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REG.NO. BO0108001

Dissertation

Submitted to the

KLE University, Belgaum, Karnataka.

In partial fulfillment
of the requirements for the degree of

M. D. (DOCTOR OF MEDICINE)

IN

PHARMACOLOGY

Department of Pharmacology and
Pharmacotherapeutics,

J. N. Medical College,

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Endorsement by the Head Of Department, Principal/ Head of the institution

This is to certify that the dissertation entitled ***"EFFECT OF CIPROFLOXACIN, OFLOXACIN AND NORFLOXACIN ON ACUTE AND SUBACUTE INFLAMMATION IN MALE WISTAR RATS – AN EXPERIMENTAL STUDY"*** is a bonafied research work done by **THE CANDIDATE REG.NO. BO0108001.**

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ABBREVIATIONS

AA	:	Arachidonic acid
COX	:	Cyclo-oxygenase
CD	:	Cluster of differentiation
ECM	:	Extra cellular matrix
GM-CSF	:	Granulocyte macrophage colony stimulating factor
H_2O_2	:	Hydrogen peroxide
HOCl	:	Hypochlorite
HSP	:	Heat Shock Proteins
IgE	:	Immunoglobulin E
ICAM	:	Intercellular adhesion molecule
IL	:	Interleukin
IFN-γ	:	Interferon-γ
IO_2	:	Singlet oxygen
LFA	:	Leukocyte function antigen
LTB_4	:	leukotriene B_4
LTs	:	Leukotrienes
MICs	:	Minimum inhibitory concentration
ml	:	millilitre
mg	:	milligram
NSAIDs	:	Nonsteroidal anti-inflammatory drugs
NO	:	Nitric Oxide
NOS	:	Nitric oxide synthase

OH^-	:	Hydroxyl radicals
O_2^-	:	Superoxide anion radical
PGs	:	Prostaglandins
PGE_2	:	Prostaglandin E_2
PGF_1	:	Prostaglandin F_1
PID	:	Pelvic inflammatory disease
PMN	:	Polymorphonuclear leucocytes
PDGF	:	Platelet derived growth factor
PAF	:	Platelet activating factor
PECAM	:	Platelet endothelial cell adhesion molecule
PGI_2	:	Prostacyclin
RANTES	:	Regulated on activation normal T expressed and secreted
RBCs	:	Red blood cells
SEM	:	Standard error of Mean
SRS-A	:	Slow releasing substance of anaphylaxis
TNF	:	Tumour necrosis factor
TGF	:	Transforming growth factor
TXA_2	:	Thromboxane A_2
VEGF	:	Vascular endothelial growth factor
VCAM	:	Vascular cell adhesion molecule
5HT	:	5-Hydroxytryptamine
5-HPETE	:	5-Hydroperoxyeicosatetraeinoic acid
5-HETE	:	5-Hydroxyeicosatetraeinoic acid

Abstract

Objective:

The present study was planned to evaluate the effect of some fluoroquinolones viz. ciprofloxacin, ofloxacin and norfloxacin in their clinically equivalent doses in acute and subacute models of inflammation in male Wistar rats.

Materials and Methods:

Two models of inflammation were used:

1. Acute inflammation (carrageenan induced rat paw edema)
2. Subacute inflammation (foreign body induced granuloma)

Animals were divided into five groups, each group of six animals received orally either vehicle, standard (aspirin) or any one of the test drug in clinically equivalent doses. Aspirin was given half an hour before, while ciprofloxacin, ofloxacin and norfloxacin were given one hour before carrageenan injection. The rat paw volume was measured with the help of plethysmograph at different hours and percentage inhibition of edema in various treated groups was calculated.

In subacute model of inflammation, two sterile cotton pellets weighing l0mg and two sterile grass piths (25x2mm) were implanted subcutaneously, randomly in the axillae and groin, through a small incision under light halothane anaesthesia. The treatment was started on the day of implantation and was repeated every twenty-four hours, regularly, for ten days. On the eleventh day, the rats were sacrificed to obtain cotton pellets and grass piths covered with granulation tissue. Mean granuloma dry weight of cotton pellets for various groups was calculated. The sections of grass piths were stained with haematoxylin and eosin for histopathological studies.

Results:

All the three fluoroquinolones used in the present study showed significant anti-inflammatory activity in acute as well as subacute models of inflammation.

Conclusion:

The present study exhibited anti-inflammatory effects of fluoroquinolones. These findings suggest that fluoroquinolones when given in PID, gonococcal urethritis and tuberculosis by virtue of there anti-inflammatory activity may reduce complications of the disease like tubal stricture, infertility, urethral stricture etc. that occur because of significant inflammation associated with these conditions.

Key words: ciprofloxacin, ofloxacin, norfloxacin, aspirin, inflammation.

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INTRODUCTION

Inflammation is a complex reaction to injurious agents such as microbes and damaged, usually necrotic cells that consist of vascular response leading to accumulation of fluids, migration and activation of leukocytes and systemic reactions. Inflammation is fundamentally a protective response, the ultimate goal of which is to rid the organism of both the initial cause of cell injury like microbes, toxins and consequence of such injury like necrotic cells and tissue. Though the process of inflammation is brought about by vascular as well as cellular events, the former appears to contribute maximum for the pathogenesis of acute inflammation. This complex phenomenon involves endogenous chemical mediators such as histamine, 5-hydroxytryptamine, various chemotactic factors, bradykinin, leukotrienes and prostaglandins.[1]

The inflammatory response is closely interwined with the process of repair. Inflammation serves to destroy, dilute, or wall off the injurious agents, and sets into motion a series of events that try to heal and reconstitute the damaged tissue. During repair, the injured tissue is replaced through regeneration of native parenchymal cells or by filling of the defect with fibrous tissue (scarring) or most commonly by a combination of these two processes.[1]

Inflammation and repair with fibrosis may be potentially harmful. Fibrosis may lead to disfiguring scars or fibrous bands that cause intestinal obstruction or tubal block leading to infertility or urethral stricture. For this reason, our pharmacies abound with anti-inflammatory drugs, which ideally would control the harmful sequelae of inflammation yet not interfere with its beneficial effects.[1]

Many non steroidal anti-inflammatory drugs (NSAIDs) like aspirin, phenylbutazone, indomethacin etc are in clinical use but, all of these are not completely devoid of adverse effects.[2] Hence the search for safer and better anti-inflammatory agents other than NSAIDs continues. However, drugs which are not structurally related to NSAIDs like penicillamine,[2] allopurinol etc.,[2] have been in clinical use to treat inflammatory conditions like rheumatoid arthritis, gout etc.

Since vascular mechanisms contribute maximally for the pathogenesis of inflammation,[1] logically it could be expected that vasoactive substances could influence the process of inflammation. Accordingly, adrenergic agonists,[3] calcium channel blockers[4] and calcium[5] have also been reported to posses anti-inflammatory activity in experimental studies.

Some other drugs unrelated to NSAIDs like statins which inhibit HMG-CoA reductase enzymes[6, 7, 8] and sulfonamides like sulfamethizole[9] have also been reported to posses anti- inflammatory activity in experimental models though they are not routinely used in the treatment of inflammatory disorders.

Interestingly, studies have reported that ciprofloxacin induce production of Prostaglandin E_2 (PGE_2) which has been shown to exert immunomodulatory activity[10] and result in dysregulation of production of proinflammatory cytokines.[11] PGE_2 may act to attenuate cytokine induced inflammatory response.[12] In addition , ciprofloxacin on its own has shown to reduce expression of adhesion molecules[13] and suppress release of pro inflammatory cytokines.[14, 15] Invitro study has shown that ciprofloxacin significantly decreased Staphylococcus aureus Newman induced nasal inflammation by inhibiting interleukin(IL)-8 synthesis.[16]All these properties explain that ciprofloxacin may have anti inflammatory properties.

However invivo models have shown that ciprofloxacin induce inflammation of tendon eventually leading to its rupture in rats,[17] induce skin inflammation due to phototoxicity by increasing PGE_2 & 6 keto prostaglandin F_1(PGF_1) alpha from dermal fibroblasts.[18] In addition PGE_2 which is induced by ciprofloxacin has also been reported to possess pro-inflammatory activity like induction of Cyclo-oxygenase (COX) 2,[10] pro inflammatory cytokines (IL-23, IL-17),[19] P19 (Ubiquitous transgenic expression of P19 leads to multi organ inflammation and premature death), P40 [19]and produce clinical signs of inflammation like fever, hyperalgesia, edema etc.[1] Therefore, the effect of ciprofloxacin on inflammation remains controversial and also there is scanty information regarding the effects of other fluoroquinolones on inflammation.

Fluoroquinolones are used in the treatment of neisseria gonorrhoea and chlamydia infections which are one of the major causes for pelvic inflammatory disease (PID) in women and urethritis in men. In both these infectious conditions an inflammatory reaction develops followed by repair with fibrosis that results in tubal block and sterility in women and urethral stricture in males respectively as a sequelae.[20]

Fluoroquinolones are used as second line drugs in the treatment of tuberculosis.[21] This disease, which is caused by Mycobacterium tuberculosis is associated with inflammation, fibrosis and calcification which can lead to significant complications like pulmonary fibrosis, constrictive pericarditis, hydrocephalus, tubal stricture leading to infertility, peritoneal adhesions leading to intestinal obstruction.[22]

Since these infectious conditions are associated with significant inflammation leading to fibrosis, it could therefore be hypothesized that drugs possessing anti-inflammatory property to some extent may overcome the inflammatory reaction to infection and also its sequelae.

In view of the use of fluoroquinolones in PID & tuberculosis(as second line drug) and its controversial reports on inflammation, the present study was planned to explore the influence of ciprofloxacin and related fluoroquinolones like ofloxacin and norfloxacin on acute and subacute models of inflammation by comparing it with control and anti-inflammatory activity of aspirin (standard anti-inflammatory drug) in male Wistar rats.

OBJECTIVES

1. To investigate the influence of ciprofloxacin, ofloxacin and norfloxacin on inflammation in:

   i. Acute (carrageenan induced rat paw edema) model and

   ii. Subacute (foreign body induced granuloma) model,

   and to compare it with control and aspirin (standard anti-inflammatory drug).

REVIEW OF LITERATURE

A. INFLAMMATION:

The word "Inflammation" is derived from the latin word Inflammacio, which means "to set a fire".[23] Description regarding clinical features of inflammation can be seen in Egyptian papyrus (dated around 3000 BC), but it was Celsus, a Roman Encyclopedist of the first century AD, who for the first time listed the four cardinal signs of inflammation: rubor, tumor, calor and dolor (redness, swelling, heat, and pain). Though not precise, this concise description of inflammation generated keen interest among other investigators leading to the present status of knowledge of inflammation. A fifth clinical sign, loss of function (functio laesa), was later added by Virchow.[24]

In 1793, the Scottish surgeon John Hunter enunciated after a first hand study of injured tissue on the battlefield, that inflammation was a salutary process and not a disease. Subsequently, a German Pathologist, Julius Cohnheim (1870), gave a microscopic description of inflammation. Noting the initial changes in blood flow, the subsequent edema caused by increased vascular permeability, and the characteristic leukocyte emigration, he wrote descriptions of inflammation.[24]

In the 1880s, the Russian biologists Elie Metchnikoff discovered the process of phagocytosis. He concluded that the purpose of inflammation was to bring phagocytic cells to the injured area to engulf invading bacteria. At that time Metchnikoff contradicted the prevailing theory that the purpose of inflammation was to bring in factors from the serum to neutralize the infectious agents. It soon became clear that both, cells (phagocytes) and serum factors (antibodies) were critical for defense against microorganisms, and in recognition of this Metchnikoff and Paul Ehrlich (who developed the humoral theory of immunity) shared the Noble Prize in 1908.[23,24]

Barbour Henry linked the process of inflammation to fever and allergic processes and thus contributed immensely in understanding the pathophysiology of inflammation. The discovery of the fundamental concept of chemical mediators by Thomas Lewis (1913-1993), can be considered a milestone in the history of inflammation because not only did it help in identifying the other inflammogens but it also formed the basis for the development of newer anti-inflammatory agents.[24]

Inflammation is the local response of living mammalian tissues to injury due to any agent. It is a body defense reaction in order to eliminate or limit the spread of injurious agent, followed by removal of the necrosed cells and tissues.[25]

The agents causing inflammation maybe as under:

1. Infective agents like bacteria, viruses and their toxins, fungi, parasites.
2. Immunological agents like cell-mediated and antigen- antibody reactions.
3. Physical agents like heat, cold, radiation, mechanical trauma.
4. Chemical agents like organic and inorganic poisons.
5. Inert materials such as foreign bodies.[25]

Depending upon the duration and mode of the onset, the inflammatory reaction can be classified as acute, sub-acute or chronic.

Acute inflammation is characterized by a sudden onset and has a short course (a few minutes to several hours). The signs of inflammation are present along with constitutional symptoms. The microscopic picture comprises of exudation of fluid, plasma and leukocytic emigration.[26]

On the other hand, **sub-acute inflammation** is said to last for one to six weeks (or more) and is usually seen in tubular structures like appendix or fallopian tube. It is characterized by vascular exudative changes of acute inflammation and proliferative changes of chronic inflammation. Here the exudate consists chiefly of eosinophils, lymphocytes, plasma cells, histiocytes and fibroblasts.[26]

Chronic inflammation is known to last from months to years and is characterized by proliferation of connective tissue and blood vessels, with the presence of lymphocytes, plasma cells and histiocytes, but the absence of polymorphs.[26]

Irrespective of the type of injury, the pathogenesis of acute inflammatory response comprises of:

I. Vascular changes: alterations in vessel caliber resulting in increased blood flow (*vasodilation*) and structural changes that permit plasma proteins to leave the circulation (*increased vascular permeability*).

II. Cellular events: emigration of the leukocytes from the microcirculation and accumulation in the focus of injury (*cellular recruitment and activation*). The principal leukocytes in acute inflammation are neutrophils (polymorphonuclear leukocytes). [1]

I. Vascular Changes:

Alteration in the microvasculature (arterioles, capillaries and venules) is the earliest response to tissue injury. These alterations include haemodynamic changes and changes in vascular permeability.

Haemodynamic Changes: Irrespective of the type of injury, immediate response is of transient vasoconstriction followed by persistent progressive vasodilation. Progressive vasodilation, inturn, may elevate the local hydrostatic pressure resulting in transudation of fluid into the extracellular space. This is responsible for swelling at the local site of acute inflammation. Stasis or slowing is followed by leucocytic margination or peripheral orientation of leucocytes along the vascular endothelium. The leucocytes stick to the vascular endothelium briefly, and

then move and migrate through the gaps between the endothelial cells into the extravascular space. This process is known as emigration.[25]

Changes in Vascular Permeability: In the initial stage, the escape of fluid is due to vasodilation and consequent elevation in hydrostatic pressure. This is transudate (does not contain protein) in nature. But subsequently, the characteristic inflammatory oedema, exudate (rich in proteins and inflammatory cells), appears by increased vascular permeability of microcirculation. [1]

Mechanism of increased vascular permeability include

1. Contraction of endothelial cells mainly in venules resulting in development of temporary gaps between endothelial cells which results in vascular leakiness. It is mediated by release of histamine, bradykinin and other chemical mediators.

2. Retraction of endothelial cells occurs due to re-organisation of the cytoskeleton of the endothelial cells that causes reversible retraction at the intercellular junctions. This change is mediated by cytokines such as interleukin-1(IL-1) and tumour necrosis factor(TNF)-α.

