Review of Literature
Chapter 2

Review of Literature

2.1 Inflammation

Inflammation is defined as the local response of living mammalian tissue to injury due to any agent\[^{17}\]. It is a common clinical condition said to be protective response intended to eliminate the initial cause of cell injury as well as the necrotic cells and tissues resulting from original insult\[^{18}\]. Inflammation manifests usually in the form of painful swelling associated with some changes in skin covering the site and usually the process may interfere with physical function of inflamed part. The cardinal signs viz. redness, swelling, heat, pain and loss of function are brought about by complex actions of various inflammogens e.g. histamine, bradykinins, prostaglandins (PGs), leukotrienes etc. The inflammatory response could be suppressed logically, by inhibiting the activity of these endogenous mediators. However, the use of antagonists of some of the mediators to suppress inflammation may not be feasible clinically. Antihistamines are rarely used as anti-inflammatory agents probably due to their clinical inefficacy, while PG synthesis inhibitors like NSAIDs are clinically effective and are widely used.
in practice.

The NSAIDs are not completely devoid of adverse effects. The gastric or intestinal mucosal damage is the commonest adverse effect attributed to their ability to block non-specifically cyclooxygenase (COX-1 and COX-2) enzyme. Inhibition of COX-1, a constitutive enzyme is said to be responsible for gastric adverse effects. Newer NSAIDs like celecoxib, rofecoxib which inhibit selectively COX-2 enzyme are supposed to be free from such toxicity, but not completely devoid of other adverse effects.

The historical highlights are:

- Clinical feature of inflammation were described in an Egyptian papyrus dated around 3000 B.C.

- Celsus: A Roman writer of first century A.D. first listed four cardinal sign of inflammation Rubor, Tumor, Calor and Dolor.

- Fifth clinical sign loss of Function (Functio laesa) was later added by Virchow.

- In 1793, the Scottish Surgeon John Hunter noted what is now considered an obvious fact: that inflammation is not a disease but nonspecific response that has a salutary effect on its host.

- Metchnikoff and Paul Ehrlich (who developed the humoral theory of immunity) shared the nobel prize in 1908.

- To these names must be added the name of Sir Thomas Lewis who on the basis of simple experiments studying the inflammatory response in skin,
established the concept that chemical substance such as histamine locally induced by injury mediate the vascular changes of inflammation.

Inflammation is the reaction of the vascular and supporting element of a tissue to injury, and result in the formation of a protein rich exudate, provided the injury has not been so severe as to destroy the area. It is a body’s defense reaction, in order to eliminate or limit the spread of injurious agent as well as to remove the consequent necrotic cell and tissue.

2.1.1 Types of Inflammation

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

Based on the course and duration, the inflammation can be called as:

1. Acute: It is characterized by local vasodilatation and increased capillary permeability. It is immediate and early response to injurious agents having three major components.

   (a) Increase in blood flow.

   (b) Structural changes that leads to plasma protein and leukocytes into circulation.

   (c) Accumulation of leukocyte in focus of injury.

2. Sub-Acute: The inflammation lasts for 1 to 6 weeks or more. The type which is neither acute nor chronic is termed as sub-acute inflammation. It lasts longer as compared to acute inflammation. Microscopically vascular,
exudative as well as proliferative changes of acute and chronic inflammation are present. Exudate chiefly consists of eosinophils, lymphocytes, plasma cells, histocytes and fibroblasts.

3. Chronic: Chronic inflammation as described by Robbins, is considered to be of prolonged duration (weeks or months) in which active inflammation, tissue destruction and attempts at healing are proceeding simultaneously. Chronic inflammation frequently begins insidiously as a low grade, smoldering after asymptomatic response and examples include rheumatoid arthritis, atherosclerosis, tuberculosis etc. Chronic inflammation is characterized by,

(a) Infiltration with mononuclear cells.
(b) Tissue destruction.
(c) Repair by connective tissue replacements.

2.1.2 Process of Inflammation

It is clearly known that the inflammation involves many mechanisms that are continuously operating during the process. The histopathological and biochemical studies of inflammation indicate that it develops in two distinct phases.

1. Vascular Phase

2. Cellular Phase

Vascular Phase

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

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

1. Transient vasoconstriction of arterioles.

2. Persistent progressive vasodilatation.

3. Slowing of the blood flow and stasis.

1. Transient vasoconstriction of arterioles:

Regardless of the stimulus, there may be transient vasoconstriction of the arterioles immediately followed by an injury probably due to antidromic nerve reflex mechanism\textsuperscript{19}. This results in reduction of blood flow to the injured areas and lasts for few minutes if the injury is mild and for several minutes, if the injury is severe as in burns.

2. Persistent progressive vasodilatation:

Immediately after transient vasoconstriction of arterioles, the progressive vasodilatation of the blood vessels, which involves mainly the arterioles and to a less extent affects other components of microcirculation like venules and capillaries. These changes appear within half an hour of injury. Initially vasodilatation is mediated by vasoactive amines but after 30 to 60 minutes it appears to be due to kinins, prostaglandins etc.\textsuperscript{22}

3. Slowing of the blood flow and stasis:

The concomitant change in vascular permeability due to released mediators leads to the movement of fluid and protein into the tissues (exudate). The escape of plasma into the tissues causes consequent rise in viscosity of blood
leading to increased resistance to flow in venules and a corresponding rise in hydrostatic pressure in capillary bed. The latter effect assists the expulsion of more plasma, leading to further increase in viscosity and blood stasis. The time scale of changes in vascular caliber is variable. With mild stimuli, stasis may not become apparent until 15 to 30 minutes have elapsed, whereas with severe injury, stasis may occur in a few minutes. 

**Cellular Events in Inflammation**

The major event at the site of inflammation is accumulation of leukocytes, to engulf the inflammogens in order to protect rest of the normal tissue. For this purpose, leukocytes reach the site of injury by marginization, pavementing, sticking, emigration and chemotaxis. Following stagnation, red blood cells stick together to form clumps by a process known as sludging. These red blood cell clumps largely occupy central axis of blood stream leaving white blood cell to move peripherally. Neutrophils are said to extrude pseudopods to separate the intracellular junctions between the endothelial cells to emigrate between them to pass through basement membrane. Finally neutrophil polymorphs penetrate the vessel wall within 2-9 minutes.

The unidirectional migration of cells towards an attractant, or more simply, locomotion oriented along a chemical gradient is called as chemotaxis. There are separate chemotactic factors for neutrophils, monocytes, eosinophils. Bacterial products, components of complement system are reported to be chemotactic agents for neutrophils and eosinophilic granulocytes. The various factors recognized to influence the process include Leukotriene B4 (LTB4), 15 hydroxyeicosa tetraenoic acid (15-HETE), platelet activating factor (PAF) and component of
complement system (C5a) \textsuperscript{26, 27}. The leukocytes, initially predominated by neutrophils, adhere to the endothelium via adhesion molecules, then leave the microvasculature and migrate to the site of injury under the influence of chemotactic agent. Phagocytosis, killing and degradation of the offending agent follows, which may lead to death of the microorganism. During chemotaxis and phagocytosis, activated leukocytes may release toxic metabolite and proteases extracellularly, potentially causing tissue damage \textsuperscript{28}.

2.1.3 Mediators of Inflammation

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

1. Vasoactive amines
   
   (a) Histamine
   
   (b) Serotonin

2. Arachidonic acid metabolites
   
   (a) Eicosanoids
      
      i. Prostaglandins
      
      ii. Leukotrienes and Lipoxine
   
   (b) Platelet activating factors (PAF)

3. Cytokine - Interleukin, tumor necrotic factor, chemokines
1. Vasoactive Amines

(a) Histamine

One of the mediators to be described earliest was the vasoactive amine, histamine. The fact that injections of histamine could reproduce many of the features of acute inflammation led Lewis to speculate that it was acting as the mediator of the inflammatory response. Histamine, which is present throughout the body tissues is formed by decarboxylation of the natural amino acid, histidine, and is stored primarily in mast cells, which are usually found along the blood vessels, and also in circulating basophils. Irrespective of nature of noxious stimulus, histamine is released from basophils and mast cells mediating various events especially in acute inflammation. Histamine release is associated with increase in blood flow, increase in microvascular permeability and oedema formation. In addition, in skin, it causes a flare response due to local axon reflex leading to vasodilatation. Histamine can also cause pain and itching. Although it
Figure 2.1: Scheme for mediators derived from arachidonic acid and sites of drug action (dashed arrows)
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does not have general chemotactic activity, histamine may be chemotactic specifically for eosinophils. In chronic inflammation, histamine although could mediate a succession of acute episodes, it can be considered as a major mediator of increased microvascular permeability. Thus in acute inflammation, histamine could cause both vasodilatation and increase vascular permeability, but in chronic inflammation would only fulfil the vasodilator role, perhaps serving to potentiate the increase in microvascular permeability caused by a second mediator.

Pharmacological analysis of the receptor involvement in inflammatory responses to histamine has shown that the vasodilatation involves both H1 and H2 receptors and microvascular permeability involves only H1 receptors. Although the individual H1 and H2 antagonists have been reported by some workers to possess the anti-inflammatory activity, the combination of H1 and H2 receptor antagonists are more effective anti-inflammatory agents as compared to any one single receptor antagonist. Recent data provide the evidence that, similarly to adenosine and \( PGE_2 \), histamine is a potent suppressor of LT biosynthesis, and support concept that histamine may play a dual role in the regulation of inflammation.

(b) Serotonin (5-HT)

5-HT is a naturally occurring amine with major effects on a variety of bodily functions. The first systemic studies with 5-HT were made independently by Page and Gaddum in 1957. Four families of 5-HT receptors (5-HT1, 5-HT2, 5-HT3, 5-HT4) comprising of 14 receptor subtypes have so far been recognized. From 5-HT1 types 5-HT1B/1D
is known to constrict cranial blood vessels and inhibit release of inflammatory neuropeptide [34].

