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Review **Article**

EVALUATION OF ANTI-INFLAMMATORY ACTIVITY THROUGH IN VITRO AND IN VIVO MODELS

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ABSTRACT:

Inflammation is a complex biological response of the immune system to harmful stimuli such as pathogens, damaged cells, or irritants. Chronic inflammation is associated with various diseases, including autoimmune disorders, cardiovascular diseases, and cancer. Anti-inflammatory agents aim to modulate or suppress inflammation, thus providing therapeutic benefits. This paper explores the in vivo and in vitro models used to study anti-inflammatory mechanisms and evaluate the efficacy of potential anti-inflammatory agents. In vitro models, such as cell cultures and cytokine assays, provide controlled environments to study specific molecular and cellular pathways. In contrast, in vivo models, including animal studies, offer insights into systemic responses and pharmacokinetics. A comprehensive understanding of these models is critical for developing effective anti-inflammatory therapies. This review highlights the advantages, limitations, and applications of both in vivo and in vitro approaches, providing a framework for selecting appropriate models in preclinical research.

Key words: Inflammation, cytokine assays, in vitro and in vivo models.

INTRODUCTION

An important physiological reaction that shields the body from wounds and infections is inflammation. On the other hand, chronic disorders can result from dysregulated or protracted inflammation, which presents serious global health issues. The goal of anti-inflammatory treatments is to lessen related harm and bring the inflammatory response back into equilibrium.

The development and evaluation of anti-inflammatory agents rely on robust experimental models that replicate the complexity of inflammatory processes. In vitro models offer precise control over experimental conditions, enabling detailed analysis of molecular and cellular mechanisms. Common in vitro techniques include cytokine profiling, cell viability assays, and co-culture systems. These models are invaluable for high-throughput screening of potential anti-inflammatory compounds.

Conversely, in vivo models replicate the dynamic and systemic character of inflammation in a living thing. The pharmacodynamic and pharmacokinetic characteristics of anti-inflammatory drugs are frequently evaluated using animal models, such as paw edema caused by carrageenan and systemic inflammation caused by lipopolysaccharide (LPS). An overview of the main in vitro and in vivo models used in anti-inflammatory research is given in this study, with a focus on their applicability in preclinical investigations. By contrasting these models, we hope to help researchers choose the best approaches to further the development of anti-inflammatory drugs.

WHAT IS INFLAMMATION:-

Inflammation is a biological function that is caused after a mechanical tissue disorder or from the reactions by the occurrence of a physical, chemical, or biological mediator in the body.

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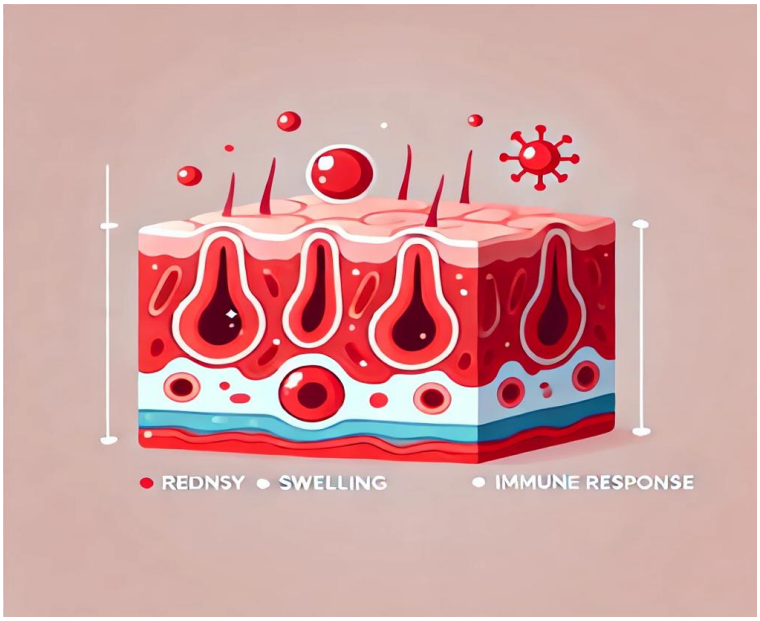

Figure1:-Inflammation picture

TYPES OF INFLAMMATION:-

There are two types of inflammations:-

- **Acute inflammation**
- **Chronic inflammation**

1. Acute inflammation

Acute inflammation is an immediate, beneficial response to injury or infection that helps the body fight off harmful microorganisms and speed up healing. It is a protective process designed to eliminate the cause of tissue damage, remove damaged cells, and initiate tissue repair. This response typically occurs quickly and lasts for a short period, ranging from a few hours to a few days.

2. Chronic Inflammation

Chronic inflammation is when the body continues to send inflammatory cells even when there's no danger. This can damage healthy cells, tissues, and organs, and lead to diseases like cancer, heart disease, and diabetes.

CAUSES OF INFLAMMATION:-

Inflammation is the body's natural response to injury, infection, or harmful stimuli. The causes of inflammation can be divided into two main categories: acute and chronic.

Acute Inflammation Causes

Acute inflammation is a short-term response that typically resolves after the harmful stimulus is removed. Common causes include:

1.Infections:

- Bacterial (e.g., strep throat, urinary tract infections)
- Viral (e.g., influenza, COVID-19)
- Fungal or parasitic infections

2.Injuries:

- Cuts, scrapes, or wounds
- Sprains or fractures
- Burns or frostbite

3.Irritants:

- Chemicals (e.g., acids, alkalis)
- Allergens (e.g., pollen, dust)

4.Toxins:

- Environmental pollutants
- Bacterial toxins

Chronic Inflammation Causes

Chronic inflammation occurs when the immune response persists, potentially leading to tissue damage. Causes include:

1. Chronic Infections:

- Untreated infections like hepatitis, tuberculosis

2.Autoimmune Disorders:

- Rheumatoid arthritis
- Lupus
- Multiple sclerosis

3.Prolonged Exposure to Irritants:

- Smoking
- Pollution
- Alcohol abuse

4.Obesity and Metabolic Disorders:

- Excess adipose tissue can release inflammatory cytokines.

5.Unresolved Acute Inflammation:

- Inadequate treatment of infections or injuries.

6.Certain Diseases:

- Diabetes
- Cancer
- Cardiovascular diseases

SYMPTOMS OF INFLAMMATION:-

Inflammation presents with a range of symptoms that depend on whether it is acute or chronic. The classic signs of acute inflammation are localized and often appear suddenly, while chronic inflammation may cause more subtle, systemic symptoms over time.