3. Direct injury to endothelial cells mediated by leucocytes resulting in cell necrosis and appearance of physical gaps.

4. Leakiness in neovascularization. Newly formed capillaries under the influence of vascular endothelial growth factor (VEGF) during the process of repair are excessively leaky.[25]

II. Cellular events:

The cellular phase of inflammation consists of two processes: [25]

1. Exudation of leucocytes; and

2. Phagocytosis.

1. Exudation of Leucocytes:

The sequence of events in the recruitment of leukocytes from the vascular lumen to the extravascular space consists of

a) Margination, adhesion to endothelium, and rolling along the vessel wall;

b) Firm adhesion to the endothelium;

c) Transmigration between endothelial cells; and

d) Migration in interstitial tissues towards a chemotactic stimulus. [1]

Rolling, adhesion, and transmigration are mediated by the binding of complementary adhesion molecules on leukocytes and endothelial surfaces. Chemical mediators (chemoattractants) and certain cytokines affect these processes by modulating the surface expression or avidity of the adhesion molecules and by stimulating directional movement of the leukocytes. [1]

a) Margination, adhesion to endothelium, and rolling along the vessel wall;

The normal axial flow consists of stream of cells comprised by leucocytes and red blood cells (RBCs) and peripheral cell free layer of plasma close to vessel wall. Due to slowing and stasis, the central stream of cells widens and peripheral plasma zone becomes narrower because of loss of plasma by exudation. This phenomenon is known as margination.[25] subsequently, leukocytes tumble on the endothelial surface, transiently sticking along the way, a process called rolling. The weak and transient adhesions involved in rolling are mediated by the selectin family of adhesion

molecules. Selectins are receptors expressed on leukocytes and endothelium that contain an extracellular domain that binds sugars (hence the lectin part of the name). The three members of this family are E-selectin (also called CD62E), expressed on endothelial cells; P-selectin (CD62P), present on endothelium and platelets; and L-selectin (CD62L), on the surface of most leukocytes. Selectins bind sialylated oligosaccharides (e.g., sialyl-Lewis X on leukocytes) that are attached to mucin-like glycoproteins on various cells. The endothelial selectins are typically expressed at low levels or are not present at all on normal cells. They are up-regulated after stimulation by specific mediators. Therefore, binding of leukocytes is largely restricted to endothelium at sites of infection or tissue injury (where the mediators are produced).[1,27]

b) Firm adhesion to the endothelium;

This adhesion is mediated by integrins expressed on leukocyte cell surfaces interacting with their ligands on endothelial cells. Integrins are transmembrane heterodimeric glycoproteins (composed of different α and β chains) that also function as cell receptors for extracellular matrix. Integrins are normally expressed on leukocyte plasma membranes in a low-affinity form and do not adhere to their appropriate ligands until the leukocytes are activated by chemokines. Chemokines are chemoattractant cytokines that are secreted by many cells at sites of inflammation and are displayed bound to proteoglycans on the endothelial surface. When the adherent leukocytes encounter the displayed chemokines, the cells are activated, and their integrins undergo conformational changes and cluster together, thus converting to a high-affinity form. At the same time, other cytokines, notably TNF and IL-1 (also secreted at sites of infection and injury), activate endothelial cells to increase their expression of ligands for integrins. These ligands include ICAM-1 (intercellular

adhesion molecule 1), which binds to the integrins LFA (leukocyte function antigen)-1 (CD11a/CD18) and Mac-1 (CD11b/CD18), and VCAM-1 (vascular cell adhesion molecule 1), which binds to the integrin VLA-4. The net result of cytokine-stimulated increased integrin affinity and increased expression of integrin ligands is stable attachment of leukocytes to endothelial cells.[1, 28]

c) Transmigration between endothelial cells;

After being arrested on the endothelial surface, leukocytes migrate through the vessel wall primarily by squeezing between cells at intercellular junctions (although intracellular movement through endothelial cell cytoplasm has also been described). This movement of leukocytes, called *diapedesis,* occurs mainly in the venules of the systemic vasculature; it has also been noted in capillaries in the pulmonary circulation. Migration of leukocytes is driven by chemokines produced in extravascular tissues, which stimulate movement of the leukocytes toward their chemical gradient. In addition, PECAM-1 (platelet endothelial cell adhesion molecule 1, also called CD31), a cellular adhesion molecule expressed on leukocytes and endothelial cells, mediates the binding events needed for leukocytes to traverse the endothelium. After passing through the endothelium, leukocytes cross vascular basement membranes by focally degrading them with secreted collagenases.[1, 29]

d) Migration in interstitial tissues toward a chemotactic stimulus.

After extravasating from the blood, leukocytes migrate toward sites of infection or injury along a chemical gradient by a process called *chemotaxis.* Both exogenous and endogenous substances can be chemotactic for leukocytes, including (1) bacterial products, particularly peptides with N-formylmethionine termini; (2) cytokines, especially those of the chemokine family; (3) components of the complement system, particularly C5a; and (4) products of the lipoxygenase pathway of arachidonic acid

(AA) metabolism, particularly leukotriene B_4 (LTB_4). These mediators are produced in response to infections, tissue damage and during immunologic reactions.[1]

Chemotactic molecules bind to specific cell surface receptors, which are members of the seven-transmembrane G-protein coupled receptor family. Binding of the chemoattractants results in G-protein-mediated signal transduction events, some of which lead to increased cytosolic calcium, which triggers the assembly of cytoskeletal contractile elements necessary for movement. Leukocytes move by extending pseudopods that anchor to the extra cellular matrix (ECM) and then pull the cell in the direction of the extension. Thus, at the pseudopod's leading edge, actin monomers are polymerized into long filaments; at the same time, actin filaments elsewhere in the cell are disassembled to allow flow in the direction of the extending pseudopod. The direction of such movement is specified by a higher density of receptor-chemotactic ligand interactions at the leading edge of the cell.[1]

The type of emigrating leukocyte varies with the age of the inflammatory response and with the type of stimulus. In most forms of acute inflammation, neutrophils predominate in the inflammatory infiltrate during the first 6 to 24 hours and are replaced by monocytes in 24 to 48 hours. Several features of leukocytes account for this sequence: neutrophils are more numerous in the blood, they respond more rapidly to chemokines, and they may attach more firmly to the adhesion molecules that are rapidly induced on endothelial cells, such as P- and E-selectins. In addition, after entering tissues, neutrophils are short-lived they die by apoptosis and disappear within 24 to 48 hours-while monocytes survive longer. There are exceptions to this pattern of cellular exudation, however. In certain infections (e.g., those caused by *Pseudomonas organisms)* the cellular infiltrate is dominated by continuously recruited neutrophils for several days; in viral infections lymphocytes may be the first

cells to arrive; and in some hypersensitivity reactions eosinophilic granulocytes may be the main cell type.[1]

2. Phagocytosis

Phagocytosis of particles is an early step in the elimination of harmful substances. It includes engulfment of invading organism and production of substances that destroy phagocytosed microbes and remove dead tissues; these leukocyte products include lysosomal enzymes and reactive oxygen and nitrogen species.

Phagocytosis consists of three distinct but interrelated steps:

(a) Recognition and attachment of the particle to the ingesting leukocyte;

(b) Engulfment, with subsequent formation of a phagocytic vacuole; and

(c) Killing and degradation of the ingested material.[30]

Termination Of Acute Inflammation:

Subsequent changes in damaged area vary with nature and duration of the injurious stimulus, type of tissue involved and the degree of destruction of tissue. Any of the following may occur:

A. Resolution.

B. Healing by scar formation with or without regeneration of lost parenchymal cells.

C. Suppuration.

D. Acute course may get converted to a chronic one.[25]

Molecular Basis of Inflammatory Events:

Chemical Mediators:

The complex process of inflammation involves an intricate network of amediators or cytokines derived locally by cells at the site of inflammation, or they may be circulating in the plasma (typically synthesized by the liver) as inactive precursors that are activated at the site of inflammation in response to an aetiological factor. Though their duration of action is short-lived, due to the efficient disposal by catabolic enzyme systems, they may be continuously produced because of persistence of causative factors.[1]

Cell-derived mediators are normally sequestered in intracellular granules and are rapidly secreted upon cellular activation (e.g., histamine in mast cells) or are synthesized de novo in response to a stimulus (e.g., prostaglandins and cytokines). Plasma-protein-derived mediators (complement proteins, kinins) typically undergo proteolytic cleavage to acquire their biologic activities. Most mediators induce their effects by binding to specific receptors on target cells. Mediators may act on only one or a very few targets, or they may have widespread actions, with differing outcomes depending on which cell type they affect. Some mediators have direct enzymatic and/or toxic activities (e.g., lysosomal proteases and Reactive Oxygen Species). Mediators may stimulate target cells to release secondary effector molecules. Different mediators may have similar actions, in which case they may amplify a particular response, or they may have opposing effects, thus serving to control the response. The actions of most mediators are tightly regulated. Once activated and released from the cell, mediators quickly decay (e.g., arachidonic acid metabolites), are inactivated by enzymes (e.g., kininase inactivates bradykinin), are eliminated

(e.g., antioxidants scavenge toxic oxygen metabolites), or are inhibited (complement-inhibitory proteins).[1]

These mediators are grouped into several categories, namely:

1. Vasoactive amines
    a. Histamine
    b. Serotonin
2. Membrane derived lipid substances
    a. Eicosanoids
    i. Prostaglandins
    ii. Leukotrienes
3. Kinins
    a. Bradykinin and related kinins
    b. Tachykinins
4. Clotting system
5. Complement system
6. Lysosomal proteases
7. Cytokines
8. Biologically derived oxidants
    a. Hydroxyl radicals (OH^-)
    b. Hydrogen peroxide (H_2O_2)
    c. Hypochlorite (HOCl)
    d. Superoxide anion radical (O_2^-)
9. Nitric Oxide (NO)
10. Others

Histamine:

Histamine a widely distributed endogenous vasoactive amine is found in most tissues and physiological fluids, the richest sources being mast cells adjacent to blood vessels, basophils, platelets and cerebrospinal fluid. It is one of the first mediators to be released by injurious stimuli, which is responsible for the immediate phase of increased vascular permeability and acts on microcirculation via H_1 receptors. It causes arteriolar dilation and increases vascular permeability of venules, causes endothelial contraction and widening of inter-endothelial junctions. Thus histamine contributes to formation of edema and it appears to be an important mediator in the pathogenesis of acute inflammation.[1] Release of histamine is calcium dependent and is also stimulated by anaphylotoxins, complement-3a (C3a) and complement 5a (C5a), substance P, cytokines like interleukin-1 (IL-1) and interleukin-8 (IL-8), Platelet Factor-4 and secreted (Rantes) cytokine.[31, 32] Inflammogenic activity of histamine is brought about mainly by H_1 and partially by H_2 receptors. Certain drugs like d-tubocurarine have also been shown to release histamine.[1, 25]

5-Hydroxytryptamine (Serotonin, 5HT):

5-Hydroxytryptamine is an endogenous, preformed, biogenic amine having potent effects on blood vessels and smooth muscles. It is widely distributed in plants and tissue and is found in high concentrations in mammalian pineal gland, platelets and enterochromaffin cells. It is also found in mast cells of rodents and in venom of bees and wasps. Release of serotonin from platelets is stimulated by platelet activating factor (PAF) and causes vasodilation mainly through 5HT1 receptors by

1. Acting on endothelial cells to release nitric oxide which relaxes smooth muscles.
2. Inhibiting noradrenaline release from sympathetic nerve terminals and

3. A direct relaxant action on smooth muscles

Like histamine, it increases capillary permeability and hence appears to be a mediator of acute inflammation.[1, 25]

Kinins:

Kinins are extremely potent polypeptides released from cleavage of α_2 globulin, fraction of plasma kininogen. This normally, inactive system is triggered by contact activation of Hageman factor to release bradykinin. Kinins can be defined as an endogenous substance which produces slow contraction of guinea pig ileum, relaxation of rat duodenum, fall in blood pressure due to vasodilation and an increase in capillary and venular permeability. It is a potent vasodilator and induces edema formation and evokes pain and reflexes by acting on nerve endings. Its vasodilator action is partly due to the release of prostacyclin (PGI_2) and nitric oxide (NO) release from endothelial cells.[33] It also enhances leucocyte margination. Kinins which are mainly present in human plasma, urine, pancreatic and salivary secretion have been implicated in modulating the migration of white blood cells (WBC) and tissue cells and in the release of PAF, neuropeptides and prostaglandins during the inflammatory reaction. Of the two distinct receptors for kinins, B_1 and B_2, the B_1 receptors which are present in normal vascular smooth muscle are upregulated in inflammation.[34] These B_1 receptors are also found on inflammatory cells such as macrophages and can elicit production of inflammatory mediators like IL-1 and TNF-$\propto$.[35]

Clotting System:

The clotting system is activated by the Hageman factor. Although the primary function of the clotting system is to maintain vascular integrity, it also plays an important role in microbial resistance and inflammation. Fibrinopeptides which are

released during conversion of fibrinogen to fibrin contribute significantly to inflammatory reaction by their chemotactic property.[36]

Complement System:

The complement system is an integral part of the body's humoral defence mechanism and is also a primary mediator of the inflammatory process. It designates a major complex of plasma proteins, which comprises a group of proteases that circulate in zymogen form. They interact sequentially to affect a variety of inflammatory events, either directly or through interplay with other factors. Complement components (C1- C9) are present in inactive form in plasma and an elaboration of the biologic function of complement components leads to an increased vascular permeability, chemotaxis and opsonization. C5a can be generated in extra-vascular tissue fluid in response to inflammatory stimuli.[37, 38] C3a, C5a and to a lesser extent C4a increase the vascular permeability and cause vasodilation mainly by releasing histamine from mast cells. C5a is also known to activate the lipoxygenase pathway of arachidonic acid metabolism in neutrophils and monocytes to promote the release of mediators and is also implicated in the role of leucocyte adhesion to endothelium by activating the leucocytes and increasing the avidity of surface integrins to their endothelial ligands. C3b is mainly involved in opsonization and phagocytosis by neutrophils and macrophages. The chemotactic effect of complement system particularly C3 and C5 and the complement activating effects of neutrophils can set up a self perpetuating cycle of neutrophil emigration.[1, 25]

Lipid Derived Mediators (The Arachidonic Acid Metabolites) (Eicosanoids)

The arachidonic acid metabolites are the best studied lipid mediators and virtually all cells involved in inflammatory response have the capacity to generate one

or the other form of Arachidonic Acid (AA) derivatives such as prostaglandins(PG), thromboxane A_2 (TXA_2), prostacyclin (PGI_2) and leukotrienes. The AA can be metabolized by two pathways-the cyclooxygenase pathway, which gives rise to prostaglandins and lipoxygenase pathway, which gives rise to leukotrienes (LTs). While the cyclooxygenase enzyme is found in many tissues, the lipoxygenase enzyme is found in leucocytes and their derivatives.[33]

Prostaglandins:

During the inflammatory process, prostaglandins (PGs) are generated at the site of inflammation and cause hyperalgesia. PGE_2, a potent pyrogenic substance is known to produce vasodilation, increase blood flow and synergize with other factors to increase vascular permeability and cause hyperalgesia. They also potentiate the action of kinins in increasing the vascular permeability. Similarly, PGI_2 also causes vasodilation and pain. It potentiates carrageenan induced edema and hyperalgesia in rats, while in rabbit skin it can induce hyperaemia and augment plasma exudation in response to permeability inducing stimuli such as bradykinin. Thromboxane A_2 (PG peroxide) which causes platelet aggregation and vasoconstriction is a highly unstable product and gets rapidly converted to thromboxane B_2. It is mainly derived from platelets.[33]

