin 6

Graph-10: **Effect of Ranolazine on serum IL-1β levels in CFA induced chronic inflammation**



Post hoc analysis by Dunnet's test: Disease control group vs aspirin group **p≤0.005, Disease control group vs Ranolazine group ****p≤0.005, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, IL-1β= Interleukin 1 beta

Graph-11: **Effect of Ranolazine on serum TNF-α levels in CFA induced chronic inflammation**



Post hoc analysis by Dunnet's test: Disease control group vs aspirin group *p≤0.05, Disease control group vs Ranolazine group *p≤0.05, Boneferonii's test: aspirin group vs Ranolazine group- ns-non significant, n=6 in each group, TNF-α= Tissue Necrosis factor alpha

2. c. Histopathological images of CFA induced RA of ankle joint:

The histopathological examination of section of ankle joint in disease control rats showed cartilage destruction and vast inflammatory infiltrates, while aspirin and ranolazine treated rats showed much less cartilage destruction and scanty infiltration. This finding indicates ranolazine exhibit anti-inflammatory activity in chronic inflammation.

Figure-11: Histopathological image of CFA induced ankle joint in Disease control group

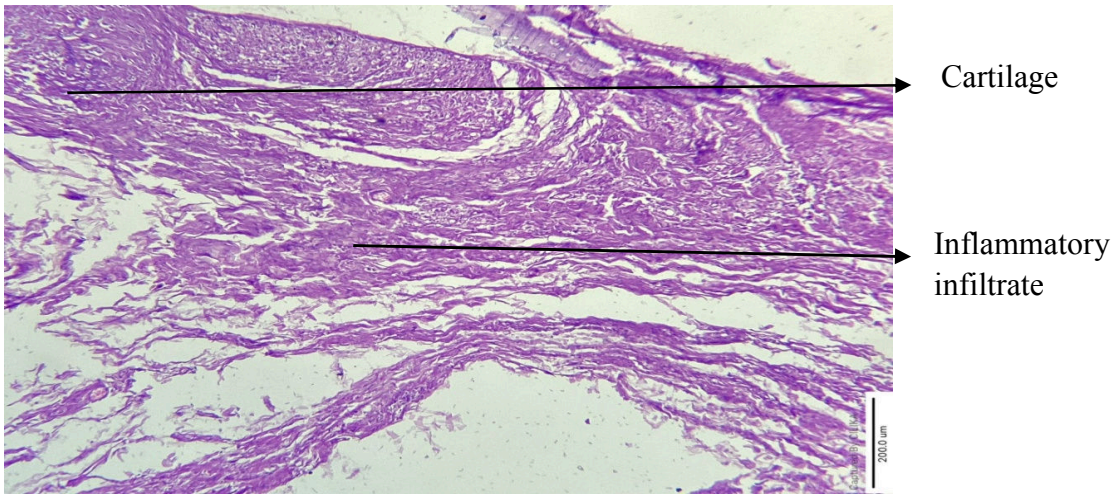


Figure-12: Histopathological image of CFA induced ankle joint in aspirin treated group

