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Review Article

Overview of In Vitro Anti-Inflammatory Models

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ABSTRACT

Inflammation is a complex biological response linked to numerous health issues. To better understand its mechanisms and develop treatments, laboratory models are crucial. This article explores key lab tests, including egg albumin denaturation, antiproteinase activity assessment, and heat-induced hemolysis, used to evaluate anti-inflammatory compounds. The discussion delves into the molecular aspects of inflammation, such as cytokines, chemokines, prostaglandins, and reactive oxygen species, and their roles in inflammatory processes. Laboratory models offer a controlled, cost-effective, and ethically sound approach for initial research on inflammation. However, their limitations in replicating real-life environments necessitate the use of complementary methods, such as 3D cultures and organoids, to boost accuracy. This article emphasizes the value of combining various laboratory models to gain a deeper understanding of inflammation and develop effective treatments. By refining these techniques, researchers can accelerate the discovery of new treatments and combat inflammatory diseases.

INTRODUCTION

Inflammation serves as a fundamental protective response of the body. In the life sciences, biological entities are often seen and detailed as they engage in activities that lead to various results, which can sometimes be completely contrary; these activities employ a common language and are defined by the associated entity. For instance, the phrase "lactate dehydrogenase activity" refers to a process that includes the conversion of lactate into pyruvate and the reverse process of changing pyruvate back to lactate. The phrase "phagocytosis" refers to the mechanism in which a phagocytic cell surrounds and decomposes bacteria, alongside how a phagocytic cell absorbs bacteria and subsequently reproduces them through lysing the phagocyte, or the "exploitation" of the host cell by the bacteria [1]
Immune response that responds to harm, illness, or injury. It involves heightened blood circulation to the affected regions, the release of chemical signals, and the stimulation of immune cells [2]
The response of living mammals to various forms of injury is referred to as inflammation. It represents a tangible protective mechanism. In

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biological studies, organisms are often observed and detailed while engaging in actions that yield diverse results, some of which may directly oppose one another. These activities are categorized in a comparable manner, based on the specific organism involved. For example, the phrase "lactate dehydrogenase activity" describes a function that involves transforming pyruvate into lactate and the reverse transformation of lactate into pyruvate. The term "phagocytosis" describes the mechanism by which a phagocyte surrounds and completely dismantles bacteria, alongside the method in which a phagocyte consumes bacteria and subsequently reproduces them via phagocyte lysis, which denotes the "consumption" of the host cell by bacteria. Initially, the damaged cells and tissues are eliminated to lessen or control the spread of the harmful agent [3] In the human body, inflammation is an essential defensive response that promotes healing [4]. Swelling has historically been accepted as a normal part of recovery. It was considered an adverse reaction that could be harmful to humans until the late 19th century. However, research demonstrating the critical function of inflammation in the body's defence and healing systems was not initiated until the 1800s by Metchnikoff and his colleagues. Inflammation's significance in healing and repair, as well as its negative effects, are now becoming increasingly recognised. Currently, inflammation is thought of as a whole sequence of events that begins with the initiation of a reaction, continues through the development of noticeable symptoms, and ends with the recovery and return to normal of tissue or organ structure and function. Sometimes, however, there is no resolution, and the patient is left with a chronic inflammatory disease that might last a lifetime. This include inflammatory conditions such as rheumatoid arthritis, osteoarthritis, retinitis, inflammatory bowel disease, psoriasis, multiple sclerosis, and atherosclerosis [5]