Leukotrienes:

These lipoxygenase pathway products, mediate virtually every step of inflammation. Rather than being preformed, they are generated in mast cells and therefore called as secondary mediators. LTB4, LTC_4, LTD_4 and LTE_4 are the types of leukotrienes which are derived from a common precursor, LTA_4. LTB_4 is a potent

inflammatory mediator. It induces polymorphonuclear leukocyte chemotaxis, chemokines degranulation and adherence to endothelial cells. LTC_4, LTD_4 and LTE_4 which together constitute slow releasing substance of anaphylaxis (SRS-A) induce vascular permeability as well as cutaneous vasoconstriction and have been implicated in the pathogenesis of various inflammatory diseases. In addition, LTB_4 appears to play a role in interferon production.[39] 5-Hydroperoxyeicosatetraeinoic acid (5-HPETE) and its alcoholic metabolite 5-HETE (hydroxyeicosatetraeinoic acid) stimulate the super oxide generation in guinea pig and human neutrophils by augmenting intracellular calcium level that facilitates the protein kinase-C activity. HETE, the other major product of lipoxygenase pathway is found to be chemotactic for polymorphonuclear leucocytes (PMN) and eosinophils. More recently, neutrophil derived trihydroxy metabolite of AA, called lipoxin, has been reported to have both proinflammtory and anti-inflammatory effects.[1] They inhibit neutrophil chemotaxis but stimulate monocyte adhesion and motility.[40]

Platelet Activating Factor (PAF):

A phospholipid derived mediator, PAF is generated and released from sensitized mast cells by the action of immunoglobulin E (IgE). It is synthesized by platelets, monocytes, neutrophils, eosinophils, renal mesangial and medullary cells and vascular endothelial cells.[2] Cytosolic calcium increases with PAF due to influx of calcium through membrane bound calcium channels and also due to mobilization of intracellular calcium secondary to inositol 1,4,5, triphosphate formation. Its release is stimulated by chemotactic peptides, thrombin, collagen, autacoids and by PAF itself. Besides platelet aggregation, PAF causes vasoconstriction but in low doses it causes vasodilation and increased venular permeability. It helps in leucocyte adhesion, degranulation and oxidative burst, and is chemotactic to neutrophils, monocytes and

eosinophils. It boosts the synthesis of other mediators particularly eicosanoids and in higher doses produces hyperalgesia. A role for PAF in inflammation is supported by the ability of synthetic PAF antagonists to inhibit inflammation in some experimental models.[1, 25]

Cytokines:

Cytokines like interleukins, IL_1 and IL_2, TNF-α, and interferons are polypeptides and soluble immunoglobulins which are produced by activated T-lymphocytes (lymphokines) and macrophages (monokines). They are the main cytokines involved in inflammation. IL_1 regulates the systemic inflammatory response by stimulating the synthesis of acute phase proteins, by increasing blood neutrophils and by causing fever in addition to its local effects like erythema, edema and chemoattraction. It brings about increased leucocyte adherence, prostanoids and PAF synthesis, nitric oxide production and release of platelet derived growth factor (PDGF). It synergizes with TNF-α for many of its actions and the latter not only stimulates its synthesis but also causes aggregation, primes neutrophils in acute phase protein synthesis and helps in adhesion molecule expression.[25, 28]

Chemokines:

The chemokines are a family of small (8-10 kD), structurally related proteins that act primarily as chemoattractants for different subsets of leukocytes, resulting in their recruitment to the site of inflammation. Chemokines mediate their activities by binding to specific G-protein-coupled receptors (eg: CXCR4 and CCR5) on target cells. CXC and CC are two major groups' chemokines which play a major role in inflammation.

CXC chemokines have one amino acid separating the conserved cysteines and act primarily on neutrophils. IL-8 is typical of this group; it is produced by activated

macrophages, endothelial cells, mast cells, and fibroblasts, mainly in response to microbial products and other cytokines such as IL-1 and TNF.CC chemokines have adjacent cysteine residues and include monocyte chemoattractant protein 1 (MCP-1) and macrophage inflammatory protein 1α (MIP-1α) (both chemotactic predominantly for monocytes), RANTES (regulated on activation normal T expressed and secreted) (chemotactic for memory CD4+ T cells and monocytes), and eotaxin (chemotactic for eosinophils). [1]

Nitric Oxide (NO):

Nitric oxide (NO) is a highly reactive mediator which is synthesized endogenously by the L-arginine dependent enzyme, nitric oxide synthase (NOS). Recent studies have suggested that increased NO release by activated macrophages may contribute to inflammation and tissue injury.[41] It activates guanylyl cyclase enzyme thereby indirectly influencing calcium ion concentration. Numerous pathophysiological processes like atherosclerosis, endotoxaemia and ischaemic reperfusion injury have been associated with production of NO. A common feature of these conditions is localized generation of excess reactive oxygen intermediates, in particular, superoxide anion and hydrogen peroxide.[42] NO has also been implicated in carrageenan induced increase in vascular permeability and edema formation.[43] Many cytokines such as IL-1, lipopolysaccharides(LPS) and gamma radiation can stimulate macrophages for NO synthesis.[44] Paradoxically NO and superoxide anion may also function as a defence against oxidative stress by reducing intracellular levels of reactive intermediates.[43]

Oxygen Free Radicals in Biological Systems:

Reactive oxygen species is a collective term used to include not only oxygen radicals (O_2^-, OH^-) but also some oxygen derivatives which do not contain unpaired

electron such as H_2O_2, singlet oxygen (IO_2) and hypochlorous acid (HOCL).[45] A major source of O_2^-, H_2O_2, OH^- and HOCL is the respiratory burst produced by neutrophils, macrophages and other cells in response to particulate and non particulate stimuli. Reactive oxygen species are toxic not only because of their intrinsic activity as mediators of inflammation and their ability to modulate inflammatory processes but also due to their synergistic activity with serum proteases.[46] The superoxide radicals have been implicated with increased vascular permeability and cell death.[25]

Neuropeptides:

Another class of compounds with the capacity to modulate inflammatory events are neuropeptides, produced from neuroendocrinal tissue and neural tissues. The mammalian tachykinins comprise three related peptides-substance P, neurokinin A and neurokinin B. They are implicated with vasodilation, increased vascular permeability, mast cell degranulation, eicosanoid production, enhanced neutrophil adhesion and chemotaxis. Substance-P has been implicated in nociception in rats and mice, and also with early phase of carrageenan induced edema.[47] The inflammatory response of substance-P is suggested to be mediated by activation of cholinergic neurons or by histamine release.[48, 49]

Lysosomal Enzymes:

Lysosomes release a variety of agents during inflammation. They are:

1. Cationic or Basic Proteases:

They act directly on the blood vessels to increase the permeability and serve as chemotactic agents for monocytes.

2. Acid Proteases:

They degrade the bacteria within the phagolysosomes.

3. Neutral Proteases:

One of their many functions is to cleave C_3 to C_5 to form active proteases. They activate kininogen to kinin and directly act on blood vessels to increase their permeability.

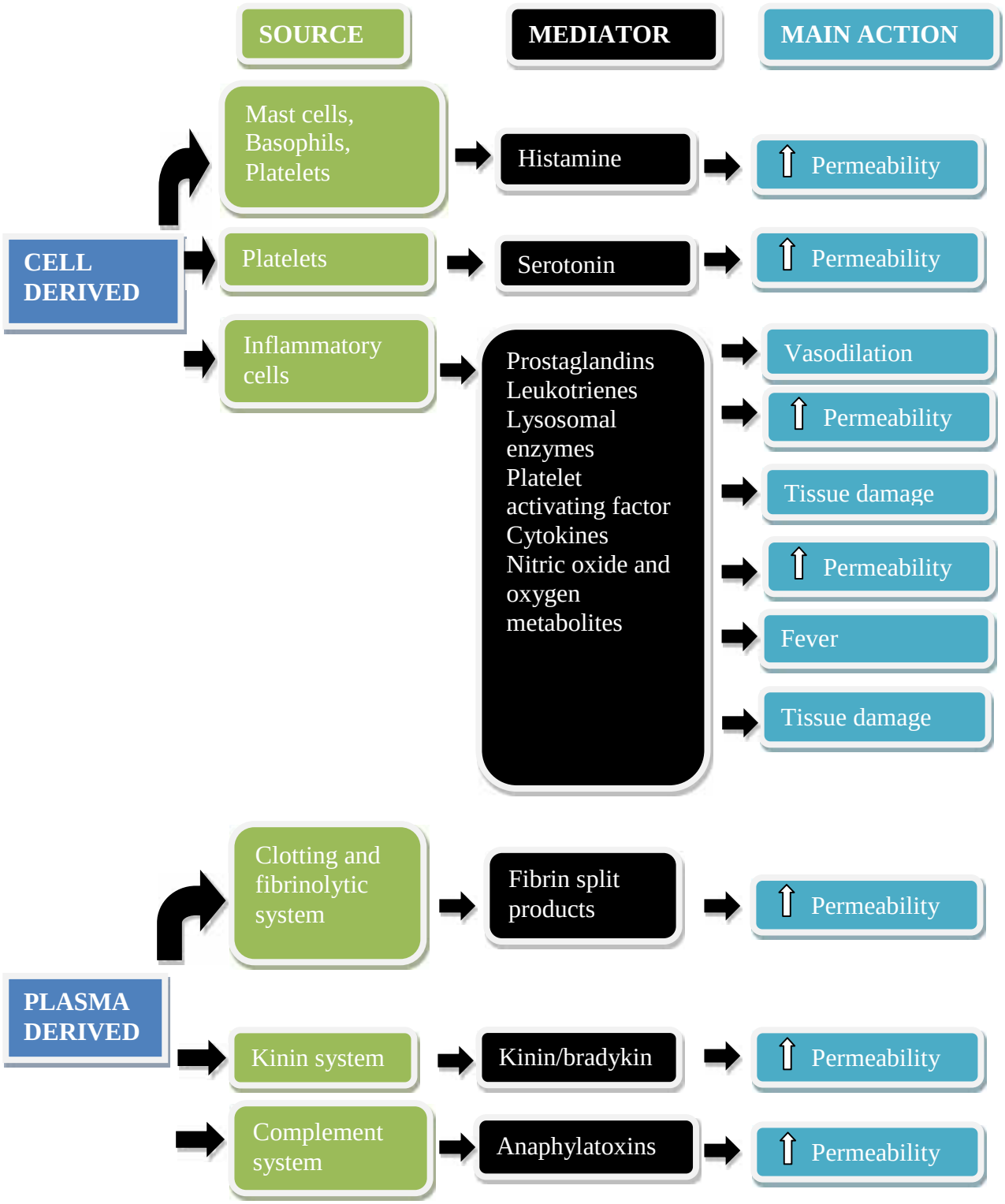
Lysosomal constituents which have thus been incriminated with numerous effects are held in check by serum and tissue fluid antiproteases such as α-antitrypsin, deficiency of which leads to sustained action of proteases.[1, 25]

Others:

There are many other endogenous and exogenous substances involved in the pathogenesis of inflammation. Growth factors like platelet derived growth factor and transforming growth factor (TGF) are said to be chemotactic and resemble cytokines in their functions. Certain extracellular matrix compounds too have been shown to possess chemotactic activity.[1] In addition, adenosine, the hydrolysed metabolite of ATP has been demonstrated to possess both pro and antiinflammatory effects.[5O]

The different mediators and events occurring in acute inflammation have been depicted below.[25]

MEDIATORS OF INFLAMMATION



B. SCREENING OF ANTI-INFLAMMATORY AGENTS

The widespread use of anti-inflammatory agents to treat common clinical inflammatory conditions has evoked much interest in their laboratory evaluation. The various methods of evaluation have been described in noted text books.[51, 52] A variety of models have been introduced to assess the anti-inflammatory activity of the various drugs. They measure the cardinal signs of inflammation viz. rubor (redness), dolor (pain), calor (heat), tumor (swelling) and functio laesa (loss of function). Some other methods work by modifying events in inflammation.

I. DEPENDING ON MODIFICATION OF SIGNS OF INFLAMMATION, THE VARIOUS METHODS ARE:

1. ERYTHEMA (Redness):

This early stage of an acute inflammatory process is reproduced in the guinea pig with the study of cutaneous erythema produced by UV rays (Ultraviolet rays). The technique was first described by Adams in the year 1960.[51]

Albino guinea pigs are pretreated with the test drug 30 min before exposure to the UV rays for 20 seconds to the depilated skin. After 2 hours, the degree of erythema is estimated visually on a scale of 0-4, preferably by a blind observer. The main draw-back of the test is the inability to get accurate measurement of the reaction and the variability of response among animals.

Phenylbutazone, aminopyrine, aspirin and sodium salicylate were found to be effective by this method.

Similarly, in human volunteers specific irritant like 5% tetrahydrofuryl-nicotinate in the form of a cream can be rubbed on the skin to produce erythema in order to evaluate anti inflammatory agents.[53]

However both these methods are not commonly employed.

2. EDEMA (Swelling):

This is one of the most popular methods to screen anti-inflammatory agents. It can be easily performed as a routine test, is cheap and reproducible.

Pedal inflammation (edema) is produced by injection of various phlogistic (irritating) agents into the hind paw of the rat. Various irritants used are carrageenan, mustard, dextran, egg-white, compound 48/80, yeast, serotonin, histamine, formalin, kaolin, substance-P, nystatin, glass powder, cobra venom, naphthoylheparamine etc. Carrageenan which was first used by Winter C.A, E.A Risley and G.W.Nuss is still the most widely used irritant.[51] It is injected in the volume of 0.05 ml of 1% normal saline into one of the hind paws. The test drugs are given appropriately prior to carrageenan and paw edema is measured plethysmographically at regular intervals of time. Paw edema can also be measured gravimetrically by isolating and weighing edematous organs like paw or ear after sacrificing the animal. However this can produce misleading results because it is difficult to obtain time response effect of drugs. Plethysmography still remains the most popular method because of reliability and reproducibility of results. Though rats are commonly used, mice can also be used for this experiment.

Mouse ear edema test:

This method was described by Brown and Robson in 1964.[52] Ear edema is produced by application of xylol to mouse ear and 40 minutes later the difference in weight between xylol treated and untreated ear is measured to quantify the edema.

3. HEAT (Fever):

Method to produce fever was described by Sheth and H.L. Borison in 1960.[52] Fever is produced by intracerebroventricular administration of purified bacterial lipopolysaccharide (extract of E. Coli) to cats and body temperature is recorded by

means of a thermocouple implanted in the retroperitoneal space. Yeast and TAB vaccine can also be used instead of bacterial lipopolysaccharide.

4. PAIN (Dolor):

Pain being a cardinal sign of inflammation, suppression of inflammatory pain indirectly indicates their anti-inflammatory activity. Various tests are commonly employed to evaluate analgesic activity of the drug.

One of the most commonly used methods is the Randall Selitto technique, 1957.[52]

Measured pressure is applied on the rat paw, in which inflammation is produced by local injection of Brewer's yeast. The study of the response in the healthy paw permits a direct comparison of the effect of drug on inflammatory and non-inflammatory pain. The test seems even more sensitive when carrageenan is used instead of brewer's yeast.[52]

Pain can be induced by different stimuli like wet heat, dry heat, radiant heat, chemical irritants, mechanical pressure and by use of electrical stimulus. Various equipments like analgesiometer, Eddy's hot plate and Janssen's warm water bath[51] are used where radiant heat, direct heat and wet heat are used as the noxious stimulus respectively.