Much of the recent work has been focused on vascular and neurological studies with 5-HT. Currently it appears that 5-HT is a mediator of acute inflammation in rats and mice only. Unlike most inflammatory mediators, 5-HT does not increase local blood flow.

It is now appreciated that 5-HT is a natural constituent of rodent mast cells but not those of most other animal species or man. The absence of 5-HT from mast cells in non-rodent species largely precludes a role for this amine in inflammation in these species.

5-HT is widely distributed in high concentration mainly in the intestine, blood, spleen and nervous system. 5-HT are similar to histamine but it is a less potent mediator of increased vascular permeability and vasodilation than histamine [35].

2. Arachidonic Acid Metabolites (AA-Metabolites)

(a) Eicosanoids Oxidative metabolism of arachidonic acid is increased in inflamed tissues. In body PGs, thromboxane (TXA) and leukotrienes (LTs) are all derived from eicosa (referring to 20C atom) tri/tetra/pentaenoic acids. Therefore they can be collectively called eicosanoids. There are two principle enzyme pathways of arachidonic acid oxidation and production involved in inflammatory process. They are:

- The cyclooxygenase which produces prostaglandins, and
- The 5-lipoxygenase, which produces leukotrienes and lipoxins.

Of all, the various cyclooxygenase products formed during inflammation, \( \text{PGE}_2 \) and prostacyclin (\( \text{PGI}_2 \)) are the most important. Both
these products are potent vasodilators and hyperalgesic agents and since they have been detected at sites of inflammation, it is believed that they contribute to the erythema, oedema and pain, which are characteristic of the inflammatory response \(^{36}\). The selective inhibition of cyclooxygenase by NSAIDs explains the anti-inflammatory, analgesic and antipyretic activity of this class of drugs.

Of all the leukotrienes, LTB4 is the strongest candidate as an inflammatory mediator. It is one of the most potent leukotactic substance known and its presence in inflamed tissues could represent a local control mechanism for the accumulation of inflammatory leukocytes.

i. Prostaglandins (PGs):

The PGs are a group of oxygenated, polyunsaturated, C20 fatty acids containing a characteristic cyclopentane ring between C8 and C12. PGs are not stored in tissues but they are bio-synthesized from fatty acid precursors such as phospholipids. High levels of PG were found in inflammatory exudates. PGs are reported to induce vasodilatation, increased vascular permeability, mast cell degranulation and leukocyte accumulation \(^{37}\). Although all the major immune inflammatory cell types except lymphocytes are capable of generating PGs after an appropriate stimulus, in general, macrophages seems to be the major source. PGD2 major metabolite of the cyclooxygenase pathway in mast cells, it causes vasodilation and potentiates edema formation \(^{38}\). PGE series are powerful vasodilators which produce long lasting erythema following intradermal injections. \(PGE_2\) plays a major role in the production of oedema, erythema and pain of inflamm-
Recent evidence suggests that \textit{PGI}_2 also contributes to this response \cite{39}. \textit{PGE}_2 is also said to be one of the most potent pyretic agent known \cite{10}. \textit{PGI}_2 and \textit{PGE}_2 potentiate the oedema and pain producing effects of other mediators such as histamine, bradykinin and complement component C5a. This marked potentiation of the response to histamine and bradykinin has been observed in several species viz., guinea pig, rat, rabbit and man \cite{11}. \textit{PGI}_2 can potentiate the carrageenan induced oedema and hyperalgesia in rats. Polymorphonuclear leukocytes appear to be responsible for the local release of thromboxane (TXA2) during carrageenan induced inflammation in rats \cite{12}.

\textbf{ii. Leukotrienes (LTs):}

The leukotrienes (LTs) were so named because they were originally demonstrated in “leukocytes” and they contain a conjugated “triene” system in their structure; unlike PGs, they do not contain a ring system. Like PGs, LTs are also not stored in tissues, but are biosynthesized from phospholipids upon cell stimulation. LTs have biological actions that are often more potent than those of previously known mediators such as histamine \cite{43}. Following stimuli, LTB4 is released which possess maximal chemotactic, chemokinetic and immune response by direct action on leukocytes \cite{44}. Leukotrienes stimulate leukocytes to release lysosomal enzymes, mediating the inflammatory process and hypersensitivity reaction and are referred to as slow reacting substance of anaphylaxis (SRS-A). It is now generally accepted that a minute amount of LTC4, LTD4 and LTE4 makes up the material for SRS-A \cite{45}. 
(b) Platelet Activating Factor (PAF):

PAF is a phospholipid mediator formed by different cells including eosinophils, macrophages, platelets, neutrophils and vascular endothelium. Intradermal injection of PAF duplicate many of the signs and symptoms of inflammations, including increased vascular permeabilities, hyperalgesia, edema, and infiltration of neutrophils. Beside platelet stimulation, PAF causes vasoconstriction and bronchoconstriction and is 100 to 10,000 times more potent than histamine in inducing vasodilation and increased vascular permeability. This suggests the importance of PAF antagonist platelet derived growth factor suppose to produce fibroblast, chemotaxis, proliferation like primary effects in rheumatoid arthritis. PAF was first discovered as a powerful platelet stimulating agent released from antigen stimulated rabbit buffy coats. PAF activates most inflammatory cells and induces a variety of in-vivo effects related to inflammation, particularly to immediate hypersensitivity and accordingly to bronchial asthma. In experimental studies PAF have been shown to induce platelet aggregation, broncho-constriction and hypotension. Bronchoconstriction is accompanied by a reduction of the total number of circulating platelets and neutrophils (leukocytes) and by increased vasopermeation.
3. Cytokines:

Among the several polypeptides influencing the inflammatory process are cytokinins, produced by a variety of cell types; and tumor necrosis factor produced mainly by lymphocytes and macrophages. Interleukin-1 (IL-1) was discovered in 1972 by Gery and Waksman. Later on, other members of this cytokinin have been recognized viz. IL-alpha and IL-1 beta. Interleukin-8 (IL-8), a potent neutrophil activating and chemotactic agent is known to be released from monocytes in presence of lipolipopolysaccharide. IL-8 is known to be implicated in the pathogenesis of a large number of neutrophil driven inflammatory disease like skin diseases, lung diseases and joint diseases. Interlukine-1 and tumor necrosis factor (TNF) are produced by activated macrophages. Both of these cytokines increase eicosonoid production. TNF causes aggregation and activation of neutrophils. Chemokines are a family of small structurally related proteins that primarily act as activators and chemoattractant for leukocytes.

4. Kinins:

Kinins are family of small peptides formed in blood and biological fluids by the action of Kallikreins on large protein substances. The kininogens exert a variety of biological actions by activating at least two different receptor types, B1 and B2. Several of the biological effects of bradykinins are mediated by endogenous agents such as PGs, histamine and/or 5-HT and possibly catecholamines and rennin. Recently kinins have been shown to stimulate the collagen synthesis and cell proliferation in human fibroblasts. In this way, by acting not only accurately on membrane receptors, but possibly also on intracellular sites, (on cytoplasmic or nuclear components) kinins appear to participate in the acute and chronic phases of the inflam-
Kinins have been reported to produce vasodilatation, local oedema, and pain and increase vascular permeability. Kinins may also modulate migration of white blood cells that take part in the inflammatory process. Kinins are among the most potent activators of PG release. It has also been shown that kinins promote the release of prostacyclin from vessels, the heart, the kidney and from cell cultures (rat adipocytes, human endothelial cells) possibly by interacting with membrane phospholipases. Kinins have also been shown to modify membrane currents and intracellular ion concentration, particularly Ca2+.

5. Clotting System:
The action of thrombin on fibrinogen molecules release the fibrinopeptides. These are potential inflammatory mediators. They induce vascular leakage and these are chemotactic to neutrophil. Plasminogen activator released from the endothelium and other tissue generates plasmin from plasminogen. Plasmin breaks down fibrin, and thus byproducts released, increase vascular permeability and induce chemotaxis.

6. Complement System:
The complement system consists of plasma proteins, which play an important role in both immunity and inflammation. The significant role of complement system is in the fraction of biological active byproducts, which can act as inflammatory mediators. The byproducts include C3 fragments, C5 fragments and C-Kinins. The major inflammatory effect of complement system byproducts are increased vascular permeability, chemotactic attraction of leukocytes and killing of pathogens by phagocytosis. The C5-derived peptide have potent effect on leukocytes in-vitro and promote neutrophil
endothelial interaction in-vivo which leads to neutrophil accumulation and associated oedema formation.

7. Nitric oxide and biologically derived oxidant:

(a) Nitric oxide:
In the past ten years several research fields have converged to show that the tiny molecule nitric oxide (NO), a reactive gas, functions both as a signaling molecule in endothelial and nerve cells and as a killer molecule by activated immune cells. Nitric oxide is one of the inflammatory mediators generated by macrophages in response to presence of endotoxin, a lipopolysaccharide found in the cell wall of gram negative bacteria.

(b) Biologically derived oxidant:
Free radicals are chemical species with one or more unpaired electrons in their outer orbital. Their production is essential for normal metabolism but they are theoretically destructive unless controlled. These oxidants include superoxide anion radical (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hypochlorous acid (HOCl), hydroxyl radical (OH$^-$) and possibly singlet oxygen (O$_2$). Molecular oxygen (O$_2$) is in fact a biradical, having two unpaired electrons of parallel spin. The univalent reduction of oxygen produces the “superoxide anion radical”. This is denoted as O$_2^-$, the dot signifying an unpaired electron. A major source of O$_2^-$, H$_2$O$_2$, OH$^-$ and HOCl is the respiratory burst produced by neutrophils, sensitized monocytes, macrophages and eosinophils, in response to particulate and non-particulate stimuli (e.g. bacteria, aggregated IgG, opsonised particles, complement, interleukin-1).
The sites of oxidant production are the phagosome and/or the plasma membrane depending on the stimulus but $H_2O_2$, $O_2^-$ and $OH^-$ can normally be detected in the extracellular environment.