Classic Symptoms of Acute Inflammation (Localized)

1. Redness : Due to increased blood flow to the affected area.
2. Swelling : Caused by fluid accumulation in the tissue.
3. Heat : The inflamed area feels warm due to increased blood supply.
4. Pain :Resulting from pressure on nerves and the release of inflammatory chemicals.
5. Loss of Function : The affected area may become difficult to use due to pain or swelling.

Systemic Symptoms of Acute Inflammation

In severe cases, inflammation may affect the whole body, leading to:

- Fever
- Fatigue
- Malaise (general discomfort)
- Elevated white blood cell count

Symptoms of Chronic Inflammation (Systemic)

Chronic inflammation is more subtle and may cause symptoms such as:

1. Fatigue: Persistent tiredness or lack of energy.
2. Aches and Pains: Muscle or joint pain, often diffuse.
3. Digestive Issues: Bloating, diarrhoea, or constipation in cases like inflammatory bowel disease.
4. Weight Changes: Unexplained weight loss or gain.
5. Low-Grade Fever: Often unnoticed but persistent.
6. Frequent Infections: Weakened immune function due to prolonged inflammation.

MODELS USED TO STUDY ANTI -INFLAMMATORY ACTIVITY:-

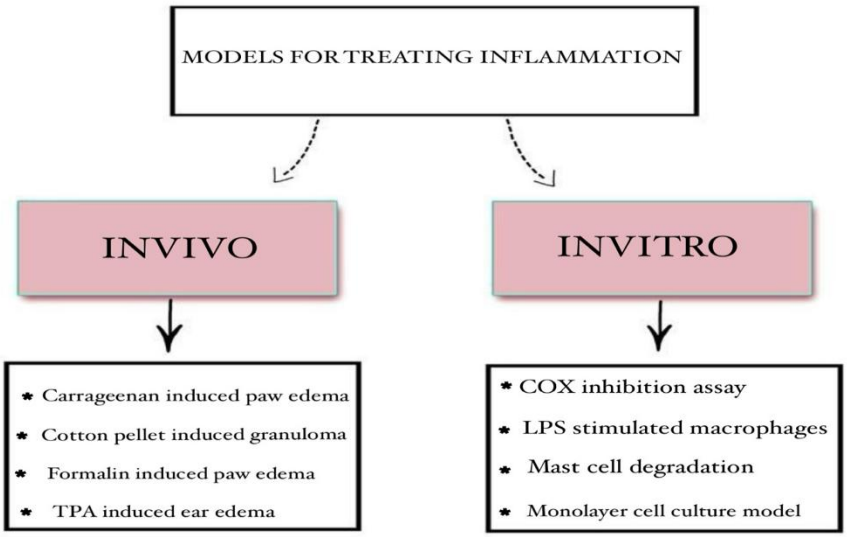

Figure 2:-invivo and Invitro models

WHAT ARE INVIVO AND INVITRO MODELS:

In vivo models:
Definition: In vivo refers to experiments or studies performed within a living organism, such as animals or humans.
Examples:
- Testing drug effects in mice or humans.
- Observing disease progression in a live animal model.

Advantages:
- Provides a realistic environment, reflecting complex biological interactions.
- Enables the study of systemic effects, such as metabolism and immune responses.

Disadvantages:
- Ethical concerns, especially in animal studies.
- Higher costs and longer timelines.
- Variability between species may limit direct applicability to humans.

In Vitro Models
Definition: In vitro refers to experiments conducted outside a living organism, typically in a controlled environment like test tubes, petri dishes, or cell cultures.
Examples:
- Studying cell behavior in culture dishes.
- Testing chemical effects on isolated tissues or enzymes.

Advantages:
- High level of control over experimental conditions.
- Cost-effective and faster than in vivo studies.
- Avoids ethical concerns related to animal use.

Disadvantages:
- Limited ability to replicate the complexity of a whole organism.
- Results may not always translate directly to in vivo conditions

ABOUT ANIMAL MODELS:-
Animal models are non-human species used in scientific research to study biological processes, diseases, and treatments in ways that mimic human systems. They serve as a bridge between in vitro (test-tube or cell-based)

studies and human clinical trials, helping scientists understand complex physiological and pathological mechanisms.

Purpose of Animal Models:

- **Understanding Diseases:** To study disease mechanisms and progression.
  1. **Testing Treatments**: Evaluating the safety and efficacy of new drugs, therapies, or medical devices.
  2. **Biological Insights:** Exploring basic physiological processes, such as reproduction, immunity, and metabolism.
  3. **Genetic Studies:** Investigating the role of specific genes in health and disease.
- **Types of Animal Models**
  1. **Natural models:** Animals that naturally develop diseases similar to human conditions (e.g., dogs with diabetes).
  2. **Induced Models:** Conditions are experimentally induced using chemicals, surgery, or environmental factors (e.g., rodents exposed to carcinogens).
  3. **Genetically Modified Models:** Animals genetically engineered to express or suppress specific genes to mimic human diseases (e.g., transgenic mice for Alzheimer's research).
  4. **Homologous Models:** Animals that closely replicate human diseases with similar symptoms and mechanisms (e.g., monkeys for Parkinson's disease).
  5. **Orphan Models:** Used for studying rare diseases or conditions unique to animals that may have human applications.
- **Commonly Used Animals**
  1. Rodents (Mice and Rats): Most widely used due to their genetic similarity to humans, short lifespans, and ease of handling.
  2. Zebrafish: Ideal for genetic and developmental biology studies.
  3. Non-Human Primates: Used for studying neurological and infectious diseases.
  4. Pigs: Common in cardiovascular and organ transplantation research.
  5. Dogs and Cats: Used in veterinary research and for studying specific conditions like heart disease.

IN VIVO MODELS TO STUDY ANTI-INFLAMMATION:-

1. Carrageenan induced paw edema:-

The carrageenan-induced paw edema model is a widely utilized experimental method for studying acute inflammation and evaluating the efficacy of anti-inflammatory agents in rodents, particularly rats and mice. This model involves the subcutaneous injection of carrageenan, a sulfated polysaccharide, into the hind paw, leading to a measurable inflammatory response characterized by edema (swelling).

- **Principle:**

The underlying principle of this model is that carrageenan injection induces a biphasic inflammatory response:

1. Early Phase (0–2 hours post-injection): This phase is primarily mediated by the release of histamine, serotonin, and bradykinin, leading to increased vascular permeability and fluid exudation.
2. Late Phase (3–6 hours post-injection): Dominated by the production of prostaglandins, particularly PGE2, and the infiltration of neutrophils, resulting in sustained edema and inflammation.

The extent of paw swelling serves as an indicator of the inflammatory response, allowing researchers to assess the anti-inflammatory potential of test compounds by measuring their ability to reduce this edema.