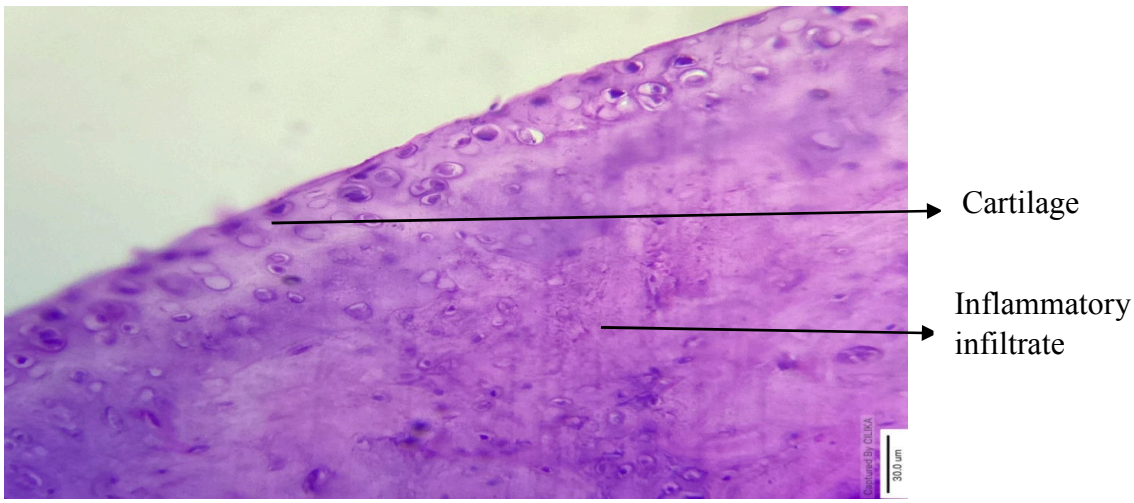
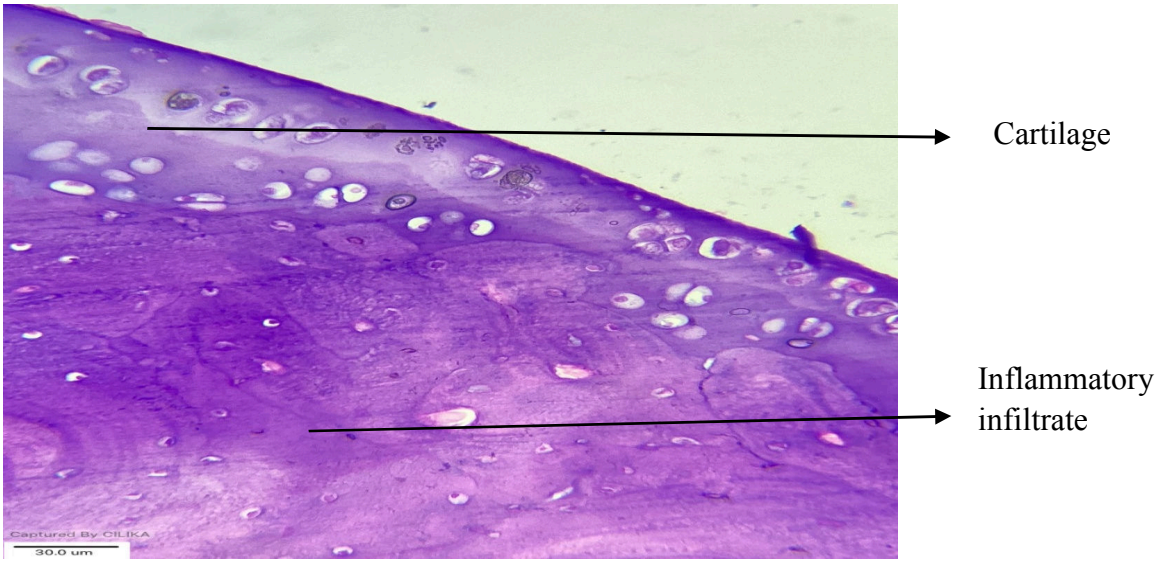


Figure-13: Histopathological image of CFA induced ankle joint in Ranolazine treated group



In the disease control the cartilage destruction and vast inflammatory infiltrates occupied, while in aspirin and ranolazine treated rats are much less cartilage destruction and scanty infiltrates are seen.

DISCUSSION

In the present study, Ranolazine-a piperazine derivative was investigated for its anti-inflammatory effects on chronic inflammatory models like a foreign body (cotton pellet) and CFA induced models in male Wistar rats. The evidence on the effects of Ranolazine on granulation tissue, paw edema, inflammatory cytokines and histopathology is conclusive in acute and sub-acute models; however, the effect of Ranolazine on chronic inflammatory model was not studied before, which has become the main objective of our present study. Through our present study and previous studies establishes the anti-inflammatory effects of Ranolazine and it can be used as an alternative or add on the therapy in the management of chronic inflammatory conditions like RA.