Chemical irritants such as oxytocin, benzoquinone and hydrochloric acid are injected intraperitoneally to induce pain. Acetic acid is commonly used to induce writhing.

Use of mechanical pressure by applying bull dog clamp to the base of tail of mice and rats as described by Bianchi C. and Francheschni, in 1954[51] is also used for rapid screening of analgesic drugs.

Charlin used pododolorimeter in which electrical stimulus is applied to the foot pad of mice/rats to elicit pain.[51] The rectodolorimeter can be used to apply

stimulus to the rectum. Electrical stimulus can also be applied to the incisor tooth pulp of the guinea pig.

Opioid analgesics which need not have peripheral analgesic action but act at the central level can also be screened by tests like measured caudal compression, pethidine potentiation, lenticular opacity, nalorphine antagonism and oxytocin cramping methods.

Non opioid analgesics which are quite effective in relieving pain are evaluated by writhing test and by use of rectodolorimeter and pododolorimeter.

For rapid screening, hot water bath and tail clip methods are used.

Hot water bath described by Janssen, P.A.J et. al. 1963[51] can be used to assess the analgesic activity. Here the bath is maintained at 55°C ± 0.5°C. The lower 5cm part of the tail is marked and this part of the tail is immersed in hot water bath and the reaction time i.e. the time taken from introducing the tail into the bath to the time taken for the withdrawal of tail is noted. An increase in the reaction time indicates analgesic activity.

Although highly predictive of clinical analgesic activity, these methods are still relatively crude. Further they also fail to distinguish between addicting and non-addicting analgesics.

Human volunteers and non-human primates are now being increasingly employed. In humans, analgesics are evaluated either against experimentally induced pain (radiant heat, ischaemia induced with sphygmomanometer cuff, intraperitoneal bradykinin) or against endogenous pain (post puerperal pain, post operative pain and pain due to malignancy). A cross over double blind technique, using a placebo or a standard drug is essential in evaluation of analgesics in man to overcome the influence of subjective component of pain.[52]

5. LOSS OF FUNCTION:

Weisinger D., in 1964,[51, 52] employed rats with Mycoplasma L4 induced arthritis and measured the grip function of the inflammed articulation. Phenylbutazone, aminopyrine, indomethacin and gold preparations were found to improve joint motility.

Instead of mycoplasma L4, silver nitrate can be injected in the tibiotarsal joint. Cortisone and benzydamine favour return of joint motility.[52]

Intra-articular talc injections can be given in pigeons as an inflammogen.

II. DEPENDING UPON MODIFICATION OF EVENTS IN INFLAMMATION:

Burns of standard duration (27 seconds) and of standard intensity (55°C) are produced in anaesthetized rats. Shaved abdomen of the rat is placed in contact with the end of a closed hollow, brass cylinder, through which thermostatically controlled water is circulated. Resultant edema can be measured by excising and weighing a standard area of the skin.[52]

Leakage of circulating protein bound dye can also be used to measure the ability of the drugs to suppress the effect of endogenous mediators on vascular permeability. The permeability enhancing substance, such as bradykinin or histamine is injected intradermally into the abdomen or flank and the intensity or the area of dye-staining is estimated. Quantitative estimation can be done by excising the area of the skin and extracting the dye.[52]

Phlogistic action of abdominal radiation on intestine has been standardized by Willoughby, 1960.[52] Amount of dye leaking into the gut is then visually estimated or extracted for the sake of comparison between control and treated groups.

Arthus reaction is the other test which helps in studying the increased vascular permeability. The animals may be actively sensitized by injecting antigens like egg

albumin or horse serum or passively sensitized by injecting corresponding antibodies. An intradermal injection of antigen is made and a rapidly developing inflammatory reaction is assessed by estimating the dye leakage or edema. The technique was first described by Marks V and Smith M.J.H, 1960.[52] Salicylate and cortisone effectively suppress the reaction.

INFLAMMATORY EXUDATE FLUID:

Peritonitis and pleurisy induced by irritant substances in the rat, permit the experimental reproduction of a phenomenon that is more typically exudative. This is followed by the second phase of granulation.

Peritonitis and pleurisy can be produced by injecting 1 ml of 1.5% formalin solution, intraperitoneally, as described by Teotino et al, 1963[51] or by injection of evans blue into the pleural cavity according to the method of Weisbach et. al. 1963.[51] The animals are sacrificed 4-8 hours later and the ascitic fluid /pleural fluid is collected and measured immediately.

Reliability of the volume estimations and the fairly prolonged period (24 hours) during which exudate collects are the main advantages of this procedure. A special feature is the ability to reveal which phase of the vascular reaction is being influenced by a particular drug. Thus histamine antagonists inhibit exudate formation only in the first hour or so while salicylates do so in the delayed phase i.e. between second and twelfth hours.

EXPERIMENTAL ARTHRITIS:

Formalin induced arthritis was first described by Selye and since then kaolin, mustard oil, mycobacterium butyricum suspended in heavy paraffin oil (Freund's adjuvant) etc. have been tried. Urates are used to produce inflammation in dog joints. Freund's adjuvant method is by far the commonest technique used. Injection of 0.05

ml of Freund's adjuvant, intradermally, beneath the plantar surface, into the foot pad of rats produces local inflammatory lesions (primary lesions) and then 10-15 days later, secondary lesions in remote areas like forepaws, ears, tail and contralateral foot. This method was described by Ward J.R and R.S. Jones.[52] Before injecting the Freund's adjuvant, the thickness of the foot pad is measured with calipers and then compared with the thickness at the end of 15 days. The test drug is fed daily for 15 days.

The advantage of this method is the resemblance it bears with chronic arthritis in humans. Acetyl salicylic acid, cortisone, paramethasone, prednisolone are some drugs showing effect against primary and secondary lesions.

Wiesenger, 1964 described a different type of arthritis which can be produced in the rat by means of intravenous injection of Mycoplasma arthritis isolated from Murphy Sturm lymphosarcoma of rats.[52] Many NSAIDs have been found to be effective.

EMIGRATION OF LEUKOCYTES :

Some of the models described earlier which appear to be less popular include:-

a. An antigen / irritant is injected intradermally or subcutaneously into a sensitized animal and the area is excised after six hours for histological examination to quantify any particular type of leukocyte.
b. Clark-Sandison rabbit ear chamber has been used to study adhesion of leukocytes to vascular endothelium and their subsequent emigration.
c. Boyden's method devised by Alison F., M.R, Smith and W.B Wood, 1955 is used to study the rate of movement of leukocytes.[52]

GRANULATION TISSUE FORMATION:

Granuloma formation method was first described by D' Arcy et al. 1960,[51] wherein, sterilized cotton pellets weighing 7-10mg are implanted subcutaneously, in male albino rats, under anaesthesia. Treatment is given daily throughout the study. The granulomas are dissected out on the fifth day for quantification. The cotton pellets are weighed after overnight drying at 60°C.

However the technique has been suitably modified by using other suitable form of foreign bodies like grass piths, plastic rods, etc. and prolonging the study for 10 days.[54] The grass piths can be immersed in 10% formalin for subsequent microscopic studies.

However, cotton pellet induced granuloma reproduces a stage of inflammation and production of granulation tissue which is fairly similar to clinical granulomatous condition. Also a good correlation exists between the degree of activity of the test drug and clinical usefulness found in rheumatoid arthritis and other chronic inflammatory diseases.

GRANULOMA POUCH METHOD:

Granulomas can also be produced by injecting 25 ml of air into the dorsal, subcutaneous tissue of the rat, followed by a chemical irritant such as croton oil, at the same site, by the technique of Fisher J.W, 1961.[51] Extent of ensuing inflammation is measured 4-14 days later, either by estimating the thickness of the pouch wall (granulation tissue) or volume of the fluid exudate within the abscess. This method is an effective technique to determine cellular exudation and its sequelae, emigration and proliferation of fibroblasts, vascular endothelium and formation of scar tissue.

Glucocorticoids are found to be effective.

Hyaluronidase inhibition is the other method which is used but is very uncommon.

C. ANTI-INFLAMMATORY DRUGS

Anti-Inflammatory Drugs can be classified as follows:

I. THE PROSTAGLANDIN INHIBITORS (NSAIDs) [55]

A. Non selective COX inhibitors (Conventional NSAIDs)

1. Salicylates - Aspirin, Diflunisal.
2. Pyrazolone derivatives - Phenylbutazone, Oxyphenbutazone.
3. Indole derivatives - Indomethacin, Sulindac.
4. Proprionic acid derivatives - Ibuprofen, Naproxen, Ketoprofen, Fenoprofen, Flurbiprofen.
5. Anthranilic acid derivatives - Mephenamic acid.
6. Aryl-acetic acid derivatives - Diclofenac.
7. Oxicam derivatives - Piroxicam, Tenoxicam.
8. Pyrrolo-pyrrol derivative - ketorolac.

B. Preferential COX-2 inhibitors

Nimesulide, Meloxicam, Nabumetone.

These drugs act as anti-inflammatory by inhibiting COX enzyme and thereby blocking the synthesis of PG's which lead to inflammation. Aspirin inhibits COX irreversibly by acetylating one of its serine residues; while other NSAIDs are competitive and reversible inhibitors.

The common beneficial actions shared by these drugs due to PG synthesis inhibition are:

- ❖ Analgesia.
- ❖ Antipyresis.
- ❖ Anti-inflammatory (high dose of aspirin).
- ❖ Anti-thrombotic (Low dose of aspirin).

- Closure of ductus arteriosus.

Though, these are the most common anti-inflammatory drugs used clinically, they share certain common toxicities[55] like

- Gastric mucosal damage (more common with aspirin).
- Bleeding-due to inhibition of platelet function.
- Delay / Prolongation of labor.
- Asthma and anaphylactoid reactions in susceptible individuals.
- Limitation of renal blood flow, sodium and water retention.[55]

These are known to act on both COX-1 and COX-2. Newer agents have been developed which are COX-2 selective.[56] These are celecoxib, rofecoxib etc. which are effective as anti-arthritic and analgesic agents but, these also are not devoid of specific side effects like increase in blood pressure when used in hypertensives and increase in risk of cardiovascular problems.[33]

II. DRUGS POSSESSING ANTI-INFLAMMATORY ACTION BUT NOT USED ROUTINELY.

1. Steroids: [2, 33, 55]

- Decrease migration of neutrophils into inflammatory site.
- Decrease adhesion by inducing membranal changes in inflammatory cells.
- Modulates the adhesion molecule receptors.
- Decrease response to chemotactic factor.
- Affect production of lymphokines.
- Block the release of IL-1, IL-6, TNF-α cytokines by inhibiting macrophage and T-lymphocyte interactions.
- Block function of IL-2, CD-4 and helper T cells.

However in chronic conditions, on prolonged use, they cause various adverse reactions, which include [33, 55]

- ❖ Increased susceptibility to infections.
- ❖ Delayed healing of wounds and surgical incisions.
- ❖ Peptic ulceration.
- ❖ Osteoporosis.
- ❖ Iatrogenic cushings syndrome.
- ❖ Suppression of hypothalamo-pitutary axis

2. Hydroxychloroquine:

Inhibits cytokine secretion, lysosomal enzymes and macrophage functions.

But causes reversible retinal damage and corneal opacity.[55]

3. Gold Salts - Oral**-Auranofin,** Parenteral **- Gold Sodium Thiomalate:**

- ❖ Inhibits T-cells proliferation and IL-2 production.[55, 57, 58]
- ❖ Inhibits protein kinase-C.[57]

But, these are known to cause [55]

- ❖ Vasodilatation and postural hypotension.
- ❖ Albuminuria secondary to membranous glomerulonephritis.
- ❖ Hepatitis, peripheral neuritis, encephalopathy and pulmonary fibrosis.
- ❖ Eosinophilia.

4. Penicillamine:

- ❖ Inhibits helper T-cells and angiogenesis.
- ❖ Decreases concentration of immune complex in plasma synovial fluid. [33]

It is associated with certain toxicities like:

- ❖ Proteinuria, kidney damage.
- ❖ Bone marrow depression.

- About half the patients develop anti nuclear antibodies.
- It may precipitate systemic lupus erythematosus and myasthenia gravis.[55]

5. Sulfasalazine: [33, 55]

- Inhibits Lipoxygenase and cyclooxygenase.
- Scavenges oxygen free radicals.
- Inhibits chemotaxis and angiogenesis.

Some adverse effects reported are

- Rashes, fever, joint pain, haemolysis and blood dyscrasias.
- Nausea, vomiting, headache, malaise and anemia.
- Male infertility has been reported.

6. Cytotoxic Drugs:

a. Cyclophosphamide: [55]

- Immunosuppressant and anti-inflammatory.
- Inhibits cellular proliferation.

However it is known to cause alopecia and cystitis.[54]

b. Colchicine:

- Binds to microtubules necessary for mitosis and cellular migration.[33]

Higher doses cause kidney damage, CNS depression, intestinal bleeding and death due to muscular paralysis and respiratory failure.[55]

Chronic therapy is associated with aplastic anaemia, agranulocytosis, myopathy and loss of hair.[33, 55]

c. Methotrexate:

- Inhibits enzyme dihydrofolate reductase.
- Enhances release of adenosine which inhibits neutrophil adherence.[59]
- Inhibits chemotaxis.

However it is not recommended in patients with renal impairment, and it is reported to cause nodulosis.[55]

7. Cyclosporine:

- Inhibits transcription of IL-3, IL-4, TNF-α, Interferon (INF) - γ, Granulocyte macrophage colony stimulating factor (GM-CSF) by activated T-cells.[55]
- Suppresses T-cell activation by blocking the transcription of cytokine genes such as IL-2.[60]

It is known to impair renal function.

As most of the drugs possessing anti-inflammatory activity used in clinical practice cause mild to severe adverse effects, the discovery for newer safer anti-inflammatory agents continues.

III. DRUGS UNDER INVESTIGATION FOR POTENTIAL ANTI-INFLAMMATORY EFFECT:

I. Xenobiotic Immunosupressants:

Tacrolimus: Inhibits transcription of IL-2, IL-3, IL-4, TNF- α, IFN-γ, and GM-CSF.[60]

Rapamycin: Blocks the response induced by IL-2.[60]

Gusperimus: Binds to 2 Heat Shock Proteins (HSP-70 and HSP-90). These proteins are involved in interaction with glucocorticoid receptors and post translational processing of proteins which include mediators of immunological and inflammatory events.[61]

II. Cytokine Modulators:

1. **Monoclonal Antibodies** to Cytokines, especially to TNF-α and IL-6. [61]

2. **Blockade of Cytokine Receptors:** Interleukin-1α antagonist and Interleukin-1β antagonist, eg : IL-lra.[61]

3. **Lisofylline:**

Inhibits phosphatidic acid and thereby formation of proinflammatory cytokines.[61]

4. **Bicyclic Imidazoles:**

Inhibits biosynthesis of cytokines by acting at transcriptional level.[61]

5. **Convertase Inhibitors:** IL-lβ and TNF-α initially synthesized in inactive form and then altered by a specific converting enzyme to yield active form of cytokine. [61]

6. **IL-13**: used directly because it inhibits function of activated monocytes. [62]

: suppresses formation of proinflammatory cytokines.