- **Procedure:**
  1. **Animal Preparation:** Select healthy adult rodents and acclimate them to the laboratory environment. Ensure ethical approval and adherence to animal welfare guidelines.
  2. **Baseline Measurement:** Measure the initial paw volume or thickness using a plethysmometer or calipers to establish a baseline.
  3. **Carrageenan Injection:** Prepare a 1% carrageenan solution in sterile saline. Inject 0.1 mL of this solution subcutaneously into the plantar surface of the hind paw.
  4. **Administration of Test Compounds:** Administer the test substance or standard anti-inflammatory drug (e.g., indomethacin) at the desired dose and route (oral or intraperitoneal), typically 30–60 minutes before carrageenan injection.
  5. **Post-Injection Measurements:** At predetermined time intervals (e.g., 1, 2, 3, 4, and 5 hours post-injection), measure the paw volume or thickness to monitor the development of edema.
  6. **Data Analysis:** Calculate the increase in paw volume or thickness relative to baseline. Assess the percentage inhibition of edema in treated groups compared to the control group to determine the anti-inflammatory efficacy of the test compound.

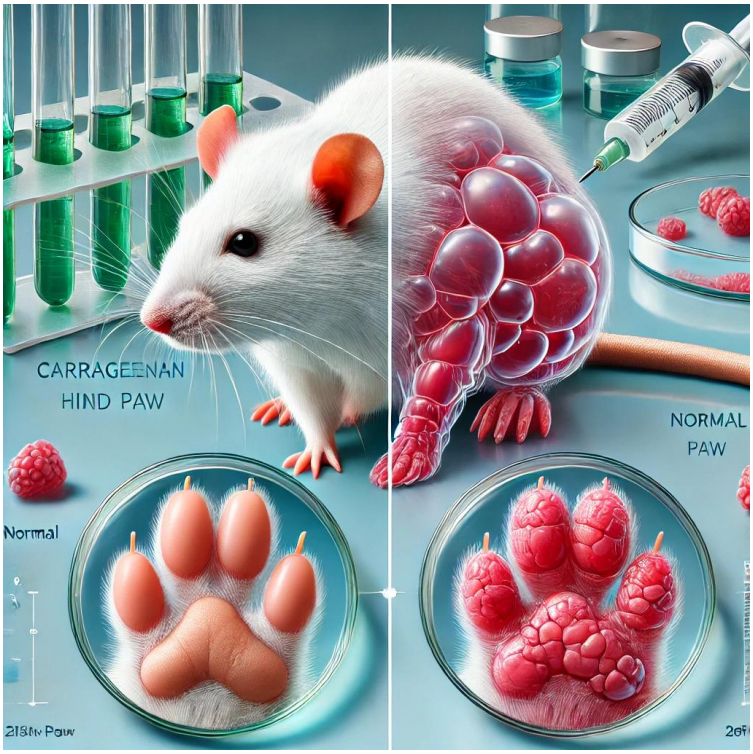

Figure .3:-carrageenan induced paw edema

Mechanism of action:-

The inflammatory response induced by carrageenan involves multiple mediators and cellular events:

Early Phase: Carrageenan triggers the release of histamine and serotonin from mast cells, and bradykinin from kininogen, leading to vasodilation and increased vascular permeability.

Late Phase: The initial mediator release stimulates the production of prostaglandins through the cyclooxygenase pathway, particularly COX-2, resulting in sustained inflammation. Additionally, neutrophil infiltration occurs, contributing to the release of reactive oxygen species and further propagating the inflammatory response.

This biphasic response provides a comprehensive model for evaluating both early and late anti-inflammatory effects of pharmacological agents.[1,2,3]

2) Cotton pellet induced granuloma:-

The cotton pellet-induced granuloma model is a widely used experimental method for evaluating the efficacy of anti-inflammatory agents, particularly in the context of chronic inflammation. This model involves the subcutaneous implantation of sterile cotton pellets in rodents, leading to granuloma formation, which serves as an indicator of the proliferative phase of inflammation.

Principle:

The principle behind the cotton pellet-induced granuloma model involves the body's response to a foreign material (cotton pellet), which leads to the formation of a granuloma. The process can be summarized as follows:

1. **Foreign Body Reaction:** When a cotton pellet is implanted into the subcutaneous tissue, it is recognized as a foreign body by the immune system, triggering an inflammatory response.
2. **Acute Inflammation:** Initially, there is acute inflammation characterized by the recruitment of immune cells such as neutrophils and macrophages, and the release of inflammatory mediators like prostaglandins, cytokines, and histamines.
3. **Chronic Inflammation and Granuloma Formation:** Over time, the persistent presence of the foreign material leads to chronic inflammation. Macrophages aggregate and transform into epithelioid cells, forming a granuloma around the cotton pellet. This granuloma serves to "contain" the irritant.
4. **Fibrosis:** Fibroblasts may also contribute to fibrosis the granuloma, further encapsulating the foreign material.

This model is used to simulate chronic inflammatory responses, allowing researchers to study mechanisms of granuloma formation and evaluate potential anti-inflammatory treatments.

Procedure:

1. **Animal Preparation:** Select healthy adult rodents (rats or mice) and acclimate them to laboratory conditions.
2. **Cotton Pellet Preparation:** Sterilize cotton pellets weighing approximately 50 mg each by autoclaving.
3. **Implantation:** Under appropriate anaesthesia, make a small incision in the dorsal region of the animal and implant the sterile cotton pellet subcutaneously. Close the incision with sutures.
4. **Treatment Administration:** Administer the test compound or standard anti-inflammatory drug at the desired dose and route, typically starting on the day of implantation and continuing for a specified period (e.g., 7 days).
5. **Granuloma Extraction:** At the end of the treatment period, euthanize the animals, carefully extract the cotton pellets along with the surrounding granulomatous tissue, and dry them at 60°C for 24 hours.
6. **Weight Measurement:** Weigh the dried granulomas to determine the dry weight, which reflects the amount of granulomatous tissue formed.
7. **Data Analysis:** Compare the granuloma weights between treated and control groups to assess the anti-inflammatory efficacy of the test compounds.