The arachidonic acid cascade involves the formation of free radicals. Mitochondria also produce $O_2^-$ and $H_2O_2$ in-vitro and are probably one of the major intracellular sources of reactive oxygen species. Small molecules such as hydroquinones, leucoflavins, catecholamines, reduced ferredoxins and pyrogallols are all known to auto-oxidise and produce $O_2^-$. Reactive oxygen species are toxic not only because of their own intrinsic reactivity and ability to act as mediators of inflammation, but also because of the modulating effect they have on their inflammatory processes, e.g. lipid peroxidation will induce enzyme leakage from cells and cellular compartments into areas where their activity is damaging. Radicals may also act synergistically with serum proteases. Hence free radicals both initiate and modulate prostaglandin cascade.

Biologically derived oxidants are capable of damaging the cells and proteins in both acute and chronic inflammatory processes hence; cells clearly require protection from auto-oxidative damage. This protection is provided in a variety of ways, viz.

- Catalytic free radical removal and
- Free radical scavenging or anti-oxidants

Despite the relatively high rate of production of super-oxide by cells during normal metabolism, the intracellular $O_2^-$ is very low. These low levels are maintained both by spontaneous dismutation of superoxide ions to $H_2O_2$ and more importantly, by its catalytic breakdown by two
superoxide dismutase (SOD) enzymes, one is cytosolic and the other mitochondrial. The anti-oxidants like Vitamin-E, caeruloplasmin can react with $O_2^-$, $OH^-$ to produce a less harmful radical species by inhibiting lipid peroxidation.

2.1.4 Termination of Acute Inflammation

After acute inflammation, subsequent changes in the damaged area vary with nature, duration of the injurious stimuli, types of tissue injured and the degree of destruction of tissue caused by the damaging agent. The subsequent changes may follow one of the four possible courses:

1. Resolution
2. Healing by scar, with or without regeneration of lost parenchymal cells
3. Suppuration
4. Chronic inflammation

2.1.5 Chronic Inflammation

Chronic inflammation is considered to be inflammation of prolonged duration (weeks or month) in which active inflammation, tissue destruction and attempts at healing are proceeding simultaneously. While it may follow acute inflammation as described earlier, chronic inflammation frequently being insidiously, as a low grade, smoldering, often a symptomatic response. Indeed, this latter type of chronic inflammation include some of the most common and disabling of human diseases, such as rheumatoid arthritis, atherosclerosis, tuberculosis and chronic lung diseases. Such inflammation arises under the following setting.
**Table 2.1: Summary of Mediators of Acute Inflammation**

<table>
<thead>
<tr>
<th>Mediators</th>
<th>Sources</th>
<th>Evoked Responses</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prostaglandins</td>
<td>Mast cells, from membrane phospholipids</td>
<td>Potentiation of other mediators, vasodilatation, pain, fever</td>
</tr>
<tr>
<td>Leukotriene B4</td>
<td>Leukocytes</td>
<td>Leukocytes adhesion, activation</td>
</tr>
<tr>
<td>Leukotriene C4, D4, E4</td>
<td>Leukocytes, Mast cells</td>
<td>Bronchoconstriction, Vasoconstriction</td>
</tr>
<tr>
<td>PAF</td>
<td>Leukocytes, mast cells</td>
<td>Vascular leakage, Chemotaxis, Bronchoconstriction, Leukocyte priming</td>
</tr>
<tr>
<td>IL-1 &amp; TNF</td>
<td>Macrophages</td>
<td>Chemotaxis, acute phase reaction, endothelial activation</td>
</tr>
<tr>
<td>IL-8</td>
<td>Macrophages, endothelium</td>
<td>Leukocyte activation, chemotaxis</td>
</tr>
<tr>
<td>Bradykinin</td>
<td>Plasma substrate</td>
<td>Pain, Vascular leakage</td>
</tr>
<tr>
<td>C3a, C5a.</td>
<td>Plasma protein via liver, macrophages</td>
<td>Vascular leakage opsonic fragment, leukocyte adhesion, activation</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>Macrophages, endothelium</td>
<td>Vasodilation, cytotoxicity</td>
</tr>
<tr>
<td>Oxygen metabolites</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Hydroxyl radical</td>
<td></td>
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<tr>
<td></td>
<td>Hydrogenperoxide</td>
<td></td>
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<tr>
<td></td>
<td>Hypochloride</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Superoxide radicals</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Vascular leakage, Chemotaxis, Endothelial damage, Tissue damage</td>
</tr>
</tbody>
</table>
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Figure 2.2: Outcome of acute inflammation

- Persistent infection by certain microorganism and certain fungi
- Prolonged exposure to potentially toxic agents either exogenous or endogenous
- Under certain condition, immune reactions are set up against the individuals own tissues, leading to autoimmune disease

In contrast to acute inflammation, which is manifested by vascular changes, edema and largely neutrophilic infiltration, chronic inflammation is characterized by,

1. Infiltration with mononuclear cells, which include macrophages, lymphocytes and plasma cells a reflection of a persistent reaction to injury
2. Tissue destruction, largely induced by the inflammatory cells
3. Attempts at repair by connective tissue replacement, namely proliferation of small blood vessels (angiogenesis) and in fibrosis
Table 2.2: Summary of Mediators of Chronic Inflammation

<table>
<thead>
<tr>
<th>Mediators</th>
<th>Sources</th>
<th>Primary Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interleukins-1,-2&amp;-3</td>
<td>Macrophages, T-lymphocyte</td>
<td>Lymphocyte activation, PG Production</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>T-lymphocytes, endothelial cells, fibroblast</td>
<td>Macrophages &amp; gametocyte activation</td>
</tr>
<tr>
<td>TNF-2</td>
<td>Macrophages</td>
<td>Prostaglandin production</td>
</tr>
<tr>
<td>Interferon</td>
<td>Macrophages, T-lymphocytes, endothelial cells</td>
<td>Many</td>
</tr>
<tr>
<td>PDGF</td>
<td>Macrophages, endothelial cells, fibroblast, platelets</td>
<td>Fibroblast chemotaxis proliferation</td>
</tr>
</tbody>
</table>

2.2 Development of Anti-Inflammatory Agents

Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the most widely used therapeutics, primarily for the treatment of pain and inflammation, especially arthritis. From a historical viewpoint, the first NSAID with therapeutics benefit was aspirin, which has now been used for more than 100 years as an NSAID. The overall worldwide production of about 50000 tons a year reflects the importance of this substance even today.

In the 1970s, a scientific breakthrough occurred with the elucidation of the molecular mechanism of aspirin and other NSAIDs. Vane, Samulson and Bergstrom succeeded in showing that these anti-inflammatory substances block the biosynthesis of prostaglandins (PGs), which contribute to a variety of physiological and pathophysiological function.
The Figure 2.3 summarizes the biosynthesis of PGs, the initial step in the biosynthesis of prostanoids is the liberation of arachidonic acid (AA) from the phospholipid of the cell membrane catalyzed by phospholipase A2. The following decisive step is the biotransformation of AA by cyclooxygenase. In a bifunctional action, this first generate the unstable PGG2 the cyclooxygenase reaction itself, which is then immediately converted into PGH2 by the same enzyme in a peroxidase reaction. As shown in the Figure 2.3 the final product of AA metabolism is PGs, thromboxanes and prostacyclin.

PGs are produced by most cells and are also present in tissue, which explain their broad spectrum of biological responses. PGs mediate a number of characteristic feature of the body’s response to tissue injury to inflammation. The outstanding effects of the PG include their cytoprotective properties in the gastrointestinal tract and control of renal function in the kidney. PGE$_2$ is the most important PG which mediates the typical symptoms of inflammation- rubor, calor, tumor, dolor and functio laesa. Dilatation of small blood vessels initiates the development of redness and heat; the increase in vascular permeability causes the characteristic swelling of tissues. Moreover, PGs sensitized peripheral nerve ending and nociceptor to transmit pain signal to the brain and spinal cord.

In addition to the well accepted pro-inflammatory role of PGs, there is also evidence of anti-inflammatory activity in certain COX-2 derived PGs in vivo, an experiment recently reported by Gilroy et al. Like aspirin all other NSAIDs such as ibuprofen, ketoprofen and naproxan develop their mode of action by blocking cyclooxygenase. Therefore administration of NSAIDs for example to treat inflammatory diseases such as osteoarthritis or rheumatoid arthritis, un-
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avoidably leads to a lack of prostaglandins required for the physiological function mentioned above. The therapeutic effects and side effect of this class of anti-inflammatory drugs are closely related to their biochemical mechanism of action.

As consequences, long term NSAID users suffer from a high incidence of GI irritation or in the worst cases from the development of life threatening GI ulcer and bleeding. This lesion can lead to increase morbidity in the patient. 

![Arachidonic acid cascade](image)

**Figure 2.3: Arachidonic acid cascade**
Administration of NSAIDs may also lead to renal disorder and have hypertensive effects, due to reduced production of PGs such as $PGI_2$ $PGE_2$ and PGD2, in the regulation of renal blood circulation, the rate of glomerular filtration is reduced; especially in patient with reduced renal function, this leads to retention of water, hypertension and in some case to renal failure [65][69].

The inhibition of cyclooxygenase in thrombocytes results in decreased production of thromboxane A2 this phenomenon prolongs bleeding time and leads to inhibition of platelet aggregation. A severe side effect of NSAIDs in bronchoconstriction with resultant asthmatic events. The reduced amount of bronchodilating $PGE_2$ on the one hand and in the shift in the metabolic pathway from the cyclooxygenase pathway to the 5-lipooxygenase pathway on the other hand seems to be responsible for the bronchoconstriction effect of NSAID [65]. The latter pathway metabolizes ‘overflow’ AA, which cannot be transformed by the blocked cyclooxygenase pathway. The resulting leukotriene act as bronchoconstrictors [65].

Because of these problems major target of drug research is the development of NSAIDs with anti-inflammatory and analgesic activity but without side effects.

The historical highlights are [70]

- In England in the mid 18th century Reverend Edmund Stone described in a letter to President of Royal Society “an account of success of the bark of the Willow in the cure of Agues (Fever)”.