III. Others:

a. Calcium Channel Blockers : Verapamil.[4]

b. Vitamin -E : Probably acts by scavenging singlet molecular oxygen.[63]

: Inhibits biosynthesis of prostaglandins.

c. Adrenergic agonists :

i. Clonidine.[64]

ii. Adrenaline :

iii. Nor-Adrenaline :

iv. Isoprenaline

→ Decreases vascular permeability and Acts by causing vasoconstriction.[3]

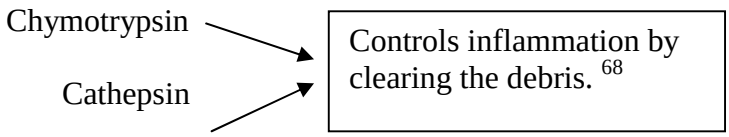
v. Salmeterol : Inhibits inflammatory mediator release.[65]

vi. Salbutamol : Inhibits early phase of carrageenan inflammation in murine model of pleurisy.[66]

d. Adrenergic Antagonists:

i. Phenoxybenzamine.[67]

e. Proteolytic Enzymes:



f. Ascorbic Acid: Is an antioxidant and therefore prevents damage by free radicals.[69]

g. Fenflumizole: Inhibits COX.[70]

h. ¥-Leinoleic Acid.[71]

i. Tetracycline Derivatives: Minocycline.[72]

j. Super oxide dismutase.[73]

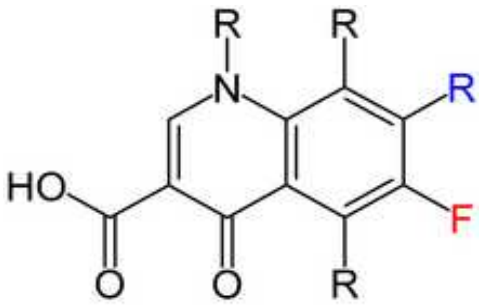
k. Mycophenolate Mofetil Immunosuppressant.[74]

D. DRUG USED IN THE PRESENT STUDY

Quinolones:

Quinolones contain a carboxylic acid moiety at position 3 of the primary ring structure. The first quinolone, nalidixic acid, was isolated as a by-product of the synthesis of chloroquine. It has been available for the treatment of urinary tract infections for many years. Addition of fluorine substituent at position 6 resulted in generation of fluoroquinolones which have better spectrum activity, favourable pharmacokinetic profile and better safety when compared to older compounds. The introduction of first fluorinated quinolone, norfloxacin was rapidly followed by development of other members of this group, such as ciprofloxacin, which has wide clinical application. Many of the newer fluoroquinolones contain piperazine moiety at position 7. [2]

Structure of quinolone antibiotics: [75]



R is usually piperazine

Fluorine substitution in fluoroquinolones

Classification of Quinolone Antibiotics: [75]

The quinolones can be classified into four generations based on antimicrobial activity. First-generation agents, which are used less often today, have moderate gram-negative activity and minimal systemic distribution. Second-generation quinolones have expanded gram-negative activity and atypical pathogen coverage, but limited gram-positive activity. These agents are most active against aerobic gram-negative bacilli. Ciprofloxacin remains the quinolone most active against

Pseudomonas aeruginosa. Third-generation quinolones retain expanded gram-negative and atypical intracellular activity but have improved gram-positive coverage. Finally, fourth-generation agents improve gram-positive coverage, maintain gram-negative coverage, and gain anaerobic coverage.

First generation

Nalidixic acid, Cinoxacin

Second generation

Class I: Lomefloxacin, Norfloxacin, Enoxacin.

Class II: Ofloxacin, Ciprofloxacin.

Third generation

Levofloxacin, Sparfloxacin, Gatifloxacin, Moxifloxacin.

Fourth generation

Trovafloxacin.

Mechanism of Action:

The quinolone antibiotics target bacterial DNA gyrase and topoisomerase IV. For many gram-positive bacteria (such as S. aureus), topoisomerase IV is the primary activity inhibited by the quinolones. In contrast, for many gram-negative bacteria (such as E. coli), DNA gyrase is the primary quinolone target.[2] Inhibition of DNA gyrase prevents the relaxation of positively supercoiled DNA that is required for normal transcription and replication. Inhibition of topoisomerase IV interferes with separation of replicated chromosomal DNA into the respective daughter cells during cell division.[76]

The DNA gyrase of E. coli is composed of two 105,000-dalton A subunits and two 95,000-dalton B subunits encoded by the gyrA and gyrB genes, respectively. The A subunits, which carry out the strand-cutting function of the gyrase, are the site of

action of the quinolones. The drugs inhibit gyrase mediated DNA negative supercoiling at concentrations that correlate well with those required to inhibit bacterial growth (0.1 to 10 mg/ml). Mutations of the gene that encodes the A subunit polypeptide can confer resistance to these drugs. Topoisomerase IV also is composed of four subunits encoded by the parC and parE genes in E. coli. Fourth generation fluoroquinolones act at DNA gyrase and topoisomerase IV, this dual action slows development of resistance. Eukaryotic cells do not contain DNA gyrase. However, they do contain a conceptually and mechanistically similar type II DNA topoisomerase that removes positive supercoils from eukaryotic DNA to prevent its tangling during replication. Quinolones inhibit eukaryotic type II topoisomerase only at much higher concentrations (100 to 1000 mg/ml).[2]

Spectrum of activity:

All fluoroquinolones are bactericidal in nature and exhibit concentration dependent bacterial killing. Bactericidal activity becomes more pronounced as the serum drug concentration increases to approximately 30-fold the minimum inhibitory concentration (MICs).[75] Ciprofloxacin is the most commonly used fluoroquinolone and described as the type agent.[33] Fluoroquinolones were originally developed because of their excellent activity against gram-negative aerobic bacteria; they had limited activity against gram-positive organisms. Several newer agents have improved activity against gram-positive cocci. MICs for gram-negative cocci and bacilli, including Enterobacteriaceae, pseudomonas, neisseria, chlamydiae, mycoplasmas, haemophilus, and campylobacter, are 1-2 mcg/mL and often less. Streptococci and enterococci tend to be less susceptible than staphylococci, and efficacy in infections caused by these organisms is limited. Ciprofloxacin is the most active agent of this group against gram-negatives, P aeruginosa in particular. Levofloxacin, the L-isomer

of ofloxacin, has superior activity against gram-positive organisms, including S. pneumoniae. Gatifloxacin, gemifloxacin, and moxifloxacin have improved activity against gram-positive organisms, particularly S. pneumoniae and some staphylococci. Gemifloxacin is active in vitro against ciprofloxacin-resistant strains of S. pneumoniae. Fluoroquinolones also are active against intracellular pathogens such as Legionella species and some mycobacteria, including Mycobacterium tuberculosis and M avium complex.[76]

Resistance:

During fluoroquinolone therapy, resistant organisms emerge about once in 10^7-10^9, especially among staphylococci, pseudomonas, and serratia. Resistance is due to one or more point mutations in the quinolone binding region of the target enzyme or to a change in the permeability of the organism. Resistance to one fluoroquinolone, particularly if it is of high level, generally confers cross-resistance to all other members of this class.[76]

Pharmacokinetics:

The quinolones are well absorbed after oral administration and are distributed widely in body tissues. Peak serum levels of the fluoroquinolones are obtained within 1 to 3 hours of an oral dose. Oral doses in adults are 200 to 400 mg every 12 hours for ofloxacin, 400 mg every 12 hours for norfloxacin and pefloxacin, and 250 to 750 mg every 12 hours for ciprofloxacin. Bioavailability of the fluoroquinolones is greater than 50% for all agents and greater than 95% for several. The serum half-life ranges from 3 to 5 hours for norfloxacin and ciprofloxacin to 20 hours for sparfloxacin. The volume of distribution of quinolones is high, with concentrations of quinolones in urine, kidney, lung and prostate tissue, stool, bile, and macrophages and neutrophils higher than serum levels. Quinolone concentrations in cerebrospinal fluid, bone, and

prostatic fluid are lower than in serum. Pefloxacin and ofloxacin levels in ascites fluid are close to serum levels, and ciprofloxacin, ofloxacin, and pefloxacin have been detected in human breast milk. Most quinolones are cleared predominantly by the kidney, and dosages must be adjusted for renal failure. Exceptions are pefloxacin and moxifloxacin, which are metabolized predominantly by the liver and should not be used in patients with hepatic failure. None of the agents is removed efficiently by peritoneal dialysis or hemodialysis.[2]

Therapeutic Uses:

1. **Urinary tract infections**

A seven to ten day course of orally administered norfloxacin or ofloxacin has been successful in the treatment of uncomplicated pyelonephritis. In the treatment of acute uncomplicated pyelonephritis in non-pregnant women, similar efficacy has been shown for levofloxacin, in a dosage of 250 mg per day for seven to 10 days, and ciprofloxacin, in a dosage of 500 mg twice daily for 10 days. Ciprofloxacin, in a dosage of 500 mg twice daily, is used in the treatment of complicated urinary tract infections and pyelonephritis, with cure rates of 93 percent and 91 percent, respectively.[2, 21, 75]

2. Prostatitis

Quinolones are effective in the treatment of prostatitis because of their excellent penetration into prostatic tissue. Norfloxacin, ciprofloxacin, levofloxacin, and ofloxacin given for four to six weeks, have shown eradication rates of 67 to 91 percent.[21, 75]

3. Pelvic Inflammatory Disease

Ciprofloxacin or ofloxacin can be used in the treatment of Neisseria *gonorrhoeae* urethritis and cervicitis. Recently, gatifloxacin was reported to be as

effective as ofloxacin against *N. gonorrhoeae*. A seven-day course of ofloxacin or sparfloxacin has been found to be as effective as doxycycline in the treatment of *C. trachomatis* infections. Finally, ciprofloxacin has been reported to be as effective as trimethoprim-sulfamethoxazole for treating chancroid caused by *Haemophilus ducreyi.*[20, 75]

4. Tuberculosis

Ciprofloxacin and ofloxacin are used as second line drugs in the treatment of tuberculosis.[21]

5. Gastroenteritis

Norfloxacin or ciprofloxacin has been found to be comparable to trimethoprim-sulfamethoxazole in the treatment of traveler's diarrhea caused by *Shigella species*, enterotoxigenic *E. coli*, or *Campylobacter jejuni*. Ciprofloxacin and ofloxacin are used in the treatment of enteric typhoid fever. Norfloxacin has been found to be superior to both trimethoprim-sulfamethoxazole and doxycycline in the treatment of *Vibrio cholerae* infection.[21, 75]

6. Respiratory Diseases

Fluoroquinolones are used in the treatment of acute bacterial sinusitis, acute bronchitis, community-acquired pneumonia and nosocomial pneumonia. Commonly used fluoroquinolones include ciprofloxacin, levofloxacin, sparfloxacin, ofloxacin, gatifloxacin, moxifloxacin and trovafloxacin.[21]

7. Fluoroquinolones in Biologic Warfare

Bacillus anthracis (anthrax) spores have recently been deployed as a biologic weapon. Fluoroquinolones have a role in postexposure prophylaxis and chemotherapy for specific agents that could be used in biologic warfare. Fluoroquinolones are

indicated for prophylaxis or treatment of anthrax, cholera, plague, brucellosis, and tularemia. Ciprofloxacin is the drug of choice for postexposure prophylaxis for anthrax until sensitivities are available. Although penicillin resistance has only rarely occurred in the natural setting of anthrax, the former Soviet Union developed a *B. anthracis* strain that was resistant to both penicillin and tetracycline.[75]

Adverse Reactions:

Fluoroquinolones are extremely well tolerated. The most common effects are nausea, vomiting, and diarrhea. Occasionally, headache, dizziness, insomnia, skin rash, or abnormal liver function tests develop. Photosensitivity has been reported with lomefloxacin and pefloxacin. QT_c prolongation may occur with gatifloxacin, levofloxacin, gemifloxacin, and moxifloxacin. Ideally, these agents should be avoided or used with caution in patients with known QT_c interval prolongation or uncorrected hypokalemia; in those receiving class IA (eg, quinidine or procainamide) or class III antiarrhythmic agents (sotalol, ibutilide, amiodarone); and in patients receiving other agents known to increase the QT_c interval (eg, erythromycin, tricyclic antidepressants). Gatifloxacin has been associated with hyperglycemia in diabetic patients and with hypoglycemia in patients also receiving oral hypoglycemic agents. Fluoroquinolones may damage growing cartilage and cause an arthropathy. Thus, these drugs are not routinely recommended for patients under 18 years of age. However, the arthropathy is reversible, and there is a growing consensus that fluoroquinolones may be used in children in some cases (eg, for treatment of pseudomonal infections in patients with cystic fibrosis). Tendinitis, a rare complication that has been reported in adults, is potentially more serious because of the risk of tendon rupture. They should be avoided during pregnancy in the absence of specific data documenting their safety.[76]

Drug interactions:

1. Plasma concentration of theophylline, caffeine and warfarin are increased by ciprofloxacin, norfloxacin and pefloxacin due to inhibition of metabolism: toxicity of these drugs can occur.[55]

2. NSAIDs may enhance the CNS toxicity of fluoroquinolones, seizures are reported.[55]

3. Antacids, sucralfate and iron salts given concurrently reduce absorption of fluoroquinolones. [55]

4. Decreased absorption of quinolones occur if didanosine or multivalent cations are administered concomitantly or less than two hours before or after a quinolone.[75]

5. May lead to hypoglycemia and/or hyperglycemia if used concomitantly with antidiabetic agents (oral hypoglycemics or insulin).[75]

6. May prolong QTc if used concomitantly with antiarrhythmics (e.g., class IA and III agents) or with cisapride.[75]

Table 1: Summary of Quinolone Antibiotics.[75]

Quinolone Antibiotics	**Antimicrobial spectrum**	**General clinical indications**
First generation Nalidixic acid, Cinoxacin.	Gram-negative organisms (but not Pseudomonas species).	Uncomplicated urinary tract infections.
Second generation **Class I:** Lomefloxacin, Norfloxacin, Enoxacin. **Class II:** Ofloxacin, Ciprofloxacin.	Gram-negative organisms (including Pseudomonas species), some gram-positive organisms (including *Staphylococcus aureus* but not *Streptococcus pneumoniae*) and some atypical pathogens.	Uncomplicated and complicated urinary tract infections and pyelonephritis, sexually transmitted diseases, prostatitis, skin and soft tissue infections.
Third generation Levofloxacin, Sparfloxacin, Gatifloxacin, Moxifloxacin.	Same as for second-generation agents plus expanded gram-positive coverage (*S. pneumoniae*) and expanded activity against atypical pathogens.	Acute exacerbations of chronic bronchitis, community-acquired pneumonia.
Fourth generation Trovafloxacin.	Same as for third-generation agents plus broad anaerobic coverage	Same as for first-, second- and third-generation agents plus intra-abdominal infections and nosocomial pneumonia.

Table 2: Fluoroquinolones used in present study.[55]

	Ciprofloxacin	**Ofloxacin**	**Norfloxacin**
Oral bioavailability (%)	60 – 80	85 – 95	35 – 45
Plasma protein binding (%)	20 – 35	25	15
Vol. of distribution (L/kg)	3-4	1.5	2
Percentage metabolized	20	5-10	25
Elimination t1/2 (hr)	3-5	5-8	4-6
Routes of administration	Oral, i.v.	Oral. i.v.	Oral
Dose (mg BD)	Oral - 250 – 750 i.v.- 100 – 200	Oral - 200 – 400 i.v. - 200	Oral – 400

METHODOLOGY

Adult healthy male Wistar rats weighing 175 ±25 g were obtained from the central animal house, J.N.Medical College, Belgaum and were acclimatized to 12:12 h light - dark cycle for 10 days prior to the day of experimentation. They were maintained on standard rat chow pellet (Amrut Brand) and water ad libitum. The study was approved by the IAEC (Institutional animal ethics committee) constituted as per the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision on Experiments on Animals), New Delhi.