The foreign body induces inflammation, pro-inflammatory cytokines release and granulation tissue formation in rats, in a way similar to humans. The pro-inflammatory cytokines is brought about by foreign body by inflammatory process due to the immune system dys-regulation. Complete Freund's adjuvant (CFA) is considered to be a better antigen for edema induction compared to other chemicals, lesser toxicity, and lower mortality rate. As the CFA model for 21 days mimics the natural pathology of human chronic inflammation diseases rheumatoid arthritis, it was used for chronic inflammation induction in the current study. A state of chronic inflammation develops in the rats after 14 days of foreign body and CFA injection, and this considered the start of the disease. Also, 24 hours post injection is considered as the best time point for measuring the paw edema to confirm the induction of inflammation.

The results of present study showed significant reduction in the dry weight of granuloma and paw edema in Ranolazine treated group and aspirin treated group as compared to disease control group at the end of the study. These findings are consistent with the results of the previous acute and sub-acute study conducted in rats using foreign body and carrageenan induced inflammatory models[12].

With the progression of paw edema and granulation tissue, there was significant movement reduction in the disease control group compared to the treated animals. Such a drastic granuloma formation and movement disorder observed in the studies done by Zhang W, Lyu J et.al.[115.] This can be explained on the basis, that CFA induced paw edema

is decreased as a result of granulation tissue formation and movement of hand paw reduced[116].

The current investigation found that Ranolazine monotherapy was effective at preventing chronic inflammation; however, the values were comparable to aspirin therapy, indicating that Ranolazine can reduced edema and granuloma formation, which is consistent with earlier research.

At the end of 21 days, the results of our study showed significant reduction in the serum level of CRP, IL-6, IL-1β and TNF-α in Ranolazine treated group and aspirin treated group as compared to disease control group. The results of previous animal study done in sub-acute models of inflammation showed reduction in the serum level of inflammatory markers: CRP, IL-6, IL-1β and TNF- α level[12]. Our study also confirmed the beneficial effect of Ranolazine on the chronic inflammatory markers like CRP, IL-6, IL-1β and TNF-α level.

Ranolazine as monotherapy is effective in reducing the inflammatory responses and is proved to be a potent anti-inflammatory drug. The anti-inflammatory efficacy of Ranolazine is used in the management chronic stable angina pectoris.[118] According to a recent review, it has found that the anti-inflammatory efficacy of Ranolazine are due to various mechanisms. Ranolazine at the therapeutic level acts by inhibiting late phase of inward sodium channels. At higher concentrations ranolazine acts by inhibiting rapid delayed rectifier potassium current, fatty acid oxidation, glucose oxidation, reduces lactic acid production[119], effects on endothelial nitric oxide synthase (eNOS) phosphorylation , improves glycemic control via exerting vasodilatory action on the pre-capillary arterioles to recruit muscle microvasculature[120], decreases inflammatory mediators IL-1 β and TNFα, and increases anti-inflammatory PPARy as well as the antioxidant Cu/Zn-SOD in astrocytes in culture[121].

Results of our study showed that ranolazine is effective in reducing inflammatory markers in chronic models of inflammation and it is consistent with the results of consistent with the results of animal study conducted by Naveena R at.al. Where in expression of IL-1, TNF-α in the serum was reduced.[12]

In our study, histopathological examination of grass pith granulation tissue of Ranolazine treated rats showed improvement in the granulation tissue infiltration as

compared to that disease control group. This can be attributed to decrease in the levels inflammatory cell infiltration, congestion, neutrophil infiltration, lymphocyte infiltration, macrophages, and fibrosis. This report suggests that Ranolazine can control inflammatory response due to decrease in the pro inflammatory cytokines.[119]

In Ranolazine treated rats histopathological examination of ankle joint tissue showed improvement in the inflammatory reactions- reduction in the joint space, congestion, synovial ulceration, periarticular inflammation, periarticular lymphocyte, granulation and edema as compared to disease control group. And also, there was reduction in the fibroblasts in the chronic inflammation. This was associated with a reduced in number of fibroblasts in chronic inflammatory model.