- The active ingredient in the Willow bark was a bitter glycoside Salicin first
isolated in the pure form in 1829 by Leroux who also demonstrated its antipyretic effect.

- Sodium salicylate was first used for the treatment of rheumatic fever and as an antipyretic in 1875.

- Acetyl salicylic acid introduced into medicine in 1899 by Dreser under name of aspirin.

- Indomethacin introduced in 1963 for treatment of rheumatoid arthritis.

### 2.2.1 Cyclo-oxygenase enzyme

In 1990’s, it was established that COX, the enzyme that catalyses the conversion of arachidonic acid to prostaglandin, exist in two isoforms, commonly referred to as COX-1 and COX-2. Recent studies suggest that COX-2 may also play an important role in various physiological processes. Under physiological conditions, COX-1 activity predominates and serves to produce PGs that regulate rapid physiological responses such as regulating vascular homeostasis, gastric function, platelet activity and renal function. During inflammation, there is increased expression of COX-2 mRNA followed by increased COX-2 production and tissue $PGE_2$ concentration.

COX-1 is in constitutive form and expressed in GI in large quantities. In stomach COX-1 is responsible for the prostaglandin production for the mucosal defense. COX-2 is inducible form and induced by inflammatory stimuli and mitogens in macrophages/monocytes, synoviocytes. It has been suggested that COX-2 specific inhibitor should be used to describe the agents, which inhibits COX-2,
but have no effect on COX-1. Selective COX-2 inhibitor (Celecoxib and Rofecoxib) were recently introduced for relieving pain in osteo-arthritis and pain and inflammation in rheumatoid arthritis. Selective COX-2 inhibitors have minimal acute gastric toxicity in animals as compared to non-selective COX inhibitor.

A new isoform of the COX family, namely COX-3, has been discovered very recently. However, the initial findings suggested that the inhibition of COX-3 could represent a primary central mechanism by which paracetamol and other NSAIDs decrease fever. The relevance of COX-3 in humans is still uncertain.

2.2.2 Physiological function of COX-2

1. COX-2 expression in kidney

Prostaglandin, as a physiological modulator of vascular tone and of salt and water homeostasis, plays a central role in maintenance of renal blood flow. The constitutive expression of COX-2 has been defected in the rodent kidney localized to the macula dense of the juxtaglomerular and the adjacent cortical thick ascending loop of Henle, in normal rats, rabbits and dogs.

2. COX-2 expression in the central nervous system

High basal level of COX-2 is found in brain especially the granule cell and pyramidal cell layer of the hippocampus.

3. COX-2 in Reproductive function

Prostaglandins derived from COX-2 activity may be important for initiating uterine contractions during labour. During early pregnancy, COX-1 and COX-2 are expressed and may be important for implantation of the ovum.
and angiogenesis, which is important for development of placenta.

4. Monocytes and Macrophages
   Monocytes and macrophages contain both COX-1 and COX-2 enzymes. Cytokines such as IL-\(\gamma\) and TNF have a synergistic effect on induction of COX-2 while IL-1 down regulates COX-2 expression.

5. Synovial tissue
   Both COX isoforms are present in synovial tissues with rheumatoid arthritis and osteoarthritis.

6. Lungs
   Cyclo-oxygenase in the lung may be induced either locally in pulmonary structure after airway damage or as part of a systemic response to cytokines.

7. Colorectal cancer
   Increased prostaglandin production by colorectal neoplasms is associated with increased COX-2 expression. Human colorectal tumors produce elevated levels of PGs (particularly, \(PGE_2\)) in comparison with normal colonic mucosa. It is conceivable that specific COX-2 inhibitors might be used as adjuvants in treatment of tumors, as well as cancer prevention.

8. COX-2 and Gastrointestinal tract
   It has been widely demonstrated that COX-2 is constitutively expressed in a variety of tissues including brain, kidney, female reproductive tract, fetal tissues and vascular endothelium. Although COX-protein has been demonstrated in normal gastric tissue of rats (Peskar et al., 2001), rabbits and humans. Prostaglandin derived from COX-1 is considered to be cytoprotective in the gastrointestinal tract. In line with this concept, Celecoxib
and Rofecoxib were shown to cause a significantly lower incidence of upper gastrointestinal adverse effects (perforations, ulcers and bleeds) than conventional NSAIDs.

2.2.3 Existing Non-Steroidal Anti-Inflammatory Drugs

The drugs used in various acute and chronic inflammatory conditions differ greatly from each other in their chemical structures, but, in spite of this difference in their chemical structures, they all bring about relief of inflammatory symptoms. Aspirin, which has been, used an analgesic and anti-inflammatory agent since 1893, is still one of the potent anti-inflammatory agent used in the clinical practice[70]. These NSAIDs can be grouped on the basis of their chemical structure as:

1. Salicylates: e.g., Aspirin and related drugs.
2. Pyrazolone derivatives: e.g., Phenylbutazone, oxyphenbutazone and related drugs
3. Acetic acid derivatives: e.g., Indomethacin, sulindac and tolmetin
4. Anthranilic acid derivatives (Fenamates): e.g., Mefenamic acid
5. Propionic acid derivatives: e.g., Ibuprofen, Naproxen, etc.
6. Oxicams: e.g., Piroxicams and related drugs.
7. 4-aminoquinolones: e.g., Chloroquine, amidoquine, etc.
8. Gold compounds: e.g., Gold sodium thiomalate aqueous solution
9. Diaryl-substituted pyrazoles: e.g., Celecoxib
10. Diaryl-substituted furanones: e.g., Rofecoxib

11. Indole acetic acids: e.g., Etoldac

12. Sulfonanilides: e.g., Nimesulide

2.2.4 Classification of NSAIDs on the basis of COX selectivity

- Non-selectivity NSAIDs: Aspirin, Naproxen, Indomethacin, Diclofenac, Flurbiprofen, Ibuprofen, Ketorlac
- Preferential COX-2 selective NSAIDs: Nimesulide, Etodoloc, Eloxican
- First generation Coxibs: Celecoxib, Rofecoxib
- Second generation coxibs: Valdecoxib, Parecoxib, Etoricoxib, Lumiracoxib

The generation of inflammatory reactions are due to the action of a number of mediators, but the most important class of mediators directly linked to the action of anti-inflammatory drugs are the prostaglandins. The biosynthesis of prostaglandins in inflammatory processes can be effectively inhibited by many NSAIDs like salicylates and other related drugs. In addition to the inhibition of biosynthesis of prostaglandins, other possible modes of action of NSAIDs have been reviewed. They are:

1. Antagonist effects on mediators other than PGs (e.g. histamine, bradykinin).
2. Inhibition of the biosynthesis of mucopolysaccharides
3. Stabilization of the lysosomal membrane
4. Uncoupling of oxidative phosphorylation
Review of Literature

5. Inhibition of chemotaxis of cells implicated in the inflammatory process

6. Sulphydryl-disulfide stabilization

7. Inhibition of collagenase production

8. Suppression of lymphocyte function

9. Fibrinolytic activity

10. Inhibition of platelet aggregation and thereby decreasing the release of 5-HT and adenyl compounds.

11. Inhibition of hyaluronidase and thereby decreasing osmotic pressure of exudates

12. Inhibition of leukocyte migration and leukocyte phagocytosis

Of all these, the inhibition of prostaglandin biosynthesis is said to be the prime mechanism of action of NSAIDs. The other mechanisms of action may not be of much relevance or may be of secondary importance. However immunosuppressant activity of cyclophosphamide like drugs may be responsible for their anti-inflammatory activity. Aspirin like drugs share the unwanted effects. The most common is a propensity to induce gastric irritation, which may lead to ulceration, and the result is loss of blood. Gastric damage by these agents can be brought by at least two distinct mechanisms, namely due to the local irritation by these drugs in the stomach allowing back diffusion of acid into the mucosa and induce tissue damage and inhibition of biosynthesis of prostaglandins. The predominant PGs synthesized by the gastric mucosa are \( PGI_2 \) and \( PGE_2 \). These inhibit acid secretion by the stomach and promote the secretion of cytoprotective mucus in intestine. Hence the inhibition of these PGs may render the stomach
Review of Literature

more susceptible to damage.

Platelet function appears to be disturbed because aspirin like drugs prevent the formation of thromboxane A2 by platelets, a potent platelet aggregating agent. Hence this increases the bleeding time. Since a number of NSAIDs are known to inhibit the denaturation of proteins, clotting time may be prolonged with aspirin like drugs\textsuperscript{76}. Other adverse effects of aspirin like drugs include prolonged gestation and labour, renal papillary necrosis, intolerance in the form of bronchoconstriction.

2.2.5 COX-2 Inhibitors

It has been proposed that the term COX-2 specific inhibitors should be used to describe agents, which inhibit COX-2 but no effect on COX-1, over whole range of doses used and concentrations achieved in clinical usage. The U.S. Food and Drug Administration approved one of these, Celecoxib and another Rofecoxib on Dec 31, 1998 for rheumatoid arthritis and osteoarthritis\textsuperscript{77}.

Celecoxib is a 1,5-diaryl pyrazole based compound\textsuperscript{44}. Celecoxib is rapidly absorbed with a tmax of about 3 hours. It is extensively metabolized and eliminated with a half-life of 11.2 hours. Celecoxib, a cyclooxygenase inhibitor that exhibit, relative in vitro and in vivo selectivity for COX-2 over COX-1. Celecoxib is the first COX-2 specific inhibitor approved for use in osteoarthritis and rheumatoid arthritis. Celecoxib produces a significant improvement in pain and inflammation, associated with a lower incidence of upper gastrointestinal ulceration and complications. The gastrointestinal safety profile, together with sustained symptomatic relief, places celecoxib as a useful alterna-
tive for the treatment of osteoarthritis and rheumatoid arthritis, particularly in patients with a high risk of gastrointestinal events.