Acute inflammation was produced by injecting carrageenan in the hind paw of Wistar rats and subacute inflammation by implanting foreign body subcutaneously as described below.

1. Carrageenan induced rat paw edema:

Rats were divided into several groups of six each. They were starved overnight with water ad. libitum prior to the day of experiment. Control group received 0.5ml of 1% gum acacia suspension, orally, while the other groups received calculated clinical equivalent doses of either ciprofloxacin, ofloxacin, norfloxacin or aspirin in 1% gum acacia suspension, orally. Aspirin was taken as the standard anti-inflammatory drug.

Thirty minutes after aspirin and one hour after ciprofloxacin, ofloxacin and norfloxacin administration, according to the technique of Winter et al. 1962, [51] 0.05ml of 1% carrageenan in normal saline was injected into the subplantar region of one of the hind paws. A mark was put on the hind limb at the malleolus to facilitate uniform dipping at subsequent readings. The paw edema volume in millilitre was measured with the help of plethysmograph by mercury displacement method at zero hour

(immediately after injecting carrageenan) (Figure-1). The same procedure was repeated at 0.5, 1, 3, 4 and 5 hours. The difference between 0 hour and subsequent reading was taken as actual edema volume. The percentage inhibition of edema in the various treated groups was then calculated by using the formula

$$\text{Percentage Inhibition of edema} = \left(1 - \frac{\text{Mean increase in paw volume in treated group}}{\text{Mean increase in paw volume in control group}}\right) \text{X } 100$$

2. Foreign Body Induced Granuloma Method:[54]

Rats were divided into several groups of six each. After clipping the hair in axillae and groin, under light halothane anaesthesia, two sterile cotton pellets weighing l0mg and two sterile grass piths (25x2mm) were implanted randomly, subcutaneously, through a small incision (Figure- 2 and 3). Wounds were then sutured and animals were caged individually after recovery from anaesthesia. Aseptic precautions were taken throughout the experiment. The treatment was started on the day of implantation and was repeated every twenty-four hours, regularly, for ten days.

On the eleventh day, the rats were sacrificed with an overdose of halothane anaesthesia to remove cotton pellets and grass piths (Figure- 4 and 5). The grass piths were preserved in 10% formalin for histopathological studies. The pellets, free from extraneous tissue were dried overnight at 60°C to note their dry weight. Net granuloma formation was calculated by subtracting initial weight of cotton pellet (10mg) from the weights noted. Mean granuloma dry weight for various groups was calculated and expressed as mg/100 gm body weight. The percentage inhibition of granuloma dry weight was calculated using formula.

$$\text{Percentage Inhibition of granuloma dry weight} = \left(1 - \frac{\text{Dry weight of granuloma in treated group}}{\text{Dry weight of granuloma in control group}}\right) \times 100$$

Drugs used and their dosages:

The clinical doses for various drugs were converted to rat equivalent doses with the help of table devised by Paget and Barnes .[52]

1. Ciprofloxacin: (Zydus Cadila Healthcare LTD, Ahmedabad) It was administered in the dose of 67.5 mg/kg body weight equivalent to 750 mg of clinical dose orally.

2. Ofloxacin: (Zydus Cadila Healthcare LTD, Ahmedabad) It was administered in the dose of 36 mg/kg body weight equivalent to 400 mg of clinical dose orally.

3. Norfloxacin: (Zydus Cadila Healthcare LTD, Ahmedabad) It was administered in the dose of 36 mg/kg body weight equivalent to 400 mg of clinical dose orally.

4. Aspirin: (Ranbaxy Laboratories LTD, New Delhi) It was administered in the dose of 200 mg/kg body weight equivalent to 2222 mg of clinical dose orally.

5. Carrageenan: It was obtained in powder form from Sigma Co. St. Louis. Carrageenan is a mixture of polysaccharide composed of sulphated galactose units and is derived from Irish Seamoss. It was administered as a suspension in 1% warm normal saline, given in the volume of 0.05ml per rat paw.

The granulation tissue preserved in 10 % formalin was processed in the Department of Pathology, J.N. Medical College, Belgaum, and sections were stained with haematoxylin and eosin, and the granulation tissue in each group was studied microscopically.

Table 3: Drug treatment schedule for anti-inflammatory studies (acute and sub acute).

Group No	Treatment	Dose mg/Kg
*1	Control (1 % gum acacia)	0.5 ml
*2	Aspirin	200
*3	Ciprofloxacin	67.5
*4	Ofloxacin	36
*5	Norfloxacin	36

*Similar groups were included for sub acute studies and drugs were given once daily for 10 days - (n=6) in each group.

- Aspirin administered thirty minutes prior and ciprofloxacin, ofloxacin and norfloxacin administered one hour prior to carrageenan injection.
- All the drugs were administered orally as a suspension in 1% gum acacia.

Statistical analysis:

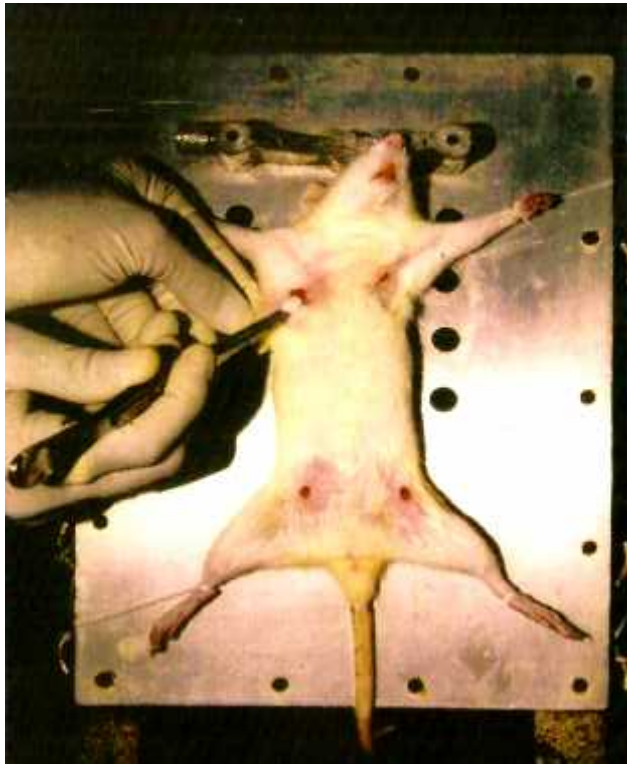
The data for all the groups was analyzed by one way ANOVA (Analysis of variance) followed by Dunnet's test using Graph pad prism software and $P \leq 0.05$ was considered significant.

Comparison between aspirin and treatment groups (ciprofloxacin, ofloxacin or norfloxacin) was done by one way ANOVA followed by Bonferroni's test using Graph pad prism software and $P \geq 0.05$ indicate that there was no significant difference between aspirin and treatment groups.

FIGURE 1

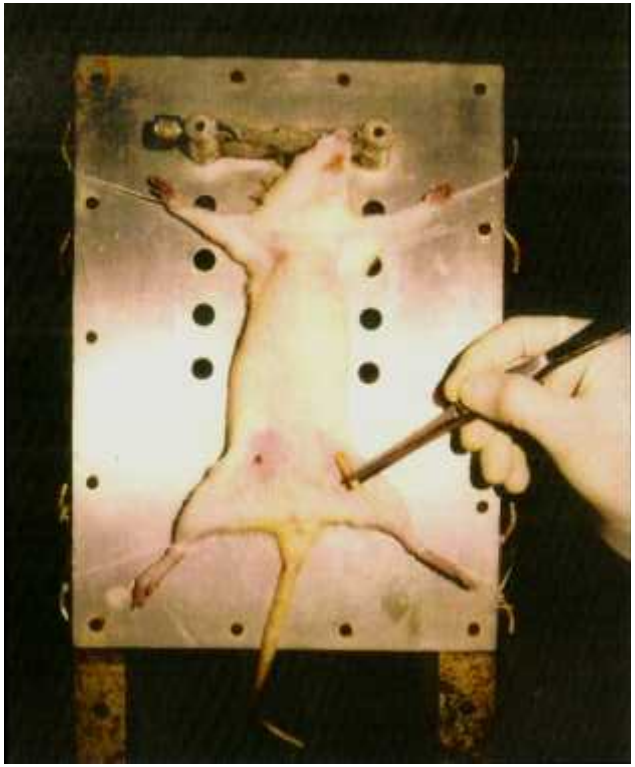
PLETHYSMOGRAPHIC MEASUREMENT OF THE RAT PAW EDEMA IN THE ACUTE INFLAMMATION STUDY

FIGURE 2



IMPLANTATION OF COTTON PELLET AS A FOREIGN BODY IN THE SUBACUTE INFLAMMATORY STUDY

FIGURE 3

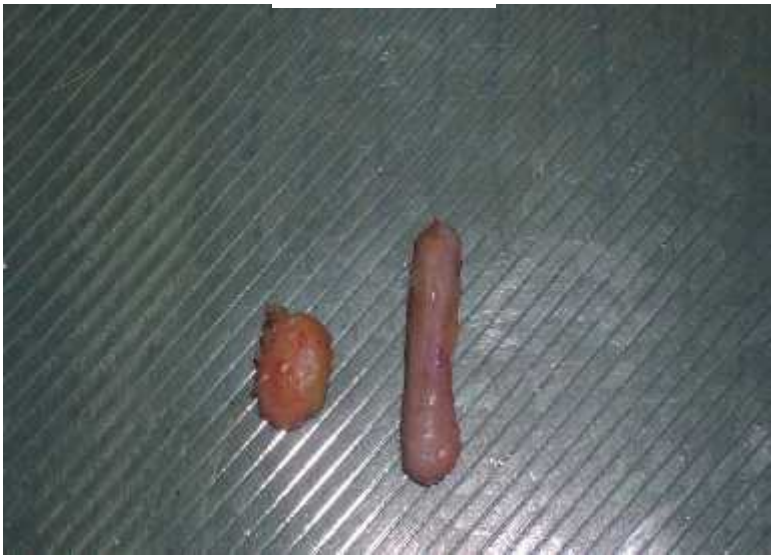


IMPLANTATION OF GRASS PITH AS A FOREIGN BODY IN THE SUBACUTE INFLAMMATORY STUDY

FIGURE 4

**DISSECTION OF GRASS PITH ON 11TH DAY,
GRASS PITH COVERED WITH GRANULATION TISSUE**

FIGURE 5



**COTTON PELLET AND GRASS PITH
COVERED WITH GRANULATION TISSUE**

RESULTS

In the present study, some fluoroquinolones like ciprofloxacin, ofloxacin and norfloxacin in their therapeutic equivalent doses, were investigated for their possible anti-inflammatory activity, in acute and subacute models of inflammation, in male Wistar rats.

CARRAGEENAN INDUCED ACUTE INFLAMMATION:

The edema volume in millilitres (ml), as measured by mercury displacement using a plethysmograph, for control group at ½ h, 1h, 3h, 4h, and 5h, was 0.35 ± 0.01, 0.51 ± 0.02 , 0.85 ± 0.02, 0.92 ± 0.01 and 0.85 ± 0.02 (Table- 4) respectively, while the corresponding mean volumes in aspirin (200 mg/kg) treated group was 0.18 ± 0.01, 0.27 ± 0.01, 0.28 ± 0.01, 0.21 ± 0.02 and 0.13 ± 0.01 respectively (Table- 4, Graph-1), with percentage inhibition 48.57%, 47.06%, 67.06%, 77.17% and 84.71% respectively indicating significant ($P < 0.01$) anti-inflammatory activity of aspirin (Table- 4, Graph- 2).

The edema volume in ml in ciprofloxacin treated group (67.5 mg/kg) at ½ h, 1h, 3h, 4h, and 5h was 0.25 ± 0.01, 0.33 ± 0.02, 0.36 ± 0.02, 0.28 ± 0.02 and 0.21 ± 0.02 respectively (Table- 4, Graph-1) with percentage inhibition 28.57%, 35.29%, 57.64%, 69.57% and 75.29% respectively suggesting significant inhibition of paw edema ($P<0.01$), indicating anti-inflammatory activity when compared with control (Table- 4, Graph- 2).

Ofloxacin in the dose of 36 mg/kg showed significant inhibition ($P<0.01$) of paw edema at ½ h, 1h, 3h, 4h, and 5h, with mean edema volume of 0.23 ± 0.01, 0.31 ± 0.01, 0.33 ± 0.01, 0.22 ± 0.01 and 0.16 ± 0.02 respectively (Table- 4, Graph-1) and percentage inhibition 34.28%, 39.21%, 61.17%, 76.08% and 81.17% respectively (Table- 4, Graph- 2) .

Also, Norfloxacin at the dose of 36 mg/kg exhibited significant inhibition $P<0.05$ at ½ h and $P<0.01$ at 1h, 3h, 4h, and 5h with mean edema volume of 0.29 ± 0.02, 0.36 ± 0.02, 0.38 ± 0.02, 0.29 ± 0.02 and 0.22 ± 0.02 (Table- 4, Graph- 1) and percentage inhibition 17.14%, 29.41%, 55.29%, 68.48% & 74.12% respectively indicating significant anti-inflammatory activity when compared with control (Table- 4, Graph- 2) .

The above results clearly indicate the anti-inflammatory activity of ciprofloxacin, ofloxacin and norfloxacin in acute model of inflammation.

Further effect of ciprofloxacin, ofloxacin and norfloxacin on carrageenan induced paw edema was compared with aspirin group. It was found that there was no statistically significant difference between ($P>0.05$) ofloxacin and aspirin group, indicating comparable anti-inflammatory activity, while a statistically significant difference was noted ($P<0.05$) between aspirin and other treatment groups (ciprofloxacin and norfloxacin) indicating that anti-inflammatory activity of ciprofloxacin and norfloxacin was less when compared to aspirin.(Table-5).

SUBACUTE INFLAMMATION (FOREIGN BODY INDUCED GRANULOMA METHOD):

The mean dry weight of ten day old granuloma, expressed as mg percent body weights, in control group was 40.83 ± 0.91, while aspirin (200mg/kg) treated group it was significantly decreased ($P<0.01$) with the mean value of 24.67 ± 1.12 and percentage inhibition of 39.58%. Similarly, ciprofloxacin (67.5 mg/kg), ofloxacin (36 mg/kg) and norfloxacin (36 mg/kg) treated group exhibited decreased granuloma dry weight ($P<0.01$) with mean value of 25.00 ± 1.03, 24.50 ± 0.88 and 27.33 ± 0.71 respectively and percentage inhibition of 38.77%, 40.00% and 33.07% respectively

indicating significant anti-inflammatory activity when compared with control (Table- 6, Graph-3 and 4).

Further mean granuloma dry weight of ciprofloxacin, ofloxacin and norfloxacin group was compared with mean granuloma dry weight of aspirin group. It was found that, there was no statistically significant difference ($P>0.05$) between them, indicating that anti-inflammatory activity of ciprofloxacin, ofloxacin and norfloxacin was comparable to aspirin in subacute study (Table-7).