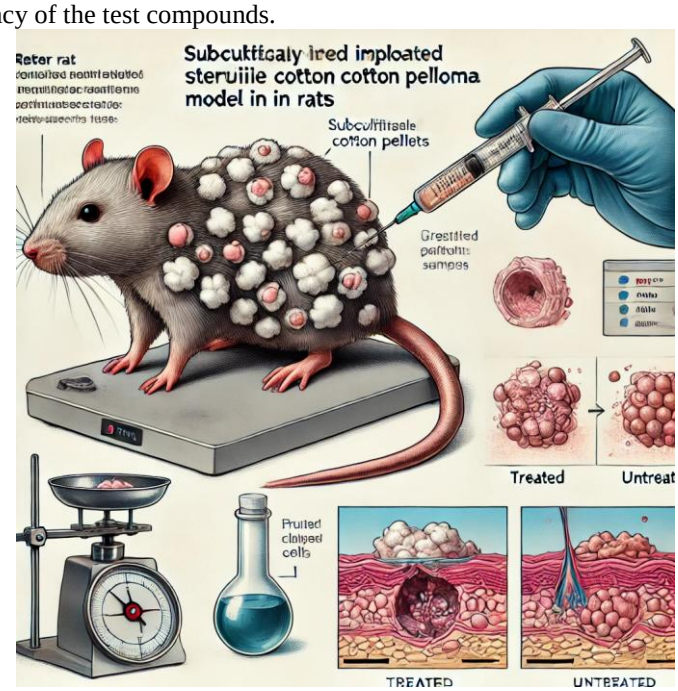

Figure 4:-cotton pellet induced granuloma

Mechanism of Action:

The cotton pellet-induced granuloma model involves a triphasic inflammatory response:

1. **Transudative hase:** Characterized by the transudation of fluid from the blood vessels into the surrounding tissue.
2. **Exudative Phase:** Involves the escape of fluid and blood cells into the tissue, leading to edema.
3. **Proliferative Phase:** Marked by the proliferation of fibroblasts and the formation of collagen, resulting in granuloma formation.

Anti-inflammatory agents may exert their effects by inhibiting one or more of these phases, thereby reducing granuloma formation.[4,5,6,7]

3) Formalin induced paw edema:-

The formalin-induced paw edema model is an established experimental method for studying inflammatory responses and evaluating the efficacy of anti-inflammatory agents in rodents, particularly rats and mice. This model involves the injection of formalin into the paw, leading to localized inflammation characterized by edema (swelling) and nociceptive behaviors.

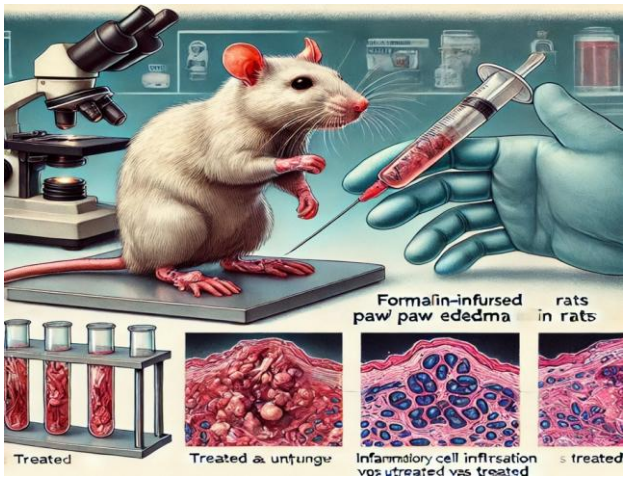

Figure.5:-formalin induced paw edema

Principle:

Formalin-induced paw edema is a widely used experimental model to study acute inflammation, nociception, and the efficacy of anti-inflammatory or analgesic agents in rodents. Its mechanism of action involves two distinct phases:

1. Early Phase (Neurogenic Pain Phase)

- **Duration:** Occurs within the first 0–10 minutes after formalin injection.
- **Mechanism:**
- Formalin directly activates nociceptors by stimulating TRPA1 (Transient Receptor Potential Ankyrin 1) ion channels on sensory neurones.
- This leads to the release of substance P and calcitonin gene-related peptide (CGRP), which contribute to pain perception and vasodilation.
- The early phase primarily reflects neurogenic pain and the activation of peripheral nociceptive fibers.

2. Late Phase (Inflammatory Pain Phase)

- **Duration:** Begins around 10–15 minutes after injection and lasts up to 60 minutes or longer.
- **Mechanism:**
- Involves an inflammatory response mediated by:
- Recruitment of immune cells (e.g., neutrophils, macrophages).
- Release of inflammatory mediators such as histamine, serotonin, prostaglandins (PGE2), bradykinin, and cytokines (e.g., TNF-α, IL-1β).
- These mediators increase vascular permeability, leading to edema and sustained pain.
- This phase reflects inflammatory pain and involves both peripheral and central sensitization.

Procedure:

1. **Animal Preparation:** Select healthy adult rodents and acclimate them to the laboratory environment. Ensure ethical approval and adherence to animal welfare guidelines.
2. **Baseline Measurement:** Measure the initial paw volume or thickness using a plethysmometer or calipers to establish a baseline.
3. **Formalin Injection:** Inject a specific concentration of formalin (commonly 1–5% formaldehyde solution) subcutaneously into the plantar surface of the hind paw, typically in a volume of 50–100 µL.
4. **Observation Period:** Monitor the animal for nociceptive behaviors (e.g., licking, biting, or shaking of the injected paw) and measure paw edema at predetermined time intervals to assess the inflammatory response.
5. **Administration of Test Compounds:** Administer the test substance or standard anti-inflammatory drug at the desired dose and route (oral or intraperitoneal), either before or after formalin injection, depending on the study design.
6. **Data Analysis:** Evaluate the extent of paw edema and nociceptive behaviors in treated and control groups to determine the anti-inflammatory and analgesic efficacy of the test compounds.

Mechanism of Action:-

Formalin induces inflammation and pain through several mechanisms:

- **Direct Nociceptor Activation:** Formalin directly stimulates sensory neurons , leading to immediate pain responses. This effect is mediated, in part, by the activation of the TRPA1 cation channel, which plays a significant role in inflammatory pain.

- **Release of Inflammatory Mediators:** Formalin injection leads to the release of various inflammatory mediators, including substance P, prostanoids, serotonin, and histamine, which contribute to vasodilation, increased vascular permeability, and edema formation.
- **Neurogenic Inflammation:** At higher doses, formalin induces neurogenic inflammation mediated by neuropeptides such as substance P, resulting in increased vascular permeability and edema.
- This model is valuable for assessing both the inflammatory and nociceptive components of pain and for evaluating the efficacy of pharmacological agents targeting these processes.[8,9,10,11]

4) TPA induced ear edema:-

The 12-O-Tetradecanoylphorbol-13-acetate (TPA)-induced ear edema model is a widely used experimental method for evaluating the anti-inflammatory properties of compounds in vivo. This model involves the topical application of TPA to the mouse ear, leading to acute skin inflammation characterized by edema, erythema, and leukocyte infiltration.

Principle:

TPA, a potent activator of protein kinase C (PKC), induces a rapid inflammatory response when applied to the skin. This response is marked by increased production of pro-inflammatory mediators, resulting in edema and other signs of inflammation. The extent of ear swelling serves as a quantitative measure of the inflammatory response, allowing for the assessment of anti-inflammatory agents.