Rofecoxib is a methylsulphonylphenyl derivative (refer structure in Figure 1.1). Rofecoxib is a long-acting drug launched with the once daily regimen. Rofecoxib selectively inhibits COX-2 in a dose-dependent manner in humans. No significant inhibition of COX-1 is observed with Rofecoxib up to doses of 100mg. Rofecoxib is also an effective analgesic in patients with primary dysmenorrhea or postoperative dental pain. The adverse effects of Rofecoxib are diarrhea, headache, nausea and upper respiratory tract infection.

Coxibs were widely prescribed drug (nearly 8 million people round the globe take these drugs) until the recent setback with the rofecoxib \textsuperscript{78, 79}, which was withdrawn from the market by innovator due to increased risk of heart attack and stroke observed with its long term use. The withdrawal of this popular NSAID has not only caused a great setback in the global market of coxibs, but has also questioned the ethics involved in the toxicity testing and sharing of information with the end user of this new class of drug.

One possible elucidation for increased cardiovascular risk with rofecoxib mainly extrapolated from animal studies is based on the biology of the COX-1 and COX-2 enzyme. COX-1 drives the synthesis of anti-aggregatory prostaglandin \((P GI_2)\). Since COX-2 inhibitors have no effect on platelet function but inhibit vascular \(P GI_2\) production, they may tilt vascular prostaglandin synthesis in favor of increased ecosonoid TXA2 which may clinically result in prothrombotic outcome.
2.2.6 COX-2 and new therapeutic targets

Apart from its role in inflammatory sites, COX-2 is involved in numerous physiological and pathophysiological functions. COX-2 is constitutively expressed in the developing kidney and brain, playing a role in their maturation and function. Additionally, COX-2 expression may be up-regulated at certain sites: in the kidney during sodium restriction, in the microglia of cognitive center within the hippocampus and cortex in AD and in intestinal adenomas and colon tumors 65, 80.

The formation of new blood vessels by angiogenesis to provide a blood supply is a major requirement for the growth of many tumors 65. While mature blood vessels express COX-1, new angiogenic cells express inducible COX-2. Based on these observations, Masferrer et al. hypothesized that tumor-derived growth factors promote angiogenesis by inducing the production of COX-2-derived PGE-2. This is supported by the fact that PGs are known to be pro-angiogenic molecules that contribute to tumor growth by inducing newly formed blood vessels (neoangiogenesis) that sustain tumor cell viability and growth.

COX-2 is expressed with in human tumor neoangiogenesis as well as in neoplastic cells present in human colon, breast, prostate and lung tissue. Celecoxib demonstrated a potent anti-angiogenic activity in a rat model of angiogenesis, in which corneal blood vessel formation is suppressed by celecoxib (Corneal micropocket model). It has been suggested that angiogenesis is inhibited through direct effects on endothelial cells by lowering the expression of angiogenic factors such as vascular endothelial growth factor (VEGF). Hence, the gate to a new field of research has opened. As monotherapeutics, anti-angiogenics are expected to
be safer and better tolerated than classic chemotherapeutic drugs and therefore an ideal candidate for prevention of cancer. A combination of anti-angiogenic drugs and cytostatic agents could have a synergistic anti-tumor activity via an impact on two independent mechanisms such as neural vascularization and neoplastic cell proliferation.

Closely related to these observations is a fact that COX-2 plays a role in the development of colon cancer. Normally COX-2 expressions is strictly regulated; however, a constitutive over expression of COX-2 seems to be important in colon carcinogenesis. In cultured human colonic fibroblasts it was shown that growth factors such as hepatocyte growth factor are involved in the progression of tumors COX-2 inhibitors are now assumed to inhibit COX-2 mediated PG synthesis which is responsible for hepatocyte growth factor expression. In addition to the well-known peripheral role of COX-2 in inflammation, recent results indicate an important role in the central nervous system. COX-2 expressed constitutively in some excitatory neurons in CNS. Moreover, expression of this isoform is markedly induced in CNS neurons by excitatory stimuli such as ischaemia and seizures so that a role of COX-2 derived PGs in certain form of neural degeneration can be assumed. It has also been shown that celecoxib maximally inhibits COX-2 in the CNS at anti-inflammatory doses. Interestingly application of COX-2 inhibitors to the spinal cords of rats, where COX-2 also is expressed prevents peripheral inflammation and hyperalgesia. Evidently COX-2 plays an important role in the CNS in inflammation and pain and it can be deduced that therapeutic effects of COX inhibitors might be caused not only by peripheral enzyme inhibition but also atleast partly by central inhibition.
COX-2 in CNS may have an ambivalent functionality in the brain since the basal production of PGs through COX-2 may participate in neuronal homeostasis, whereas the expression of COX-2 is associated with brain development. COX-2 is constitutively expressed in neurons in degenerative brain regions in AD such as the microglia of the cognitive centers within the hippocampus and cortex. Enhanced COX-2 expression in the brain may be associated with beta-amyloid protein deposition in the neuritic plaques of AD. This protein and its peptides precursors are thought to be elaborated as part of an inflammatory cascade in which microglia, a rich source of prostanoids probably participate. The role of activated microglia, which express COX-2 in cerebral inflammatory process, was recently demonstrated in the rat.

The fact that COX-2 mRNA is elevated in areas related to memory (hippocampus, cortex) and that the amount COX-2 corelates with the deposition of beta-amyloid protein represents a possible therapeutic benefit and a hopeful new strategies in the prevention or treatment of AD. Despite this interesting and optimistic outlook for future uses of COX-2 inhibitors, most of the findings are based on in vitro and in vivo assays and must urgently undergo investigation in ma. So far it is generally agreed that the constitutively expressed COX-1 enzyme is the predominant form in the GI tract, kidney and platelets, providing a rich source of physiologically important PGs. It is also evident that COX-2 is expressed at inflammatory site by leukocytes as well as by activated mesenchymal cells. The conceptual framework and properties of selective COX-2 inhibitors have been demonstrated as effective NSAIDs in blocking signs and symptoms of the inflammation.
Although this beneficial effect of specific COX-2 inhibitors is evident, some data suggest that in certain models PGs may be unexpectedly beneficial in the resolution of inflammation or tissue injury. The effect of selective COX-2 inhibitors on carrageenan pleurisy in the rat over a time course ranging from 0-48 hrs after injection of the irritant is interesting. This investigation is produced surprising results and showed that efficacy by COX-2 inhibitors strongly depends on the time course of the inflammatory process; onset of inflammation, peak inflammation and resolution. COX-2 inhibitors showed anti-inflammatory activity early on during the onset of the inflammatory response coincident with the expression of the COX-2 protein. After six hours the COX-2 inhibitors were without effect whereas dual inhibitors still show efficacy. As shown by western blotting at this point COX-2 protein was no longer present. After 48 hrs. the time of nearly complete resolution of inflammation in the model there was a second surprising increase in the COX-2 protein expression. This newly formed COX-2 protein, however, did not produced pro-inflammatory $PGE_2$ but anti-inflammatory PGs. Apart from $PGD_2$ and $PGF_{2\alpha}$, one of the proposed anti-inflammatory prostanoids assumed to be important in the resolution of carrageenan pleurisy is $15-\text{deoxy}\Delta^{12,14}PGJ_2$, a member of the cyclopentanone family.

2.2.7 COX-3 Hypothesis

During inflammation after 24 hours there was another surge of COX-2 enzyme and treating this newly formed COX-2 with selective COX-2 inhibitors 24-48 hrs after application of the irritant showed an unexpected effect on the inflammatory process, inflammation was not inhibited and still continued. It might therefore be postulated that this newly formed enzyme is not COX-2., but what is it? Is it a catalytic variant of COX-2 or can we postulate third isoenzyme a COX-3?
Review of Literature

this hypothesis is true expression of this third inducible isoform of COX could result in the typical period of remission often seen in many clinical cases of chronic inflammatory disease. Application of COX-2 inhibitors during the phase of naturally occurring remission of the inflammation could hence delay healing. Indeed, it is known that COX-2 inhibitors given over a long period lead to delayed healing.

If this hypothesis is really true, a marker for disease activity is urgently needed in order to determine the appropriate time for the use of selective COX-2 inhibitors and if a COX-3 enzyme really exists this will lead to a generation of new anti-inflammatory drug with new therapeutic targets.

2.3 Pharmacological Activities of 4-Thiazolidinones

The history of 4-thiazolidinones can be traced back to the early work on thiazoles (Figure 2.4), when in 1879 Hofmann et al., reported benzthiazole derivatives such as 2-chloro-benzthiazole. Compounds containing a simple thiazole nucleus were first reported by Hantzch et al., in a series of papers beginning from 1887. After this pioneering work, knowledge of the thiazole system developed shortly. Many thiazole derivatives were found to have biological and commercial interest. This gave impetus to biological studies of thiazole series.

Figure 2.4: Structure of Thiazole

were first reported by Hantzch et al., in a series of papers beginning from 1887. After this pioneering work, knowledge of the thiazole system developed shortly. Many thiazole derivatives were found to have biological and commercial interest. This gave impetus to biological studies of thiazole series.
Green \textsuperscript{83} in 1888, described a yellow primuline base and Dihydro-thio-p-toluidine. These were obtained by fusion of p-toluidine with sulfur. These compounds were recognized as benzthiazole derivatives. Subsequently many related compounds were prepared.

In 1935, Williams et al., \textsuperscript{84} demonstrated the existence of a simple thiazole moiety in the structure of vitamin B1 (Thiamine) (Figure 2.5). It was combined with 4-thiazolidinone with a view to increase the anti-bacterial activity \textsuperscript{85}.

The historical importance of thiazole derivatives was further emphasized during the period 1941-45, when work on the structure of penicillins (Figure 2.6) showed the presence of thiazolidine ring in it. The occurrence of thiazole derivative in nature was reported in 1952, when actithiazic acid (Figure 2.7), an antibiotic was found to be a 4-thiazolidinone derivative \textsuperscript{86} \textsuperscript{87}.