LIMITATIONS AND FUTURE RECOMMENDATIONS

We have used single dose of Ranolazine based on the clinically used human dose. Further studies can be conducted utilizing various doses of Ranolazine, to find the optimum dose for the maximum benefit. Further, our study did not estimate the efficacy of ranolazine in co administration with standard anti-inflammatory drugs like NSAIDs.

CONCLUSION

The present study showed that Ranolazine reduces chronic inflammation in foreign body and CFA induced inflammatory models in rats. The anti-inflammatory effects of Ranolazine showed by reduction in the dry weight of cotton pellet granuloma, paw edema, and decrease serum level of pro inflammatory markers. Based on the findings of this study, it can be concluded that Ranolazine may be a promising option for the use as a next line drug or add on drug in the management of chronic inflammation.

SUMMARY

The present study was conducted to evaluate the effects of Ranolazine in a foreign body induced granuloma and CFA induced chronic inflammation model. In this study, the effect of Ranolazine on granuloma formation, paw edema, inflammatory markers and histopathology of grass piths and ankle joint were evaluated. We found that treatment of chronic inflammation in rats with oral Ranolazine showed improved in the granuloma formation, paw edema, inflammatory cytokine parameters and histopathology of fibrous tissue, lymphocyte infiltration.

Key findings of the study:

- Ranolazine as monotherapy was effective at preventing the formation of dry weight granuloma in foreign body induced and paw edema in CFAs induced model.
- Ranolazine as monotherapy lowered serum CRP, IL-6, IL-1β and TNF-α level in comparison with disease control rats.
- Ranolazine monotherapy showed improvement in the histological abnormalities in the grass pith and ankle joint compared to disease control rats.

According to the findings of this study, Ranolazine maybe a viable choice for usage as a second line or add on drug in the management of chronic inflammatory conditions. Clinical trials with bigger sample sizes, as well as further research to establish the appropriate anti-inflammatory dose of ranolazine, are recommended.

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ANNEXURE – I - IAEC APPROVAL CERTIFICATE

KLEACADEMYOF HIGHER EDUCATIONAND RESEARCH
(Deemed to be University)
JAWAHARLAL NEHRU MEDICAL COLLEGE,
NEHRU NAGAR, BELAGAVI - 590010, (KARNATAKA).
INSTITUTIONAL ANIMAL ETHICS COMMITTEE.
Phone No. JNMC (0831)- 2444040

Dr.(Mrs)P.P.Patil
Chairperson, IAEC.
Prof & Head Physiology,
J.N.Medical College, Belagavi

Dr.P.A.Patil
Main Nominee - CPCSEA
Prof & Head of Pharmacology,
USM-KLE, IMP, Belagavi

Dr.(Mrs)Rekha Nayaka M.R
Member - Secretary IAEC
Asso Prof of Pharmacology
J.N.Medical College, Belagavi

CPCSEA Reg.No.: 627/PO/Re/S/02/CPCSEA

MEMBERS:

Scientist-D, RMRC,
ICMR, Belagavi.

Non-scientific Social worker,
Nidasosi.

Hon.Veternarian,
Belagavi.

Officer Incharge,
Central Animal House,
JNMC, Belagavi.

Prof of Anatomy.
JNMC,Belagavi

AHM
Link Nominee CPCSEA.
Dept of Pharmacology &
Toxicology
KLE's Coll Of Pharmacy,
Hubballi

CERTIFICATE

This is to certify that the M.D/ M.D.S/ Ph.D/ Research project Entitled : "To Evaluate the effect of Ranolazine on chronic inflammation in male Wistar rats".

Submitted by- , PG Pharmacology,
JNMC.

Has been approved by the Institutional Animal Ethical Committee Meeting held on 5.2.2021 vide Resolution No. 14/2

For sanction of 36 Male Wistar Rats.