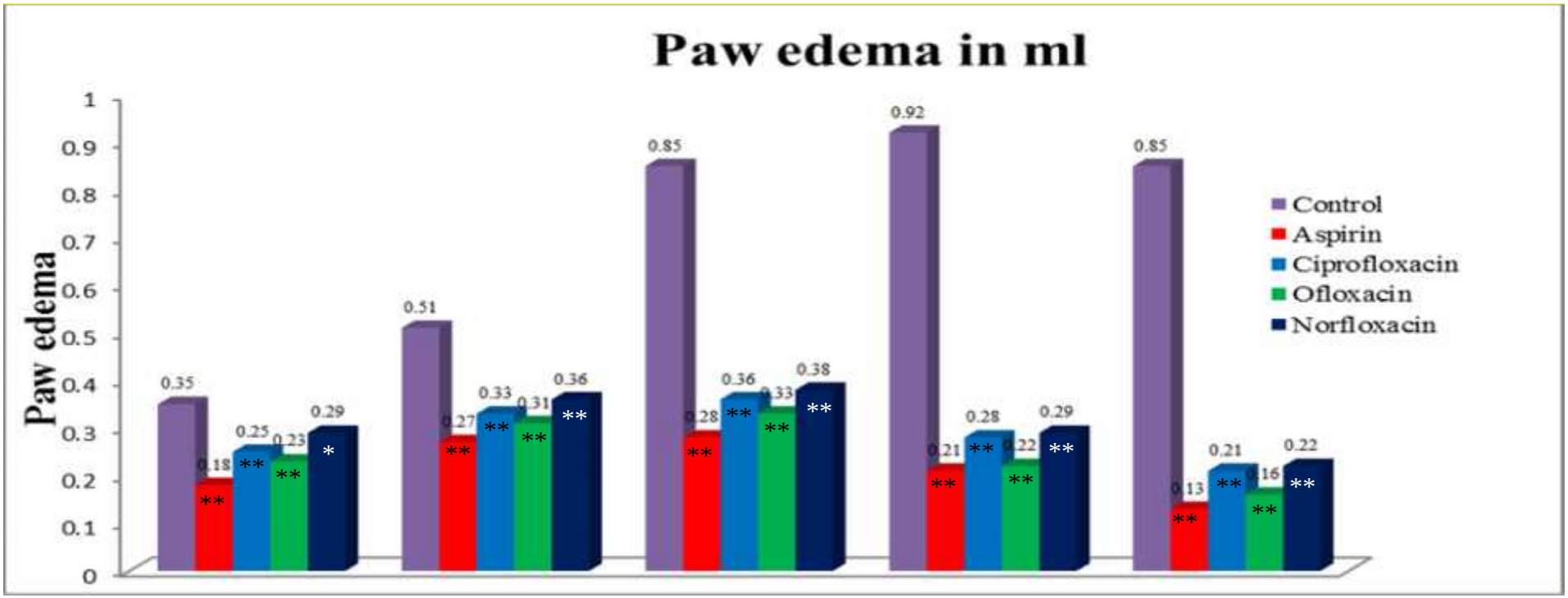
The anti-inflammatory activity of these fluoroquinolones as observed in both, acute and sub acute studies was further confirmed by histopathological studies. The sections of granulation tissues when stained with haematoxylin and eosin showed abundant fibrous tissue in the control group, while revealed reduced number of fibroblasts, decreased collagen content and fibrous tissue in the drug treated groups (Figure 6, 7, 8, 9 and 10).

TABLE 4: EFFECT OF VARIOUS TREATMENTS ON CARRAGEENAN INDUCED PAW EDEMA

Time after carrageenan injection	Control Paw edema In ml (Mean +/-SEM)	Aspirin		Ciprofloxacin		Ofloxacin		Norfloxacin		ANNOVA Result	
		Paw edema In ml (Mean +/-SEM)	Percentage Inhibition	Paw edema In ml (Mean +/-SEM)	Percentage inhibition	Paw edema In ml (Mean +/-SEM)	Percentage inhibition	Paw edema In ml (Mean +/-SEM)	Percentage inhibition	$F_{4, 25}$	P Value
½ hr	0.35 ± 0.01	0.18± 0.01**	48.57	0.25± 0.01**	28.57	0.23± 0.01**	34.28	0.29± 0.02*	17.14	25.30	<0.0001
1hr	0.51 ± 0.02	0.27± 0.01**	47.06	0.33± 0.02**	35.29	0.31± 0.01**	39.21	0.36± 0.02**	29.41	31.84	<0.0001
3hr	0.85 ± 0.02	0.28± 0.01**	67.06	0.36± 0.02**	57.64	0.33± 0.01**	61.17	0.38± 0.02**	55.29	214.4	<0.0001
4hr	0.92 ± 0.01	0.21± 0.02**	77.17	0.28± 0.02**	69.57	0.22± 0.01**	76.08	0.29± 0.02**	68.48	401.4	<0.0001
5hr	0.85 ± 0.02	0.13± 0.01**	84.71	0.21± 0.02**	75.29	0.16± 0.02**	81.17	0.22± 0.02**	74.12	269.1	<0.0001

Post hoc analysis by Dunnet's Test: * $P < 0.05$, **$P<0.01$

Graph 1: Paw edema in ml after carrageenan injection



* P < 0.05, **P<0.01

Graph 2: Percentage inhibition of Paw edema

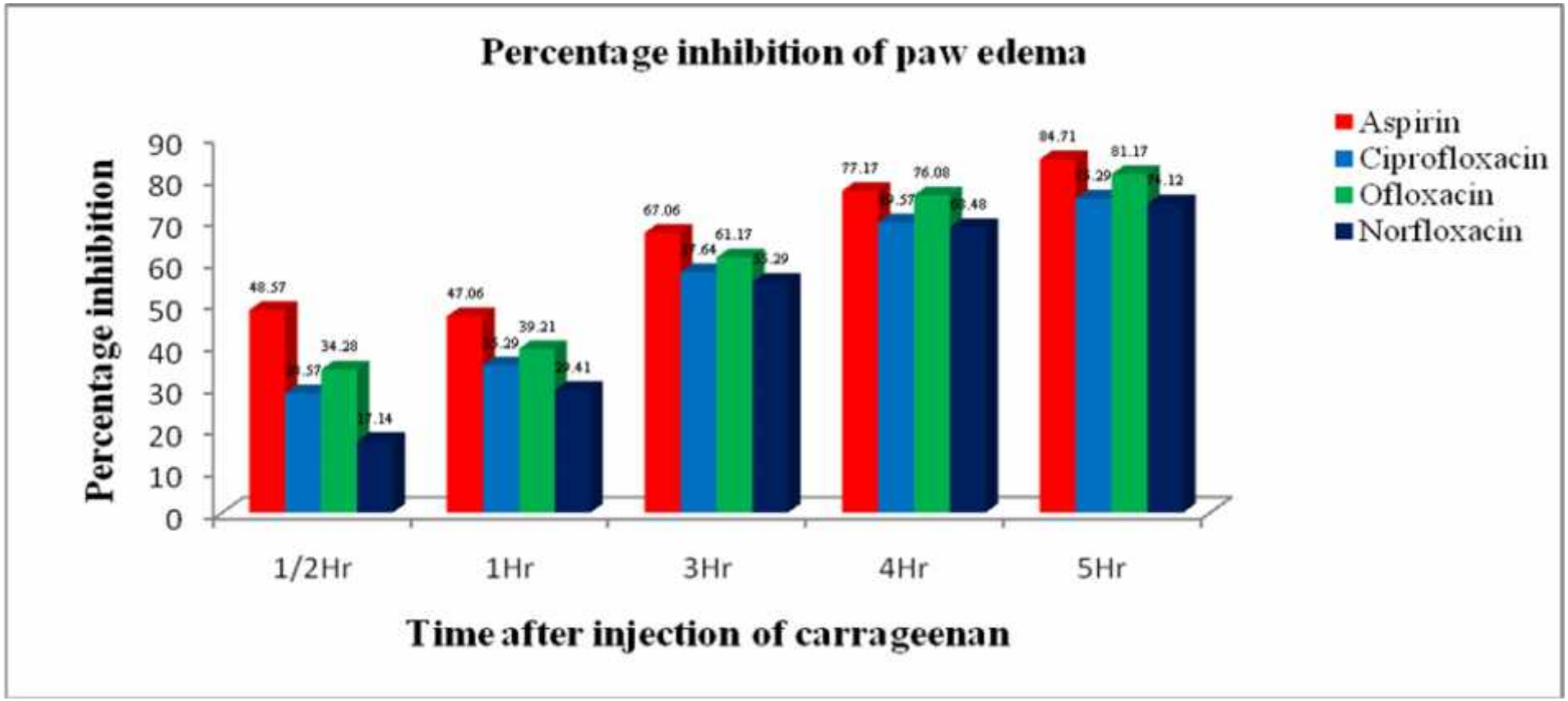


TABLE 5: EFFECT OF CIPROFLOXACIN, OFLOXACIN AND NORFLOXACIN ON CARRAGEENAN INDUCED PAW EDEMA WHEN COMPARED WITH ASPIRIN GROUP

Time after carrageenan injection	**Aspirin** Paw edema In ml (Mean+/- SEM)	**Ciprofloxacin** Paw edema In ml (Mean+/- SEM)	**Ofloxacin** Paw edema In ml (Mean+/- SEM)	**Norfloxacin** Paw edema In ml (Mean+/- SEM)	ANNOVA Result	
					$F_{3,20}$	P Value
½ hr	0.18± 0.01	0.25± 0.01	0.23± 0.01	0.29± 0.02	12.90	<0.01
1hr	0.27± 0.01	0.33± 0.02	0.31± 0.01	0.36± 0.02	8.145	<0.01
3hr	0.28± 0.01	0.36± 0.02	0.33± 0.01	0.38± 0.02	7.040	<0.01
4hr	0.21± 0.02	0.28± 0.02	0.22± 0.01	0.29± 0.02	7.387	<0.01
5hr	0.13± 0.01	0.21± 0.02	0.16± 0.02	0.22± 0.02	6.216	<0.01

Post hoc analysis by Bonferroni's Test

½hr - Aspirin Vs Ciprofloxacin P < 0.01, Aspirin Vs Ofloxacin P > 0.05 and Aspirin Vs Norfloxacin P< 0.01

1 hr - Aspirin Vs Ciprofloxacin P < 0.05, Aspirin Vs Ofloxacin P > 0.05 and Aspirin Vs Norfloxacin P< 0.01

3 hr - Aspirin Vs Ciprofloxacin P < 0.05, Aspirin Vs Ofloxacin P > 0.05 and Aspirin Vs Norfloxacin P< 0.01

4 hr - Aspirin Vs Ciprofloxacin P < 0.05, Aspirin Vs Ofloxacin P > 0.05 and Aspirin Vs Norfloxacin P< 0.01

5 hr - Aspirin Vs Ciprofloxacin P < 0.05, Aspirin Vs Ofloxacin P > 0.05 and Aspirin Vs Norfloxacin P< 0.01

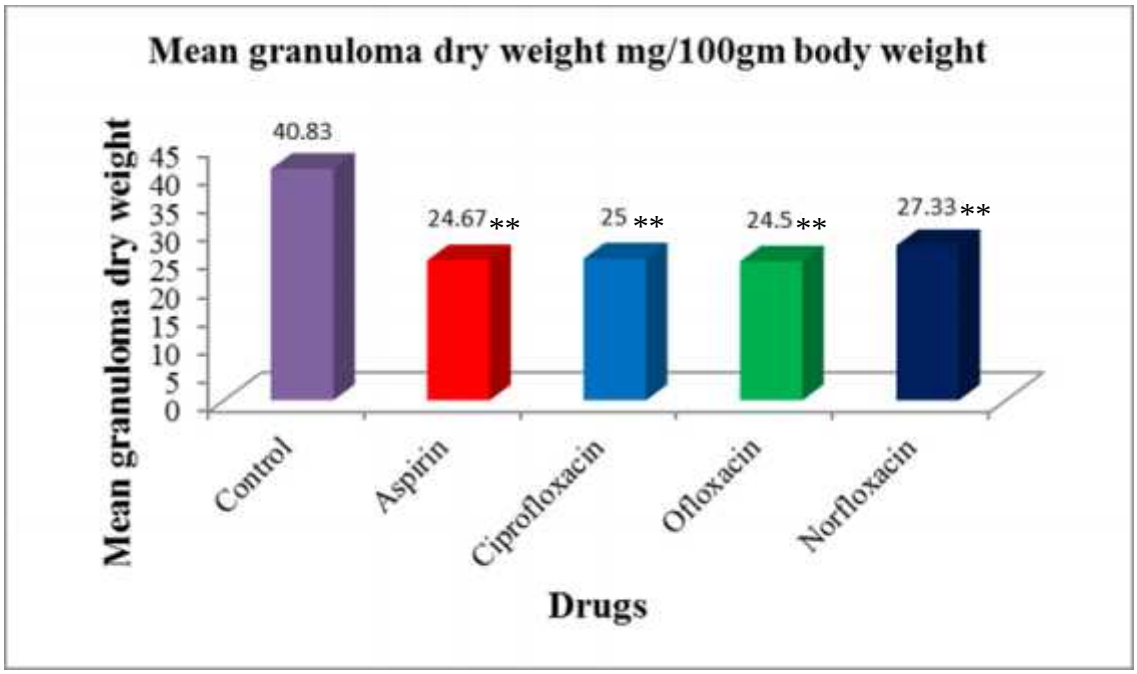
TABLE 6: EFFECT OF VARIOUS TREATMENTS ON GRANULOMA DRY WEIGHT

Sl. No	**Drug Treatment**	**Mean granuloma dry weight mg/100gm body weight (Mean+/- SEM)**	**Percentage inhibition**
1.	Control	40.83 ± 0.91	
2.	Aspirin	24.67 ± 1.12****	39.58 %
3.	Ciprofloxacin	25.00 ± 1.03****	38.77%
4.	Ofloxacin	24.50 ± 0.88****	40.00%
5.	Norfloxacin	27.33 ± 0.71****	33.07%

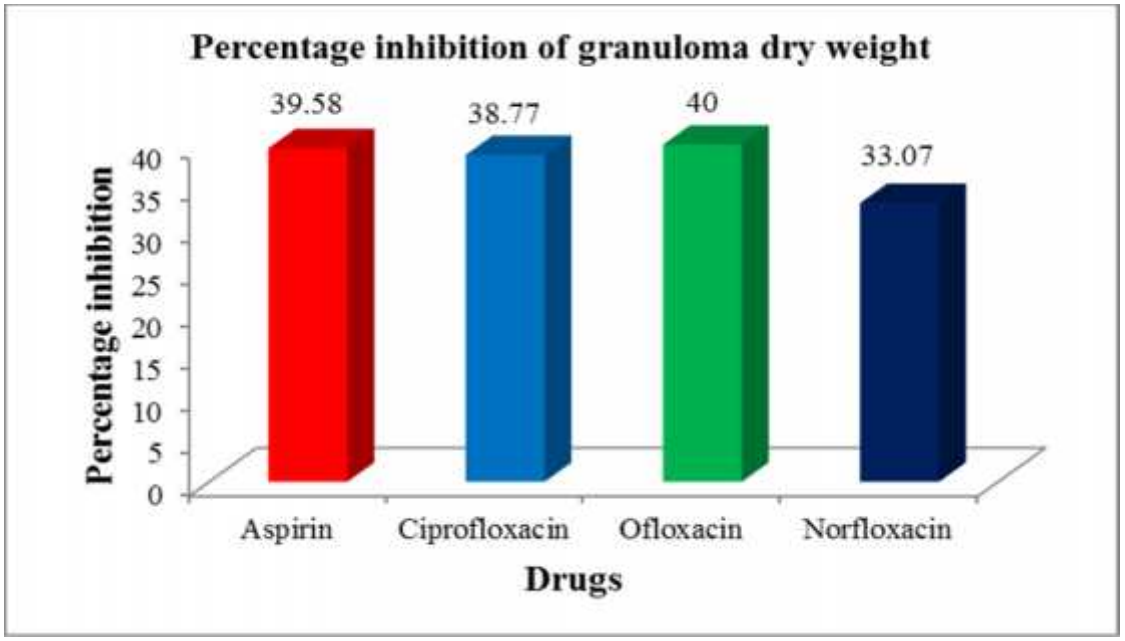
ANNOVA: $F_{4, 25} = 55.38$, $P < 0.0001$