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{thiamine.png}
\caption{Figure 2.5: Structure of Thiamine}
\end{figure}
Review of Literature

Figure 2.6: Structure of Penicillin

![Penicillin Structure](image)

Figure 2.7: Structure of Actithiazic acid

![Actithiazic acid Structure](image)
Today nobody modifies molecules in a random manner as it was prevalent until the early 1930s. Rules concerning the isosteric replacement of gradual and fairly rational modifications of molecular functions and moieties emerged at that time and expanded subsequently, to accommodate new knowledge of electronic, hydrophobic and steric conditions. Under the headings of bioisosterism more emphasis is being focused on biological rather than chemical similarities of diverse structures. This has been the most successful basis of many new medicinal agents. Such structural changes often permit estimates of the quality and type of biological activity, although predictions of potency are harder to make and chemical prognosis of the therapeutic index i.e., the probable usefulness of a drug is still largely a guess work.

All the 4-thiazolidinone derivatives synthesized, exhibited a wide range of biological activity. Most of them were developed on the basis of isosteric replacements. Some of them are cited below.

The resemblance of 3-(B-aryl ethyl) rhodanines with adrenaline provoked Buck et al. in 1931, to screen these compounds for adrenaline like activity; however due to its general insolubility not much work could be done.

Due to the presence of a sulfur atom in the thiazolidin-4-one moiety of rhodanine, its 5-substituted derivatives, were screened for antifungal activity and subsequently they were patented as fungicides.

Sedative action was claimed for 5,5-disubstituted-2-imino-4-thiazolidinones due to its similarity in structure to the barbiturates. Taking this as a ‘lead’, Erlen-
meyer et al. synthesized 5,5-dialkyl-2, 4-thiazolidinediones and suggested an investigation of the narcotic properties of the former. Some compounds in this series were found to be comparable with 5,5-diethyl-barbituric acid.

Surrey in 1948, synthesized 2-aryl-4-thiazolidinone with an amino alkyl group attached to the nitrogen atom and due to its structural resemblance with procaine, it was screened and found to have local anesthetic activity.

Trountman et al. at the same time tested several 4-thiazolidinone and its 3-ethyl homolog gave complete protection to cats against MES induced convulsions. Since, 2,4-oxazolidinedione exhibited protection against metrazole induced convulsions, correspondingly some thiazolidinone analogs were prepared and tested, which were not found to protect.

In 1950, Rao et al. prepared 4-thiazolidinones from thiosemicarbazones and found to have good anti-tubercular activity. But compounds containing a group like thiosemicarbazone were usually active at lower concentrations than the 4-thiazolidinones prepared from it, but the former caused greater fatty degeneration and hemorrhage in the liver of mice and rats.

After the discovery of sulpha drugs, its antibacterial activity gave a ‘lead’ for the synthesis of 5, 5-disubstituted-2-sulphanilamide-4-thiazolidinones.

At about the same time, in 1955, 2-benzhydryl imino-4-thiazolidine and their related compounds were reported to have central nervous system depressant activity.
In 1956, several rhodanine derivatives were patented as insecticides or nematicicides \(^{105}\).

In 1968, potential anti-tumor activity in a series of 4-thiazolidinones were reported \(^{106}\).

2, 3-bis-[\(p\)-hydroxy-phenyl]-pseudothiohydantoin and its derivatives were found to have antipyretic action on pharmacological screening \(^{107}\).

Anti-inflammatory activity was reported in 1972 for some 4-thiazolidinone derivatives \(^{108}\).

Thiazolidine-4-ones were synthesized from N-arylidine compounds prepared earlier, and the derivatives with halogen or methoxy group on phenyl ring attached to thiazolidone were reported for significant anti-fungal activity \(^{109}\).

The synthesis of 4-oxothiazolidines and their moderate anti (HIV-1) AIDS activity, anticancer activity and anti-tubercular activity was reported (Bhatt et al.,) \(^{110}\).

The synthesis of several new derivatives of thazolidine-4-ones was reported by Vashi et al. \(^{111}\) and most products displayed moderate to good anti-microbial and anti-tubercular activity.

Nailesh Joshi et al. \(^{112}\) have reported the synthesis of 2-Aryl-5-H/Methyl/carboxy
methyl-3-(4-3,4,5-trimethoxy benzamido) thiazolidine-4-one by cyclo condensation of thioglycolic acid/thiolactic acid/thiomathic acid with N-substituted benzal-4-(3,4,5-trimethoxy benzamido) benzoyl hydrazines and found them to exhibit anti-bacterial and anti-fungal activity.

Seema Mishra et al.\textsuperscript{[13]} have reported the synthesis of certain derivatives of thiazolidine-4-ones and evaluated them for anti-inflammatory, anticonvulsant, analgesic and antimicrobial properties.

The synthesis and moderate to high anti-microbial activity of thiazolidinones prepared from hydrazine thieno (3,2-d) pyrimidines was reported by Manish Shah et al.\textsuperscript{[113]}

Akhil Bhatt et al.\textsuperscript{[114]} have reported the synthesis of some thiazolidinones from 4-amino benzophenone and found them to exhibit about 94% inhibition in growth of Mycobacterium tuberculosis.

A facile route for the synthesis of certain thiazolidinone derivatives incorporated with benzothiazole moiety has been reported. Most of the test compounds exhibited spectacular activities against the test bacterial species B. subtilis, Staph. Aureus and Sal. Paratyphi.\textsuperscript{[115]}

A series of novel 4-thiazolidinones bearing lipophilic adamantyl substituent at position 2 were synthesized by Balzarini et al.\textsuperscript{[116]} with remarkable antiviral activity.

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During this review of literature survey, it became evident that thiazolidine-4-ones, as a class, exhibited a wide range of pharmacological activity.

2.4 Chemistry and Synthesis of 4-Thiazolidinones

The first report on the synthesis of 4-thiazolidinones appeared in 1906 by V. Bror Holimberg. Thiazolidin-4-ones are synthesized by the cyclisation of acyclic compounds or by inter conversion among appropriately substituted thiazolidine derivatives.

1. Cyclization of cyclic compounds

In the cyclisation reactions, the acyclic intermediate can be formed by reactions between the atoms which will subsequently be 1 and 5, 1, 2, and 3 of thiazolidin-4-one ring. In most synthesis, the intermediate which is usually isolated is an appropriately substituted alkanoic acid or its salt or its ester and ring closure occurs between the acid groups and hydrogen attached to the nitrogen between atoms 3 and 4 of the thiazolidine ring. In the formation of 2-aryl and an alkyl thiazolidin-4-one, ring closure occurs between atoms 1 and 2, atoms 2 and 3 of the thiazolidine ring.

2. Reactions of mercapto acetic acid

The reactions of thioglycolic acid with compounds of the structure R-N=C=X is a general method for the synthesis of thiazolidine-4-ones.

(a) Reactions with isothiocynate

The reaction of an isothiocyanate with mercapto acetic acid forms a derivative of rhodanine with aryl isothiocyanate. The product is
3-aryl rhodanine\textsuperscript{119}. In a variation of this method the isothiocyanate is heated with an acetic acid solution of methyl thiocyanatoacetate in the presence of a catalytic amount of lead acetate until the evolution of carbon dioxide from decomposition of cyanic acid has stopped\textsuperscript{120}.

![Reaction with isothiocyanate](image)

Figure 2.8: Reaction with isothiocyanate

(b) Reaction with Isocyanates

Isocyanate reacts with mercapto acetic acid forming derivatives of 2,4-thiazolidinedione\textsuperscript{121}.

(c) Reaction with Cyanamide

The identification of mercapto acetic acid and cyanamide as products of basic hydrolysis of pseudothiohydantoin led to the synthesis of the latter from its hydrolysis products\textsuperscript{122}. This method was used as an argument for establishing the structure of the compound as a derivative of thiazolidin-4-ones rather than thiohydantoin.

(d) Reactions with mercaptoalkanoic acid

The only extensive use of mercapto alkanoic acids is in the synthesis of thiazolidin-4-ones. The other component is a Schiff base, usually formed from an aromatic heterocyclic aldehyde. The reaction takes place in an inert solvent such as dry ether, dry benzene\textsuperscript{123,124}. With these solvents, yields are of the order of 40 to 60 percent, while in ethanolic solution the yield drops to 10%. The use of dean stark apparatus has been found to be advan-
tageous and the course of the reaction can be followed by the volume of the water collected. With an aromatic or heterocyclic aldehydes and ketones and ammonium carbonates as a source of ammonia, mercapto alkanolic acid gives thiazolidin-4-ones with a hydrogen attached to nitrogen. The reaction is believed to take place by formation of intermediates aldehyde and ketimine.

i. Mechanism of reaction

The reaction of mercaptoacetic acid with Schiff bases is most widely employed for the synthesis of thiazolidin-4-ones. In the present work, the same method was employed for the synthesis of 2,3-disubstituted thiazolidin-4-ones.

ii. Mechanism of Schiff base formation

Aldehyde and ketones react with the primary amines to form azomethines which are usually known as Schiff bases. The azomethine formation does not proceed beyond a single stage conden-
sation, the equilibrium in aqueous solution lies well over on the side of reactants; but it can be shifted towards the product by removing water. Azomethine condensation and their reversal by hydrolysis are not characteristically base catalyzed. On the acid side of neutral point, the sequence of reaction is raised, the rate of condensation may pass through maximum, at some time which seems to be related to the basicity of the adding amines.

iii. Mechanism of thiazolidin-4-one ring formation

The aromatic bound -CH=N- has two reaction centers, an electrophilic center at the methane carbon atom and the nucleophilic center at the nitrogen atom. Through this azomethine bases can react with molecules containing a replaceable hydrogen atom. Thus the reaction proceeds by the attack of mercapto acetic acid upon the -C=N- group, with the \( \text{SCH}_2\text{COOH} \) adding to carbon atom followed by the capture of proton by nitrogen and subsequent cyclization. In several cases the N-cyclised addition product

![Figure 2.10: Mechanism of thiazolidin-4-one ring formation](image)
was isolated; subsequently cyclization of certain compounds was affected by heating the open chain compound with phosphorous pentoxide in dioxane solution for 30 minutes.