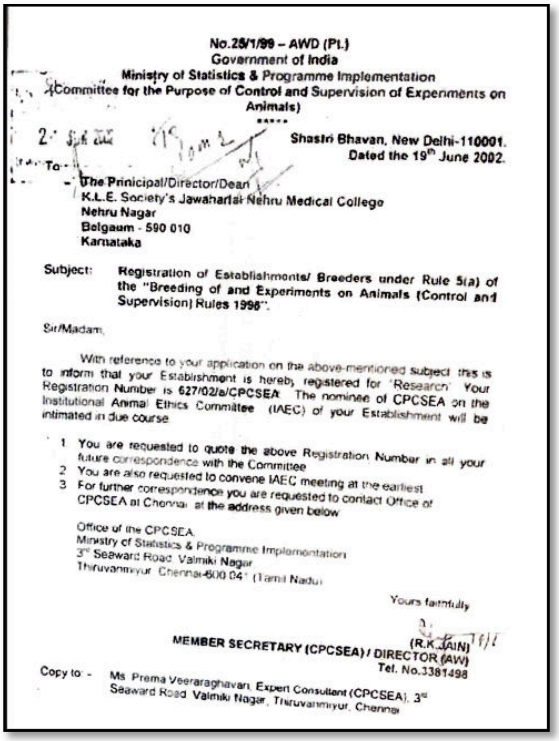
Main Nominee CPCSEA
IAEC-JNMC, Belagavi.

Signature and Name:
CPCSEA-Main Nominee

Member Secretary
IAEC-JNMC, Belagavi.

Signature and Name:
Chairman/Mem.Secretary

ANNEXURE - II - CPCSEA REGISTRATION & RENEWAL



No.25/1/99 – AWD (Pt.)
Government of India
Ministry of Statistics & Programme Implementation
Committee for the Purpose of Control and Supervision of Experiments on Animals)

Shastri Bhavan, New Delhi-110001.
Dated the 19th June 2002.

To -
The Principal/Director/Dean
K.L.E. Society's Jawaharlal Nehru Medical College
Nehru Nagar
Belgaum - 590 010
Karnataka

Subject: Registration of Establishments/ Breeders under Rule 5(a) of the "Breeding of and Experiments on Animals (Control and Supervision) Rules 1998".

Sir/Madam,

With reference to your application on the above-mentioned subject this is to inform that your Establishment is hereby registered for 'Research'. Your Registration Number is 627/02/a/CPCSEA. The nominee of CPCSEA on the Institutional Animal Ethics Committee (IAEC) of your Establishment will be intimated in due course.

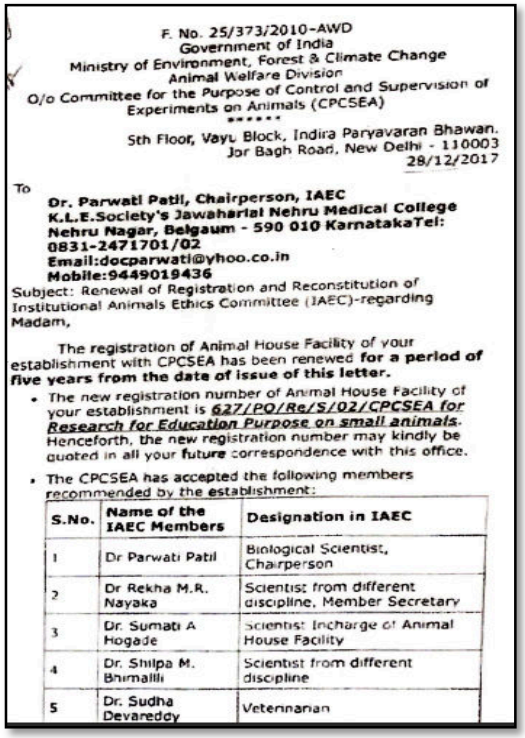
1. You are requested to quote the above Registration Number in all your future correspondence with the Committee.
2. You are also requested to convene IAEC meeting at the earliest.
3. For further correspondence you are requested to contact Office of CPCSEA at Chennai at the address given below.