Procedure:

1. **Animal Preparation:** Select healthy adult mice and acclimate them to laboratory conditions. Ensure ethical approval and adherence to animal welfare guidelines.
2. **Baseline Measurement:** Measure the initial thickness of both ears using a micrometer to establish a baseline
3. **TPA Application:** Dissolve TPA in a suitable solvent, typically acetone, to achieve the desired concentration (e.g., 2.5 µg/ear). Apply the TPA solution topically to the inner and outer surfaces of one ear (usually the right ear) using a micropipette. The contralateral ear may serve as a control.

Figure6:-TPA induced ear edema

4. **Treatment Administration:** Administer the test compound or standard anti-inflammatory drug either topically or systemically at the desired dose and route, prior to or after TPA application, depending on the study design.
5. **Observation Period:** Allow the inflammatory response to develop over a specified period, typically 4 to 6 hours post-TPA application. During this time, monitor the animals for any adverse reactions.
6. **Measurement of Edema:** At the end of the observation period, measure the thickness of both ears again using the micrometer. Calculate the increase in ear thickness as an indicator of edema.
7. **Data Analysis:** Compare the ear thickness measurements between treated and control group to assess the anti-inflammatory efficacy of the test compounds.

Mechanism of Action:

TPA induces inflammation through several mechanisms:

- **Activation of Protein Kinase C (PKC):** TPA directly activates PKC, leading to the phosphorylation of various substrates involved in inflammatory pathways.
- **Production of Pro-inflammatory Mediators:** Activation of PKC results in the upregulation of enzymes such as cyclooxygenase (COX) and lipoxygenase (LOX), leading to increased synthesis of eicosanoids like prostaglandins and leukotrienes, which contribute to edema and pain.
- **Induction of Cytokines and Chemokines:** TPA application stimulates the release of pro-inflammatory cytokines (e.g., TNF-α, IL-1β) and chemokines, promoting leukocyte infiltration into the affected tissue.
- **Oxidative Stress:** TPA-induced activation of inflammatory cells leads to the production of reactive oxygen species (ROS), contributing to tissue damage and further propagating the inflammatory response.
- The TPA-induced ear edema model is particularly useful for evaluating the efficacy of anti-inflammatory agents targeting these pathways.[12,13,14]

5) Xylene induced ear edema:-

The xylene-induced ear edema model is a widely used experimental method for evaluating the anti-inflammatory properties of compounds in vivo. This model involves the topical application of xylene to the mouse ear, leading to acute inflammation characterized by edema (swelling), erythema (redness), and increased vascular permeability.

Principle:

Xylene, an organic solvent, acts as an irritant when applied to the skin, inducing an acute inflammatory response. This response is marked by increased capillary permeability, fluid accumulation, and leukocyte infiltration into the affected tissue. The degree of ear swelling serves as a quantitative measure of the inflammatory response, allowing for the assessment of anti-inflammatory agents.

Procedure:

1. **Animal Preparation:** Select healthy adult mice, typically weighing between 18 to 21 grams, and acclimate them to laboratory conditions. Ensure ethical approval and adherence to animal welfare guidelines.
2. **Baseline Measurement:** Measure the initial thickness or weight of both ears to establish a baseline.
3. **Xylene Application:** Apply a specific volume of xylene (commonly 30 µL) to the inner surface of the right ear using a micropipette. The left ear serves as a control.
4. **Treatment Administration:** Administer the test compound or standard anti-inflammatory drug orally or via another appropriate route, either before or after xylene application, depending on the study design.

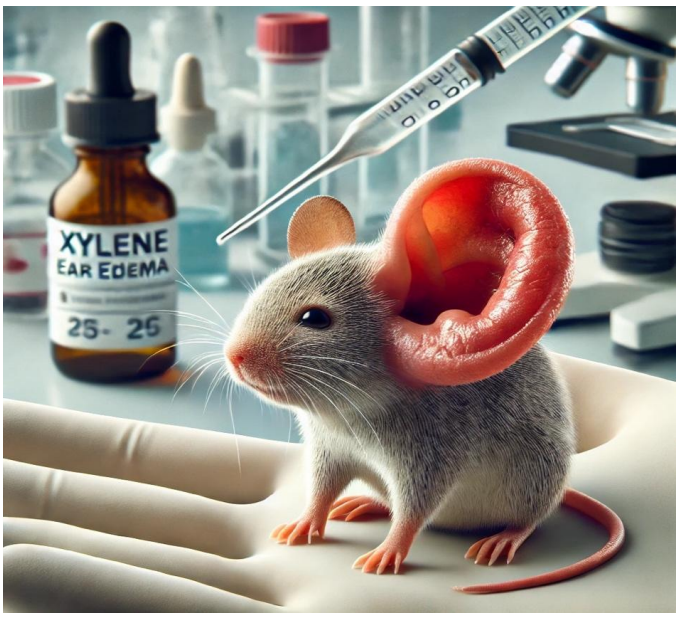

Figure .7:-xylene induced ear edema

5.Observation Period: Allow the inflammatory response to develop over a specified period, typically 30 minutes post-xylene application. Monitor the animals for any adverse reactions during this time.

6.Measurement of Edema: After the observation period, euthanize the animals humanely. Remove both ears and weigh them separately. The difference in weight between the treated and control ears indicates the extent of edema

7. Data Analysis: Compare the ear weight differences between treated and control groups to assess the anti-inflammatory efficacy of the test compounds.

Mechanism of Action:

Xylene induces inflammation through several mechanisms:

- **Increased Capillary Permeability:** Xylene application leads to the release of inflammatory mediators that increase capillary permeability, resulting in plasma exudation and tissue swelling.
- **Leukocyte Infiltration:** The irritant effect of xylene promotes the infiltration of leukocytes, particularly neutrophils, into the affected tissue, contributing to the inflammatory response.
- **Neurogenic Inflammation:** Xylene exposure may stimulate sensory nerve endings, leading to the release of neuropeptides such as substance P, which further enhance vascular permeability and inflammation.

The xylene-induced ear edema model is particularly useful for evaluating the efficacy of anti-inflammatory agents that target these acute inflammatory pathways.[15,16,17]

IN VITRO MODELS TO STUDY ANTI INFLAMMATION:-

1) cox inhibition assay:-

The COX inhibition assay is used to evaluate the effectiveness of compounds in inhibiting cyclooxygenase enzymes (COX-1 and COX-2), which are involved in the synthesis of prostaglandins, mediators of inflammation and pain.