2.5 Methods of Evaluation of Pharmacological Activity

1. Various screening methods for toxicity studies

2. Anti-inflammatory Agents

3. Analgesic Agents

4. Antipyretic Agents

5. COX enzyme inhibitory activity

1. Various screening methods for toxicity studies

   (a) Model for acute toxicity studies

   The toxicity studies were performed according to the OECD guidelines to determine the acute toxic concentrations. The OECD guidelines followed for the acute toxicity studies are Guideline no. 401, 420, 423, and 425.

   It is the principle of the test that, based on a stepwise procedure with the use of a minimum number of animals per step, sufficient information is obtained on the acute toxicity of the substance to enable its classification. The substance is administered orally to group of animals at one of the defined doses. The substance is tested using
a stepwise procedure, each step using three animals of a single sex. Absence or presence of compound related mortality of the animals dosed at one step will determine the next step i.e.;

- no further testing is needed
- dosing of three additional with the same dose
- dosing of three additional with the next higher dose or lower dose

(b) Model for subacute toxicity study (407)

The test substance is orally administered daily in graduated doses to several groups of experimental animals, one dose level per group for a period of 28 days. During the period of administration the animals are observed closely, each day for sign of toxicity. Animals which die or are killed during the test are necropsied and at the conclusion of the test surviving animals are killed and necropsied.

2. Screening methods for anti-inflammatory agents

In-vivo Methods

(a) Ear Edema in mice

(b) Paw edema in rats

(c) Granuloma Pouch Technique in rats

(d) Cotton wool Granuloma in rats

(a) Ear edema in mouse

i. Oxazolone induced Ear Edema in mice

*Purpose and Rationale*

This was first described by Evans (1971) in mice, a model for
delayed hypersensitivity that helps in quantitative evaluation of the topical and systemic anti-inflammatory agents following topical application.

Procedure
Mice of either sex with a weight of 25g are used. A fresh 2% solution of oxazolone (4-ethoxymethylene-2phenyl-2-oxazolin-5-one) in acetone is used which is applied on the inside of both ears 0.01ml under halothane anaesthesia. The mice are once again challenged after 8 days on the control ear with 0.01ml of 2% oxazolone solution. Groups of 10 - 15 animals are treated with irritant alone or with the test drugs solution. One ear remains untreated and serves as control. Inflammation occurs 24hrs. later and at this time animals are sacrificed and a disc of 8mm diameter is punctured from both sides and immediately weighed. The difference in weight is an indicator of the inflammatory edema.

Advantages
This method is suitable for both steroidal and non-steroidal anti-inflammatory agents as well as evaluation of various topical preparations.

Modification
Griswold et. al(1974) applied 3% solution of oxazolone to the left paw of mice and edema is assessed plethysmographically.
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ii. Croton-Oil Ear Edema in rats and mice

Purpose and Rationale
The method developed by Tonelli et al. (1965) as a bioassay for the concomitant assessment of the antiphlogistic and thymolytic activities of topically applied steroids.

Procedure
The mice with a weight of 22g and rats with a weight of 70g are used in this test. The irritant is prepared as Croton oil: Ethanol: Pyridine: Ethyl ether (1:10:20:69 for mice and 4:10:20:66 for rats). The test solutions are prepared in a concentration of 0.03mg/ml to 1mg/ml for mice and 3 to 10 times higher concentration for rats. The irritant is applied on both sides of the ear about 0.01ml for mice and 0.02ml for rats. The left ear is kept as control. Four hours after the application the animals are sacrificed and discs of 8mm diameter are punctured and removed and weighed immediately. The difference in weight is directly proportional to the anti-inflammatory activity.

Advantages
Useful in the evaluation of topical steroidal anti-inflammatory drugs, and also for non-steroidal anti-inflammatory agents.

iii. Xylol induced ear edema in mouse

The test compounds were suspended in CMC (0.5%) solution. The control animals received 0.5% CMC solution (vehicle). Thirty minutes after the administration of drug, the animals were treated with topical application of xylol (0.01ml on both surface of ear)
on one of the ears (either right or left). The other ear acts as the control. After thirty minutes of xylol administration the animals were sacrificed by cervical dislocation and the ears were isolated as discs of 6-8mm. The ear discs were immediately weighed and the difference in the weight of the inflamed ear and the normal ear was taken as the inflammation edema volume. Then, the percentage inhibition of edema is determined.

(b) Paw Edema in rats

*Purpose and Rationale*

One of the most commonly employed model for screening of anti-inflammatory drugs, based on the ability of such agents to inhibit the edema produced in the hind paw of the rats after injection of a phlogistic agent.

*Procedure*

Wistar rats of both sex with a body weight between 100 - 150g are used, in which the animals are starved overnight. Uniform hydration is done to each rat by giving 5ml of water by stomach tube (control) or the test drug dissolved or suspended in the same volume. Thirty minutes later animals are injected subcutaneously with 0.05ml of 1% solution of carrageenan into the plantar side of the left hind paw. The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume is measured plethysmographically immediately after injection, again at an interval of 30min, 1hr, 2, 3, 6 and eventually 24hrs.

*Evaluation*

The difference in the average values in the paw edema at different time
interval between the treated and control animal groups is calculated for each time interval and statistically evaluated. 

Advantages

The method is very simple and time response curve can be obtained.

Modifications

Various other irritants can be used to induce paw edema like,

- 0.05ml of undiluted fresh egg white.
- 0.1ml of 1% formalin.
- 0.1ml of 1-3% dextran solution etc.

(c) Granuloma Pouch Technique in rats

Purpose and Rationale

The method originally invented by Selye and was developed by Robert and Nezamis (1957) using croton oil as irritant in which antiseptic inflammation results in large volumes of hemorrhage exudates is elicited resembling the sub acute type of inflammation. 

Procedure

Rats of both sex with a body weight between 150-200g are used in groups of 10 animals. The back of the animal are shaved and disinfected and with a thin needle a pneumoderma is made in the middle of the dorsal skin by injecting 20ml of air under anaesthesia. Into this pouch, 0.5ml of 1% croton oil in sesame oil is injected avoiding any leakage of air. The air is withdrawn after 48hrs. and after 72hrs the resulting adhesions are broken. From the day of air pouch formation the animals are treated with the drug either orally or subcutaneously. On the 4th or 5th day the animals are sacrificed and the pouch is opened and exudate is collected in glass cylinders. Control has an exudate volume between 6 and 12ml, which is reduced dose dependant
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in the treated animals.

Advantages

The method is useful to estimate the potency of anti-inflammatory corticosteroids both after local and systemic administration.

Modification

Carrageenan can be used to induce exudate formation, which was demonstrated by Boris and Stevenson (1965)\textsuperscript{132}.

(d) Cotton wool granuloma in rats

Purpose and Rationale

The foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton, histologically, giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenan\textsuperscript{133}.

Procedure

Male Wistar rats with a weight of 200g are used. The back skin is shaved and disinfected with 70\% ethanol under anaesthesia and an incision is made in the lumbar region. By using blunted forceps cutaneous tunnels are formed and a sterilized cotton pellet is placed on both sides in the scapular region. The pellets are of standard size of 10 or 20mg, the animals are treated for 7 days either subcutaneously or orally. Then, they are sacrificed on the 8th day and pellets are removed, freed of extraneous tissue and dried until constant weight. The net dry weight i.e., after subtracting the weight of the cotton pellet is determined.

Evaluation
The percentage change in the granuloma weight relative to vehicle treated group is determined after getting the average weight of the cotton pellets in each group.

Advantages

This method is useful in the evaluation of steroidal as well as non-steroidal anti-inflammatory agents.

Modifications

- Tanaka et. al. (1960) implanted filter paper pellets soaked in 7% formalin solution in rats.
- Hicks (1969) implanted pellets impregnated with irritant substances, such as capsicum oleoresin.

3. Screening methods for analgesic agents

(a) Centrally acting Analgesic agents

   i. Haffner’s Tail Clip Method in Mice
   
   ii. Tail Flick or other Radiant Heat Methods
   
   iii. Tail Immersion Test (Caudal Immersion)
   
   iv. Hot Plate Methods in mice or rats
   
   v. Electrical Stimulation of the tail
   
   vi. Formalin test in rats.

(b) Peripheral Analgesic Activity

   i. Writhing Test

(a) Centrally acting analgesic agents

   i. Haffner’s Tail Clip Method

Purpose and Rationale
Method described by Takagi et. al., who observed raised tail (Straub phenomenon) in mice, who found the tail after treatment to be less sensitive to noxious stimuli (especially with opioid drugs like morphine etc.)\textsuperscript{[136]}. 

**Procedure**

The reaction time for mice weighing 18-25g for a noxious stimulus by applying an artery clip at the root of the tail is noted. The control group animals respond to the noxious stimuli by biting the clip or the tail near the location of clip. A stopwatch in 1/10 seconds increments measures the time between the stimulation onset and response. Drugs can be administered subcutaneously for fed and by oral for fasted animals. The $ED_{50}$ can be calculated by using various concentrations of drugs and then peak time of the activity can be calculated by repeating the test at 15, 30, 60 . . . minutes after drug administration.

**Advantages**

The method is simple and need no sophisticated equipment. The method is also less time consuming, leading to quick evaluation of analgesic agents.

**Disadvantages**

Only opioid analgesics can be tested and not the Non-steroidal anti-inflammatory analgesic drugs.

**Modification**

Carrageenan suspension induced in the tail for additional hyperalgesia. This method can be used for Non-steroidal anti-inflammatory agents also.
ii. Tail Flick or Radiant Heat Method

**Purpose and Rationale**

The method developed by Schumacher et al., (1940), for quantitative measurement of pain threshold in man against thermal radiation. This procedure has been used to evaluate analgesic activity in animal experiments by measuring drug induced changes in the sensitivity of mice or rats to heat stress applied to their tails.