Office of the CPCSEA,
Ministry of Statistics & Programme Implementation
3rd Seaward Road, Valmiki Nagar,
Thiruvanmiyur, Chennai-600 041 (Tamil Nadu)

Yours faithfully,

(R.K.JAIN)
MEMBER SECRETARY (CPCSEA) / DIRECTOR (AW)
Tel. No.3381498

Copy to: - Ms. Prema Veeraraghavan, Expert Consultant (CPCSEA), 3rd Seaward Road, Valmiki Nagar, Thiruvanmiyur, Chennai



F. No. 25/373/2010-AWD
Government of India
Ministry of Environment, Forest & Climate Change
Animal Welfare Division
O/o Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)

5th Floor, Vayu Block, Indira Paryavaran Bhawan,
Jor Bagh Road, New Delhi - 110003
28/12/2017

To

Dr. Parwati Patil, Chairperson, IAEC
K.L.E.Society's Jawaharlal Nehru Medical College
Nehru Nagar, Belgaum - 590 010 KarnatakaTel:
0831-2471701/02
Email:docparwati@yhoo.co.in
Mobile:9449019436

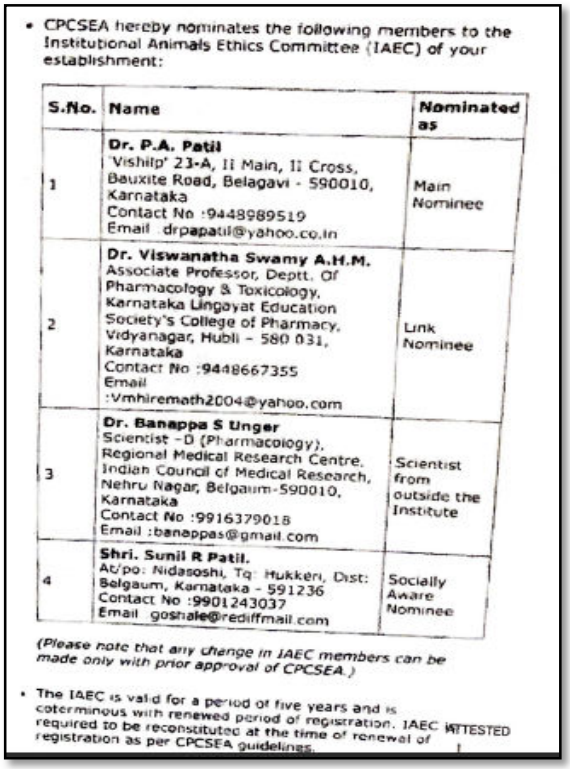
Subject: Renewal of Registration and Reconstitution of Institutional Animals Ethics Committee (IAEC)-regarding

Madam,

The registration of Animal House Facility of your establishment with CPCSEA has been renewed **for a period of five years from the date of issue of this letter.**

- The new registration number of Animal House Facility of your establishment is ***627/PO/Re/S/02/CPCSEA for Research for Education Purpose on small animals***. Henceforth, the new registration number may kindly be quoted in all your **future** correspondence with this office.
- The CPCSEA has accepted the following members recommended by the establishment:

S.No.	Name of the IAEC Members	Designation in IAEC
1	Dr Parwati Patil	Biological Scientist, Chairperson
2	Dr Rekha M.R. Nayaka	Scientist from different discipline, Member Secretary
3	Dr. Sumati A Hogade	Scientist Incharge of Animal House Facility
4	Dr. Shilpa M. Bhimalli	Scientist from different discipline
5	Dr. Sudha Devareddy	Veterinarian