Principle:

Cyclooxygenase (COX) enzymes catalyze the conversion of arachidonic acid to prostaglandins, which are critical mediators of inflammation. COX-1 is constitutively expressed in many tissues and plays a role in maintaining normal physiological functions, while COX-2 is induced in response to inflammation. Inhibition of these enzymes, particularly COX-2, is a target for anti-inflammatory drugs.

The COX inhibition assay typically measures the reduction in prostaglandin production after exposure to a test compound, using either COX-1 or COX-2 as the enzyme source.

Procedure:

1. Preparation of Reagents:

COX enzyme solution: Purified COX-1 or COX-2 enzyme.

Arachidonic acid: Substrate for COX enzymes.

Test compound: Compound to be tested for COX inhibition, prepared at various concentrations.

Detection reagent: Prostaglandin E2 (PGE2) or other prostaglandins, detected by colorimetric or fluorometric methods.

2. Assay Setup:

- Mix the COX enzyme solution with the test compound in a micro plate well.
- Add arachidonic acid to start the enzyme reaction.
- Incubate the mixture at 37°C for a specified duration (e.g., 10-15 minutes).

3. Detection:

- After the incubation, stop the reaction and add the detection reagent that reacts with the prostaglandin product.
- Measure the absorbance or fluorescence of the reaction using a micro-plate reader. The higher the signal, the more prostaglandin was produced.

4. Data Analysis:

- Calculate the percentage inhibition of COX activity by comparing the signal from treated wells to a control (no inhibitor).

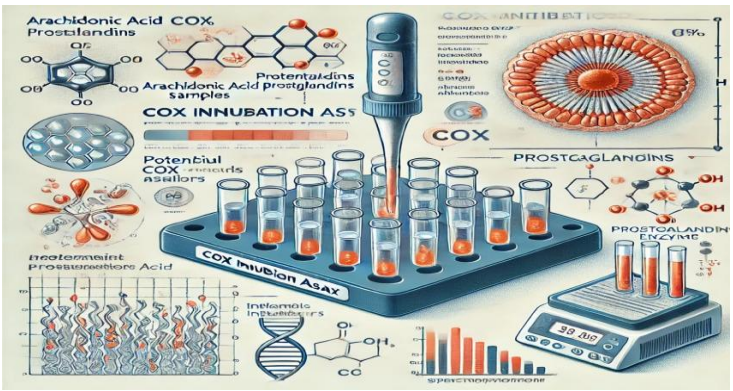

Figure 8:-cox inhibition assay

- Determine the IC_{50} value (concentration of the test compound that inhibits 50% of COX activity).

Mechanism of Action:

COX enzymes have two functional sites:

1. **Cyclooxygenase site:** This site catalyzes the conversion of arachidonic acid to prostaglandin G2 (PGG2).
2. **Peroxidase site:** This site catalyzes the conversion of PGG2 to prostaglandin H2 (PGH2), which is the precursor for other prostaglandins.

Inhibitors can bind to either of these sites, preventing the conversion of arachidonic acid to prostaglandins. Non-selective COX inhibitors block both COX-1 and COX-2, while selective COX-2 inhibitors primarily block the inducible form of the enzyme associated with inflammation.[18,19,20]

2) LPS stimulated macrophages assay:-

The LPS-stimulated macrophage assay is a commonly used in vitro model to study inflammation and test the efficacy of anti-inflammatory agents. LPS (lipopolysaccharide), a component of the outer membrane of Gram-negative bacteria, is a potent inducer of inflammation, primarily through activation of macrophages and the release of pro-inflammatory cytokines.

Principle:

LPS activates macrophages via the toll-like receptor 4 (TLR4), leading to the production of inflammatory mediators like cytokines (e.g., TNF-α, IL-6, IL-1β) and prostaglandins. These molecules play crucial roles in the immune response to infection, but excessive production can lead to chronic inflammation and tissue damage. The LPS-stimulated macrophage assay helps assess the anti-inflammatory effects of compounds by measuring their ability to inhibit LPS-induced cytokine production and other markers of inflammation.

Procedure:

1. Cell Culture Preparation:
   - Macrophage Cells: Cultivate macrophage cell lines (e.g., RAW 264.7, J774A.1) or primary macrophages (from mouse or human sources) in appropriate culture conditions (e.g., RPMI-1640 or DMEM media with 10% FBS, incubated at 37°C with 5% CO2).
2. Treatment Preparation:
   - Prepare the test compound (e.g., potential anti-inflammatory drug) at various concentrations (e.g., 1, 10, 100 µM).
   - Dilute LPS to the desired concentration, typically 1 µg/mL for stimulation.
3. Incubation with LPS:
   - Seed macrophages in a 96-well plate and allow them to adhere for several hours (usually 2–4 hours).
   - Add the test compound to the cells and incubate for a pre-determined period (e.g., 1 hour) to allow any effects on macrophage function.
   - Stimulate the cells with LPS (1 µg/mL) to activate the macrophages.
4. Cytokine Measurement:
   - After a specified period of LPS stimulation (usually 24 hours), collect the culture supernatant.
   - Measure the levels of cytokines such as TNF-α, IL-6, and IL-1β using enzyme-linked immunosorbent assays (ELISA), cytokine bead arrays (CBA), or other detection methods.

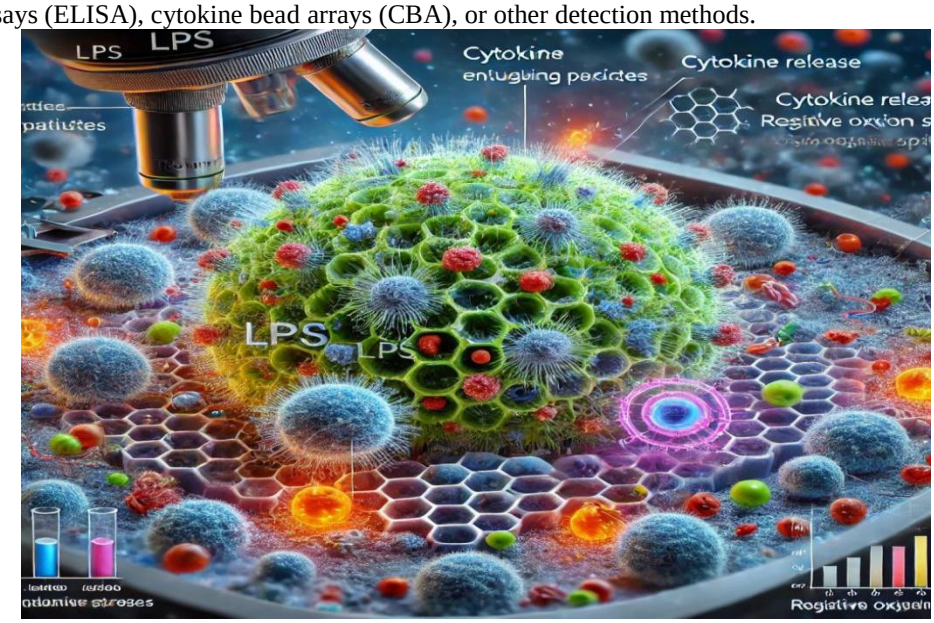

Figure 9:-LPS stimulated macrophages

5. Cell Viability (Optional):
   - Perform a cell viability assay (e.g., MTT or MTS assay) to ensure that the compound does not induce cytotoxicity.
6. Data Analysis:
   - Analyze the inhibition of cytokine production by comparing the levels of cytokines in LPS-stimulated cells with and without the test compound. A reduction in cytokine release indicates anti-inflammatory activity.