**Procedure**

Mice are placed into cages leaving the tail exposed. A beam of light is focused to the proximal third of the tail. Within few seconds the animals flick the tail aside or tries to escape. The tail flicking reaction time is measured. A cut-off of 6 sec. is taken as maximum time for selecting the animals for this test. Groups of 10 mice of both sex with a weight between 18-22g are used for each dose. Before the administration of the test compound or the standard the normal reaction time is determined. The escape reaction which is the end point is this test can be regarded as a complex reaction mediated by the brain. The test compounds and the standard are administered either orally or subcutaneously. The animals are subjected to the same procedure after 30, 60, and eventually 120min. and for each individual animal the reaction time is noted.

**Evaluation**

The average reaction time after each time interval are calculated
and compared with the pretest value by analysis of significance. At each time interval, the animals that show reaction time twice as high as or higher than pretest values are regarded as positive. Percentages of positive animals are counted at each time interval and each dose and $ED_{50}$ values are calculated.

**Advantages**

This test is useful for discriminating the centrally acting morphine like analgesics and non-opiate analgesics.

iii. Tail Immersion Test (Caudal Immersion)

**Purpose and Rationale**

The method developed selectively for morphine like compounds. To check the capability of these drugs to prolong the reaction time for the tail withdrawal reflex in rats induced by immersing the end of the tail in warm water of $55^\circ C$.

**Procedure**

Young female Wistar rats of 170 - 200g are used for this test, with its lower 5cm portion of the tail marked. This part of the tail is immersed in a cup of freshly filled water of exactly $55^\circ C$. The reaction time is recorded in 0.5sec units by a stopwatch. The tail is dried after each immersion. The reaction time is determined before and periodically after either oral or subcutaneous administration of test compounds (eg. after 0.5, 1, 2, 3 ... hrs). The cut-off time is taken as 10sec. The withdrawal time of untreated animals is between 1 and 5.5sec. and withdrawal time of >6sec. is therefore regarded as positive response.

**Evaluation**
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$ED_{50}$ values can be calculated for each compound and the time response curves can be obtained.

*Advantages*

This test is simple and useful to differentiate the central opioid like analgesics from peripheral analgesics.

iv. Hot Plate Method

*Purpose and Rationale*

The rats and mice paws are sensitive to heat at temperatures not damaging the skin. The animal responds by jumping, withdrawing the paws and licking the paws.

*Procedure*

This method was originally described by Woolfe and Mac. Donald (1944). Groups of 10 mice of either sex weighing 18 - 22g are used for this test. The commercially available electrically heated surface hot plate is used which is set at $55^\circ$C to $56^\circ$C. The animals are placed on the hot plate and the reaction time is recorded by using a stopwatch. The latency is recorded before and after 20, 60, and 90min. following oral or subcutaneous injection of the standard and test compounds.

*Evaluation*

The prolongation of the latency time is compared with the values before and after administration of the test compounds, or the values of the control with the experimental groups statistically by using t-test.

*Advantages*

Differentiation between central and peripherally acting analgesics
is easy. Suitable for evaluating centrally acting analgesics.

v. Electrical Stimulation of the Tail

*Purpose and Rationale*

This method was described by Burn et. al.(1950). Since the mice tail is very sensitive to any stimulus, this method was given where stimulus can be varied either by the duration of the electric shock or by an increase in the electric current.

*Procedure*

Male mice with a weight of 20g are placed in a special cage and pair of alligator clips are attached to the tail whereby the positive electrode is placed at the proximal end of the tail. Rectangular wave pulses of intensity 40 -50V at a frequency of 1 shock/sec. with a pulse duration of 2.5sec. is applied from a constant voltage stimulator. The normal response time of the stimuli is 3-4sec. After drug administration, the response time is registered at 15min. intervals until the reaction time returns to control level.

*Evaluation*

The AUC is calculated after plotting a graph of reaction time (y-axis) vs. time interval (x-axis).

*Advantages*

The central analgesics effect can be easily demonstrated.

*Disadvantages*

The peripheral analgesics given at higher doses can also be detected, difficulty in differentiation between central and peripherally acting analgesics.
vi. Formalin Test in Rats

**Purpose and Rationale**

This model proposed as a chronic pain model explained by Dubuisson and Dennis (1977), which is sensitive to centrally acting analgesic agents\(^\text{[140]}\).

**Procedure**

Male Wistar rats weighing 180-300g are administered with 0.05ml of 10\% formalin into the dorsal portion of the front paw. Simultaneously the drug is administered either subcutaneously or orally and each animal is placed in a clear plastic container for observation.

Pain responses are indicated by elevation or favoring of the paw or excessive licking and biting of the paw, where readings are taken at 30 and 60mi. and scored according to a pain scale.

Analgesic response or protection is shown if both the paws are resting on the floor with no obvious favoring of the injected paw.

**Evaluation**

\(ED_{50}\) values can be calculated using various doses.

**Advantages**

Almost inefficient to peripheral analgesics and helps in identification of central analgesic agents. This test also allows dissociation between inflammatory and non-inflammatory pain response and helps in rough classification of analgesics according to their site and their mechanism of action.
(b) Peripheral Analgesic Activity

i. Writhing Test in Mice

*Purpose and Rationale*

The animals react with a characteristic stretching behavior called as writhing when pain is induced by injecting irritants like phenylquinone or acetic acid into the peritoneal cavity of mice. This reaction is suitable for peripheral analgesics [11].

*Procedure*

Mice of either sex with a weight between 20-25g are injected with a suspension of phenylquinone (0.25ml of 0.02% suspended in 1% CMC suspension) intraperitoneally for groups of 5 mice used as control and test groups. Test animals were administered with the drug or standard at various pretreatment times prior to phenylquinone administration.

The mice are placed individually into glass beakers and after 5min. of phenylquinone administration, it is observed for 10min. and number of writhes is recorded for each animal.

For scoring purpose, a writhe is indicated by stretching of the abdomen with simultaneous stretching of at-least one hind limb.

*Formula*

\[
% \text{ inhibition} = \frac{\text{Avg. writhes in control group} - \text{Avg. writhes in drug group}}{\text{Avg. writhes in control group}} \times 100
\]

(2.1)

*Evaluation*

A dose range is run in which the \( ED_{50} \) is calculated along with the determination of effective compounds with an average percentage inhibition of >70%, compounds with <70% inhibition are consid-
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...ered to have minimal activity.

Advantages
Ideal for determining both centrally acting as well as for peripherally acting analgesics.

Disadvantages
Even some psychoactive agents also show activity and the reaction is not specific for irritant leading to lack of specificity and misconception of psychoactive agents to analgesics.

4. Screening methods for antipyretic agents

In-vivo Methods

(a) Antipyretic Testing in rats.
(b) Antipyretic Testing in rabbits.

(a) Antipyretic Testing in rats

Purpose and Rationale
The subcutaneous injection of Brewer’s yeast suspension is known to produce fever in rats. The decrease in temperature after administration of compounds with antipyretic activity can be achieved.

Procedure
A 15\% suspension of Brewer’s yeast in 0.9\% saline is prepared and injected in the back below the nape of the neck to the rats (10ml/kg) to produce fever. The animals with body weight of 150g are used and before injection the rectal temperature is measured using a thermocouple to a depth of 2cm into the rectum. The site of injection is massaged in order to spread the suspension beneath the skin. Room temperature is maintained at 22°C to 24°C through out the experiment. Food is withdrawn immediately after the yeast administration. 18 hours post
challenge, the rise in rectal temperature is recorded and measurement is repeated 30min later. The animals with a body temperature of at least 38°C are taken into the test and receive the test compounds or the standard drugs, by oral administration.

**Evaluation**

Rectal temperature is recorded again at 30, 60, 120, and 180 min post dosing. The maximum reduction in the rectal temperature in comparison with the control is calculated and compared with the effect of standard drug.

(b) Antipyretic Testing in rabbits

**Purpose and Rationale**

Lipopolysaccharides from gram-negative bacteria such as E. coli can induce fever in rabbits after intravenous injection. These fractions after 60min of administration cause an increase in body temperature by 1°C or more at a dose of 0.1 to 0.2 µg/kg. in rabbit the temperature increase by 2 maxima, first occurs after 70 min. and second after 3 hours.

**Procedure**

Rabbits of both sex with a weight of 3 to 5 kgs are used. These are placed in suitable cages with the thermocouples connected with auto recorder, introduced into the rectum. Animals are allowed to adapt to the cage for 60min. and administered with 0.2 ml/kg of 0.2 µg lipopolysaccharide intravenously into the rabbit ear. 60 min. later test compounds are administered either subcutaneously or orally and body temperature is monitored for at least 3 hours.
Evaluation
The decrease in body temperature for at least 0.5°C for >30min as compared with the temperature before administration of test compounds is regarded as positive effect.

Advantages
This is a more sensitive method than the yeast induced fever in rats. This method is decisive test for the absence of pyrogens in parenteral drugs by several pharmacopoeias such as USP 23 (1955).

5. COX enzyme inhibitory activity

Purpose and Rationale
Several assays were described to characterize COX-1 and COX-2 inhibitors, such as in vitro COX enzyme assay(Seibert et al.), COX-2 protein extraction and analysis a human whole blood assay using LPS-induced PGF2 production as an index for cellular COX-2 activity or whole-cell assays with transfected Chinese hamster ovary cells expressing COX-1 and COX-2 or COX-2 specific and COX-1 specific making use of PGE2 production as an index of cellular potency and selectivity of cyclooxygenase inhibitors.

The COX enzyme inhibitory assay is performed by using the experimental kit obtained from CAYMAN chemicals, USA. (Item No. 760111) The colorimetric COX(ovine) inhibitor screening assay utilizes the peroxidase component of cyclooxygenase. The peroxidase activity is assayed colorimetrically by monitoring the appearance of oxidized N,N,N,N- Tetramethylp-phenylenediamine (TMPD) at 590nm-610nm. The kit contained assay buffer (10X), Heme, COX-I (ovine), COX-2 (ovine), Arachidonic acid, Potassium hydroxide, Colorimetric substrate, 96 well plate.