Post hoc analysis by Dunnet's Test: ****P<0.01

Graph 3



Graph 4



**** P < 0.01**

TABLE 7: EFFECT OF CIPROFLOXACIN, OFLOXACIN AND NORFLOXACIN ON GRANULOMA DRY WEIGHT WHEN COMPARED WITH ASPIRIN GROUP

Sl. No	Drug Treatment	Mean granuloma dry weight mg/100gm body weight (Mean+/-SEM)
1.	Aspirin	24.67 ± 1.12
2.	Ciprofloxacin	25.00 ± 1.03
3.	Ofloxacin	24.50 ± 0.88
4.	Norfloxacin	27.33 ± 0.71

ANNOVA: $F_{3, 20} = 1.939$

$P > 0.05$

There was no statistically significant difference (P>0.05) in Granuloma dry weight between aspirin and treatment Group

PHOTOMICROGRAPHS OF GRANULATION TISSUE

(H AND E STAIN 10X)

FIGURE 6: Control

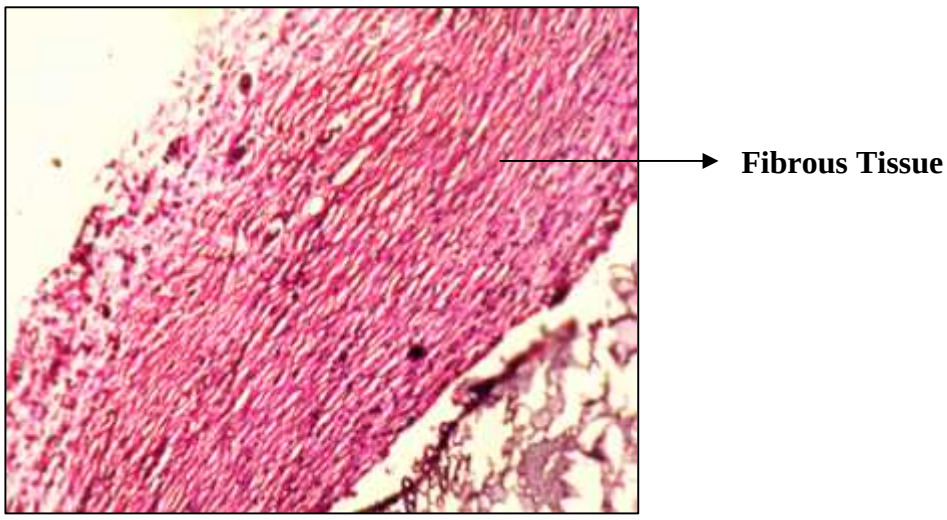
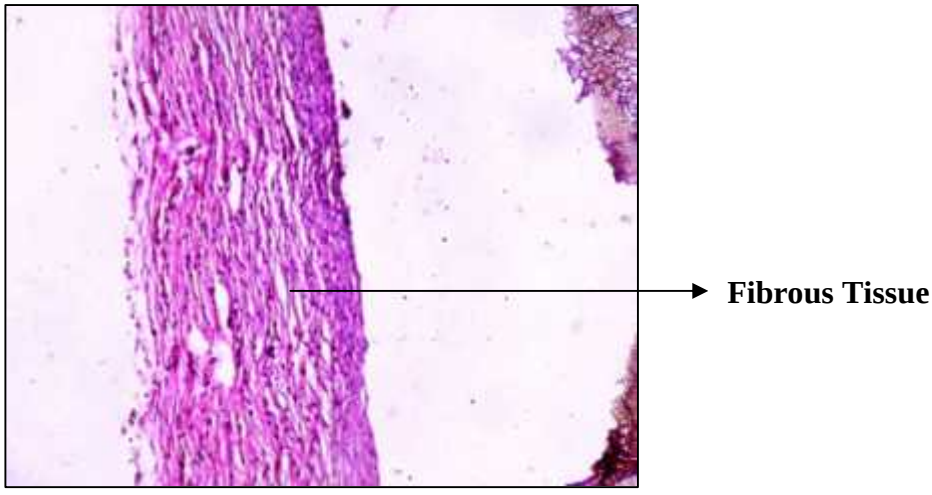


FIGURE 7: Aspirin

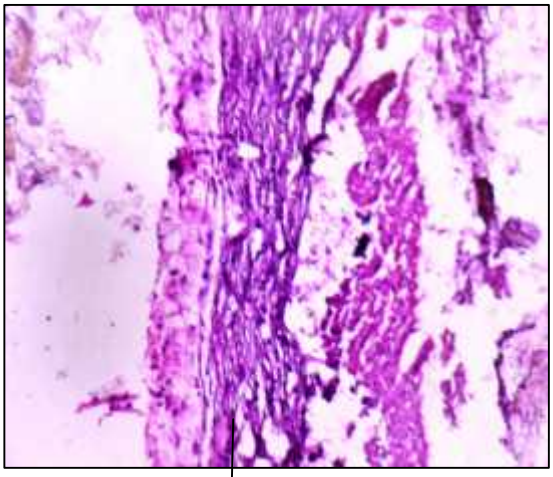


Note: Markedly reduced fibroblasts and collagen in aspirin group when compared to that of control group

PHOTOMICROGRAPHS OF GRANULATION TISSUE (H AND E STAIN)

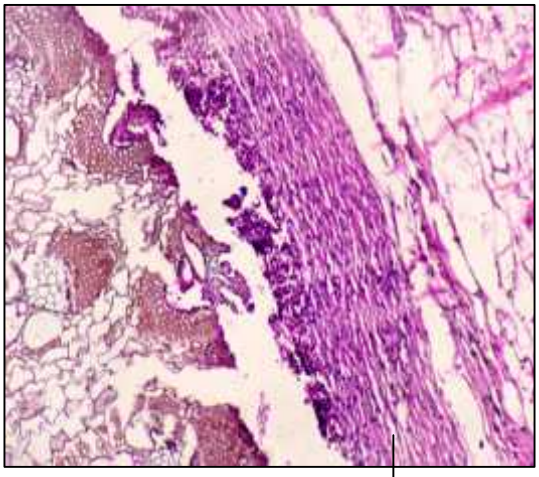
(H AND E STAIN 10X)

FIGURE 8: Ciprofloxacin



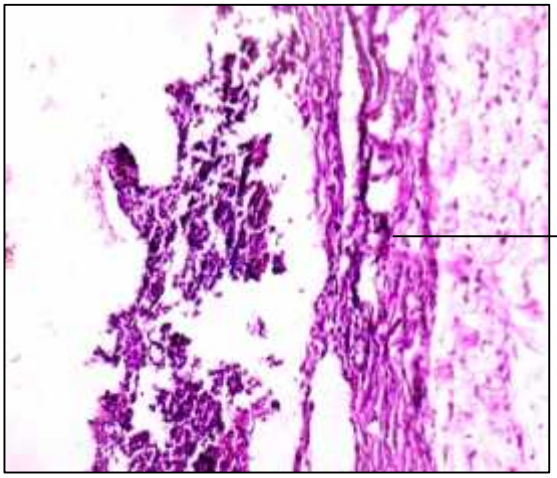
Fibrous Tissue

FIGURE 9: Ofloxacin



Fibrous Tissue

FIGURE 10: Norfloxacin



Fibrous Tissue

Note: Markedly reduced fibroblasts and collagen in ciprofloxacin, ofloxacin and norfloxacin group when compared to that of control

DISCUSSION

As mentioned in the introduction, the present study was planned to investigate the influence of some fluoroquinolones like ciprofloxacin, ofloxacin and norfloxacin on acute as well as subacute inflammation in male Wistar rats.

Results of the present study clearly indicate that all the three fluoroquinolones used in the study showed significant anti-inflammatory activity when compared with control in acute as well as subacute models of inflammation. Comparable anti-inflammatory activity was seen with aspirin and ofloxacin in acute model, while all the three fluoroquinolones (ciprofloxacin, ofloxacin and norfloxacin) showed anti-inflammatory activity comparable to aspirin in subacute models of inflammation.

Observations of the study are in agreement with the earlier reports stating that fluoroquinolones may have anti-inflammatory activity, [11, 12, 13, 14, 15, 16] while, disagree with some earlier studies wherein, fluoroquinolones have been reported to possess pro-inflammatory activity. [17, 18, 19]

In the present study mechanism of anti-inflammatory action of ciprofloxacin was not evaluated, but by previous reports it can be attributed to its property to induce production of PGE_2.[10] PGE_2 released from antigen-presenting cells, primes naive human T cells and enhance production of anti-inflammatory cytokines while inhibiting the synthesis of proinflammatory cytokines.[10] Among the four PGE_2 receptor subtypes, E-prostanoid 1(EP_1), EP_2, EP_3 and EP_4, the activation of EP_2 and EP_4 receptors leads to an increase in cyclic AMP (cAMP) levels, protein kinase A (PKA) activity and inhibits T-cell proliferation resulting in anti-inflammatory activity.[77] PGE_2 prevent the IL-18 induced expression of Inter Cellular Adhesion Molecule (ICAM), B7.2, and CD 40 on monocytes and the production of IL-12,

TNF-α, and IFN- γ in human peripheral blood mononuclear cells. [78, 79] Also studies have reported PGE_2 to inhibit P65 translocation and NFkappaB activation. PGE_2 also block IL-1beta/TNF-α stimulated ERK activation and may attenuate cytokine induced inflammatory responses.[12] All these actions may contribute to anti-inflammatory activity.

Ciprofloxacin has been reported to suppress the expression of CD14, Toll like receptor(TLR) -4, ICAM-1, B7.1, B7.2 and CD40, and the production of TNF- α induced by lipopolysacchrides in monocytes,[13] inhibits proinflammatory cytokines like IL-1, TNF α, INF γ,[14] IL- 8[15], IL- 12 and IL-18 [10] . It is also known to increase cAMP which in turn reduces release of inflammatory mediators like histamine, leukotrienes, reactive oxygen species and nitric oxide.[80] Ciprofloxacin has also been shown to inhibit advanced glycation end products-induced adhesion molecule expression on human monocytes.[81] All these actions of ciprofloxacin may contribute to its anti-inflammatory activity.

Since ciprofloxacin is considered as prototype of fluoroquinolones,[33] ofloxacin and norfloxacin's anti-inflammatory activity may be attributed to mechanism similar to that of ciprofloxacin but there is paucity of information regarding the same.

Since these drugs have significant anti-inflammatory activity, their use can be promoted in treating pelvic inflammatory disease and urethritis caused by neisseria gonorrhoea and chlamydia infections wherein they not only eliminate organisms but also may reduce complications like tubal block, sterility, dyspareunia in women and urethral stricture in males. Fluoroquinolones can be included with other first line anti-tubercular drugs in treating tuberculosis, where it can reduce complications like pulmonary fibrosis, constrictive pericarditis, hydrocephalus, tubal stricture leading to

infertility, peritoneal adhesions leading to intestinal obstruction etc., by acting both as antimicrobial and anti-inflammatory drug. However these speculations need to be confirmed clinically.

CONCLUSION

The findings of the present experimental study appear to be clinically relevant. All the three fluoroquinolones have shown significant anti-inflammatory activity in acute and subacute models of inflammation. Use of fluoroquinolones in treating PID, gonococcal urethritis and tuberculosis can result in elimination of invading organism by virtue of their antimicrobial activity, and reduce complications due to inflammation and fibrosis which are associated with these conditions by virtue of their anti-inflammatory activity.

SUMMARY

Based on the controversial reports on influence of fluoroquinolones on inflammation, the three fluoroquinolones viz ciprofloxacin, ofloxacin and norfloxacin were investigated for their anti-inflammatory activity in both acute (carrageenan induced rat paw edema) and subacute (foreign body induced granuloma) models of inflammation using male Wistar rats.

All the three fluoroquinolones when compared with control showed significant inhibition of rat paw edema in acute model and granuloma dry weight in subacute model of inflammation, indicating significant anti-inflammatory activity. Comparable anti-inflammatory activity was seen with ofloxacin and aspirin in acute study, while in subacute study, anti-inflammatory activity of all the three fluoroquinolones (ciprofloxacin, ofloxacin and norfloxacin) was comparable to anti-inflammatory activity of aspirin. Histopathological examination of grass pith revealed markedly reduced fibroblasts and collagen in treatment group which was similar to aspirin when compared to control. These results clearly indicate that all the three fluoroquinolones have anti-inflammatory property.

PID, gonococcal urethritis and tuberculosis are associated with significant inflammation and fibrosis resulting in complications like dyspareunia, urethral stricture, infertility, intestinal obstruction etc. Fluoroquinolones when given in these conditions, by virtue of their anti-inflammatory activity may reduce complications of the disease.

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CERTIFICATE

This is to Certify that the research project "EFFECT OF CIPROFLOXACIN, OFLOXACIN AND NORFLOXACIN ON ACUTE AND SUBACUTE INFLAMMATION IN MALE WISTAR RATS"

Submitted by **BO0108001** has been approved in the Institutional Animal Ethics Committee meeting held on 19th December 2008, resolution No. JNMC/IAEC/Res-2/8/2008 and was permitted to use 30 Rats/~~Mice/Rabbits~~.

You are hereby informed to strictly adhere to the protocol submitted for approval. In case the project needs to be modified later, the modified version of the protocol should be submitted to the Committee, stating valid reasons for such modifications for fresh approval.

You are required to keep the account of animals used for the project in specified proforma, Form-D.

You have to submit the brief report to the Committee after completion of the project along with **Form-D**

Dr. P.A.Patil.
Member-Secretary, IAEC,
J.N.Medical College,
BELGAUM-10.

Dr. A.Jagannadha Rao
Nominee of CPCSEA for IAEC
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Ref. No. : MDC/DOME/2158 Date: 7/10/2008

To,

BO0108001
Postgraduate student in
Department of Pharmacology,
J.N Medical College,
Belgaum.

Dear Dr. **BO0108001**

The JNMC – Institutional Ethics Committee on Human Subjects Research met on 6th October, 2008 to consider your application for approval of the research project **"EFFECT OF CIPROFLOXACIN, OFLOXACIN AND NORFLOXACIN ON ACUTE AND SUBACUTE INFLAMMATION IN MALE WISTAR RATS – AN EXPERIMENTAL STUDY".**

After review of the documents submitted by you and satisfactory explanations provided to the members, the committee has provided approval date through October 5th, 2009 at which time the study will be reviewed by the committee.

If you have any questions concerning the above, please feel free to contact the committee office.

Sincerely,

(Dr.V.D.Patil)
Chairman,
JNMC Institutional Ethics Committee on
Human Subjects Research