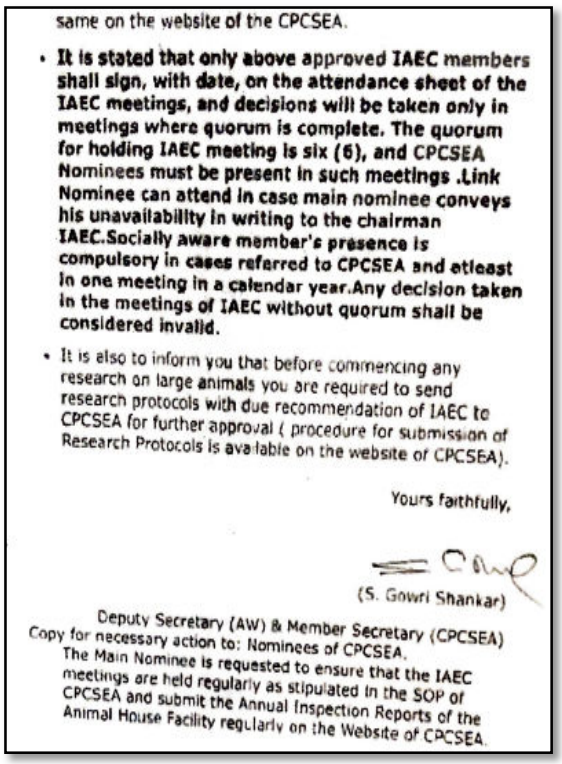
- CPCSEA hereby nominates the following members to the Institutional Animals Ethics Committee (IAEC) of your establishment:

S.No.	Name	Nominated as
1	**Dr. P.A. Patil** 'Vishilp' 23-A, II Main, II Cross, Bauxite Road, Belagavi - 590010, Karnataka Contact No :9448989519 Email :drpapatil@yahoo.co.in	Main Nominee
2	**Dr. Viswanatha Swamy A.H.M.** Associate Professor, Deptt. Of Pharmacology & Toxicology, Karnataka Lingayat Education Society's College of Pharmacy, Vidyanagar, Hubli - 580 031, Karnataka Contact No :9448667355 Email :Vmhiremath2004@yahoo.com	Link Nominee
3	**Dr. Banappa S Unger** Scientist –D (Pharmacology), Regional Medical Research Centre, Indian Council of Medical Research, Nehru Nagar, Belgaum-590010, Karnataka Contact No :9916379018 Email :banappas@gmail.com	Scientist from outside the Institute
4	**Shri. Sunil R Patil.** At/po: Nidasoshi, Tq: Hukkeri, Dist: Belgaum, Karnataka - 591236 Contact No :9901243037 Email :goshale@rediffmail.com	Socially Aware Nominee

(Please note that any change in IAEC members can be made only with prior approval of CPCSEA.)

- The IAEC is valid for a period of five years and is coterminous with renewed period of registration. IAEC required to be reconstituted at the time of renewal of registration as per CPCSEA guidelines.

ATTESTED



same on the website of the CPCSEA.

- **It is stated that only above approved IAEC members shall sign, with date, on the attendance sheet of the IAEC meetings, and decisions will be taken only in meetings where quorum is complete. The quorum for holding IAEC meeting is six (6), and CPCSEA Nominees must be present in such meetings .Link Nominee can attend in case main nominee conveys his unavailability in writing to the chairman IAEC.Socially aware member's presence is compulsory in cases referred to CPCSEA and atleast in one meeting in a calendar year.Any decision taken in the meetings of IAEC without quorum shall be considered invalid.**
- It is also to inform you that before commencing any research on large animals you are required to send research protocols with due recommendation of IAEC to CPCSEA for further approval (procedure for submission of Research Protocols is available on the website of CPCSEA).

Yours faithfully,

(S. Gowri Shankar)
Deputy Secretary (AW) & Member Secretary (CPCSEA)

Copy for necessary action to: Nominees of CPCSEA.
The Main Nominee is requested to ensure that the IAEC meetings are held regularly as stipulated in the SOP of CPCSEA and submit the Annual Inspection Reports of the Animal House Facility regularly on the Website of CPCSEA.