Mechanism of Action:

1. **Activation of TLR4**
   LPS binds to TLR4 on the surface of macrophages, triggering a downstream signaling cascade through adaptor proteins (e.g., MyD88, TRIF) and activation of NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and MAPK (mitogen-activated protein kinase) pathways.
2. **Inflammatory Mediators Release:**
   Activation of NF-κB and MAPKs leads to the expression of pro-inflammatory genes, including cytokines (e.g., TNF-α, IL-6, IL-1β) and enzymes like COX-2 (cyclooxygenase-2), which produce prostaglandins (e.g., PGE2), a key inflammatory mediator.
3. **Effect of Anti-inflammatory Compounds:**
   Anti-inflammatory compounds can inhibit the activation of TLR4 or block the downstream signaling pathways (NF-κB or MAPK), thereby reducing the production of pro-inflammatory cytokines and mediators.21,22,23

3)Mast cell degranulation:-

The mast cell degranulation assay is used to study the activation and degranulation of mast cells, which are immune cells involved in allergic reactions and inflammation. Mast cells release histamine and other pro-inflammatory mediators upon activation, making them a key target in the study of allergic diseases and inflammation.

Principle:

Mast cells contain granules filled with histamine, proteases, and other inflammatory mediators. Upon activation (e.g., by antigen binding to IgE or by other stimuli), mast cells undergo degranulation, releasing these mediators into the surrounding environment. The mast cell degranulation assay evaluates the extent of this degranulation, often by measuring the release of histamine or other substances, and helps assess the potential effects of compounds in modulating this process.

Procedure:

- **Preparation of Mast Cells:**
  - Cell Source: Use mast cell lines (e.g., RBL-2H3 or MC/9 cells) or primary mast cells derived from animal models (e.g., mice or rats).
  - Cell Culture: Cultivate cells in appropriate media (e.g., RPMI-1640 or DMEM) supplemented with fetal bovine serum (FBS) and maintain under standard cell culture conditions (37°C, 5% CO2).
- **Priming Mast Cells (if needed):**
  - IgE Sensitization: For allergic reactions, mast cells are often "sensitised" by incubating them with anti-IgE antibodies (e.g., DNP-IgE) that bind to the high-affinity IgE receptor (FcεRI) on mast cells. This primes the mast cells to respond to specific antigens (e.g., dinitrophenylated bovine serum albumin, DNP-BSA).
- **Treatment with Test Compound:**
  - Incubate the mast cells with the test compound at various concentrations for a specified period (e.g., 1–2 hours). This allows the compound to potentially influence mast cell function, either by inhibiting or enhancing degranulation.
- **Induction of Degranulation:**
  - **Stimulation:** After pre-treatment with the test compound, stimulate mast cells by adding a triggering agent. For IgE-mediated degranulation, an antigen (e.g., DNP-BSA) is added, which cross-links the IgE receptors on the mast cells, causing degranulation.
  - **Alternative Stimuli:** Some assays use non-IgE-dependent stimuli, such as compound 48/80, calcium ionophore A23187, or other chemical agents that directly induce degranulation.
- **Measurement of Degranulation:**
  - **Histamine Release:** The most common method of assessing degranulation is by measuring the release of histamine into the supernatant. Histamine can be quantified using specific assays like the fluorometric or colorimetric assay.
  - **Other Markers:** In some cases, other markers of degranulation, such as β-hexosaminidase or tryptase activity, can also be measured.

- **Data Analysis:**
  - Calculate the percentage of mast cell degranulation by comparing the histamine release in the stimulated cells (with and without the test compound) to baseline (unstimulated) cells.

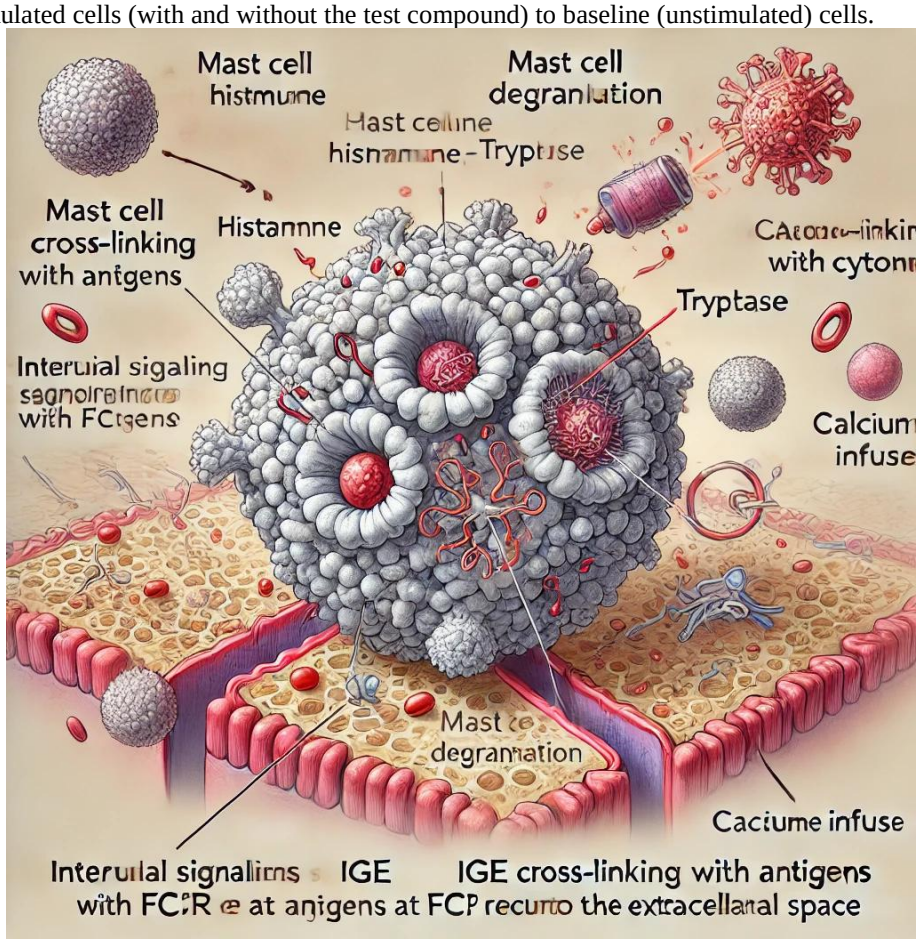

Figure .10:- mast cell degranulation

Mechanism of Action:

1. **Mast Cell Activation:**
   - Mast cells are activated when an antigen (e.g., DNP-BSA) binds to the IgE antibodies already bound to FcεRI receptors on the surface of mast cells. This cross-linking leads to the activation of intracellular signaling pathways.

2. **Signal Transduction:**
   - The cross-linking of IgE receptors triggers the activation of various intracellular signaling pathways, including the PLC/IP3 pathway, leading to the release of intracellular calcium and the activation of protein kinases (e.g., PKC, MAPK). This cascade ultimately causes mast cell degranulation.
3. **Degranulation and Mediator Release:**
   - The intracellular signaling pathways lead to the fusion of mast cell granules with the cell membrane, releasing pre-stored inflammatory mediators like histamine, cytokines, proteases, and lipid mediators (prostaglandins and leukotrienes).
4. **Effect of Inhibitors:**
   - Anti-inflammatory compounds or inhibitors can modulate these pathways by blocking the activation of receptors (e.g., blocking IgE-FcεRI interaction), inhibiting signaling molecules (e.g., PLC, PKC, or MAPKs), or stabilizing the mast cell membrane to prevent granule release.[24,25,26]

4) Mono layer cell culture model:-

The monolayer cell culture model is a widely used in vitro system where cells are cultured in a single layer on a flat surface, typically a culture dish or well plate. This model allows for the study of cellular behavior, including proliferation, differentiation, migration, and response to external stimuli, such as drugs, toxins, or growth factors.

Principle:

The monolayer cell culture model is based on the ability of adherent cells (such as fibroblasts, epithelial cells, or endothelial cells) to grow in a single layer when provided with a suitable surface (e.g., tissue culture-treated plastic) and appropriate culture conditions (temperature, CO_2 concentration, and nutrient-rich medium). Cells are typically grown in a nutrient medium, and their responses to various treatments can be studied through various assays, such as cell viability, migration, and gene expression analysis.

Procedure:

1. **Cell Preparation:**

- Select an appropriate cell line (e.g., HeLa cells, NIH-3T3 cells, or RAW 264.7 macrophages).
- Culture cells in appropriate growth medium (e.g., DMEM or RPMI-1640) supplemented with 10% fetal bovine serum (FBS) and antibiotics (e.g., penicillin-streptomycin) to prevent contamination.
- Maintain cells in a humidified incubator at 37°C with 5% CO_2.

2. **Subculture and Seeding:**

- Subculture the cells to prevent over-confluence by trypsinizing them and transferring them to fresh culture vessels.
- Seed a known number of cells (e.g., 1 x 10^4 cells per well) onto a flat surface of a multi-well plate or a petri dish.
- Allow cells to adhere and spread out to form a monolayer, typically taking 12–24 hours, depending on the cell type and seeding density.

3. **Treatment and Exposure:**

- Once the monolayer is formed, treat the cells with the experimental compounds, drugs, or stimuli. Treatment concentrations and durations will depend on the specific experiment being conducted (e.g., 24–48 hours for drug exposure).
- For migration studies, induce stress conditions or use stimuli like growth factors or chemical agents (e.g., EGF for cell migration).

Figure11:-monolayer cell culture

4. **Assays and Measurements:**

- **Cell Viability:** Assess cell viability using colorimetric assays such as MTT, MTS, or Alamar Blue, which measure metabolic activity or cell proliferation.

- **Cell Migration/Invasion:** Perform scratch wound healing assays or transwell assays to evaluate the ability of cells to migrate or invade.
- **Gene/Protein Expression:** Use PCR, Western blotting, or immunohistochemistry to assess changes in gene or protein expression due to treatment.
- **Morphological Changes:** Observe changes in cell morphology under a microscope, often used to assess differentiation or response to stimuli.

5. Data Analysis:

- Quantify results (e.g., percentage of wound closure, cell proliferation rates, or relative expression levels) using appropriate software.
- Analyze the effect of the treatment by comparing experimental groups with control groups

Mechanism of Action:

1. Cell Attachment and Spreading:

- When seeded onto a surface, adherent cells bind to the extracellular matrix (ECM) proteins (e.g., fibronectin, collagen) via integrins. This interaction triggers intracellular signaling pathways that facilitate cell spreading and migration across the surface to form a monolayer.

2. Cell Proliferation and Growth:

- Once the monolayer is established, cell growth and division are regulated by growth factors and nutrients in the medium. Cell proliferation is controlled by signals through receptors, such as the epidermal growth factor receptor (EGFR) and fibroblast growth factor receptor (FGFR).

3. Response to External Stimuli:

- Cells in the monolayer respond to external stimuli, such as cytokines, drugs, or mechanical stress, by activating signaling pathways like MAPK, PI3K-AKT, or NF-κB. These pathways can lead to various outcomes, including cell proliferation, apoptosis, differentiation, or changes in gene expression.

4. Cell-Matrix Interaction and Mechanical Forces:

- The integrity of the monolayer is maintained by adhesion molecules like E-cadherin and β-catenin, which form adherent junctions. Mechanical forces such as tension and compression, often applied during assays like migration or invasion, activate pathways such as Rho-GTPase signaling, which regulates cell shape, motility, and cytoskeletal organization.[27,28,29]

CONCLUSION:-

In conclusion, the diverse experimental models used to study inflammation and related biological processes, such as the carrageenan-induced paw edema model, cotton pellet-induced granuloma model, formalin-induced paw edema model, TPA- and xylene-induced ear edema models, COX inhibition assay, LPS-stimulated macrophage assay, mast cell degranulation assay, and monolayer cell culture model, provide invaluable tools for preclinical research.

Each model offers distinct insights into specific aspects of acute and chronic inflammation, pain, immune activation, and cellular responses. Together, they enable a comprehensive evaluation of the mechanisms underlying inflammation and the pharmacological effects of potential therapeutic agents. Their robustness, reproducibility, and relevance to human pathophysiology make them critical in the discovery and development of novel anti-inflammatory and analgesic drugs, as well as in understanding fundamental biological processes.

These models continue to play a pivotal role in bridging the gap between basic research and clinical application, advancing the field of biomedical research and the development of effective treatments for inflammatory and immune-mediated diseases.

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