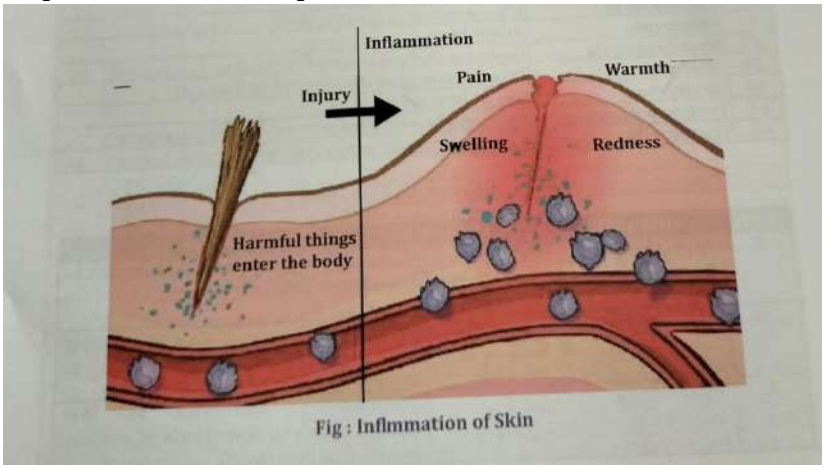

Figure: Inflammation of Skin

Signs of Inflammation:

1. Rubor (Redness): Enhanced blood circulation to the impacted region, resulting in redness.
2. Tumor (Swelling): Build-up of fluid or immune cells, resulting in swelling.
3. Calor (Heat): Enhanced blood circulation and metabolic processes, resulting in warmth or heat.
4. Dolor (Pain): Stimulation of pain receptors and secretion of pain-inducing substances.
5. Functio Laesa (Loss of Function): Reduced ability or movement in the impacted region [3] [6]

Types:

A. Acute Inflammation:

Usually lasting only a few minutes, a few hours, or at most a few days, acute inflammation is very

transient. Its main characteristics are the migration of white blood cells, mainly neutrophils, and the oedema, or fluid and plasma protein leakage. In most cases, acute inflammation follows a pretty consistent pattern, regardless of the sort of damaging agent.

The main indicators of inflammation are caused by-

a. Alterations in blood flow and vessel size (changes in hemodynamics).

b. Modifications in the permeability of blood vessels.

c. The outflow of leukocytes [7]

Causes:

1. Infection: Bacterial, viral, fungal, or parasitic infections.
2. Trauma: Physical injury, such as cuts, burns, or fractures.
3. Toxins: Exposure to chemicals, poisons, or venom.
4. Surgery: Post-operative inflammation.
5. Immune responses: Acute inflammatory responses can also occur in response to immune-mediated disorders, such as allergic reactions [8] [9]

B. Chronic Inflammation:

The term "chronic inflammation" describes an inflammatory response that lasts for weeks, months, or even years. Continuous immune cell activation, inflammatory agent release, and tissue destruction are characteristics of this illness [11] Numerous complicated diseases and conditions, such as metabolic syndromes, neurological diseases, autoimmune disorders, different types of cancer, and cardiovascular diseases, are all influenced by inflammation. Chronic inflammation, which is thought to be uncontrolled, and acute inflammation, which is thought to be regulated, are the two types of inflammation. Given its critical involvement in the aforementioned disorders, it is critical to develop techniques for managing chronic inflammation in order to create efficient therapies and remedies for various medical conditions. Differentiating between chronic and acute inflammation through molecular biology tests poses a significant challenge. To address this, our research focuses on investigating the molecular mechanisms underlying chronic inflammation and its associated conditions [12]

Types of Chronic Inflammation

1. Autoimmune diseases: Illnesses like rheumatoid arthritis, lupus, and multiple sclerosis, in which the immune system assaults healthy tissues.
2. Chronic infections: Ongoing infections, such as tuberculosis, HIV, or hepatitis, that trigger a persistent inflammatory response.
3. Environmental exposures: Prolonged exposure to pollutants, such as air pollution or pesticides, can lead to chronic inflammation.
4. Metabolic disorders: Conditions such as obesity, diabetes, and metabolic syndrome, which are characterized by chronic inflammation [10]

Objectives

1. Protection:
Protect the body from harm caused by injury, infection, or damage.
2. Elimination of Harmful Stimuli:
Remove the underlying cause of inflammation, such as pathogens, toxins, or foreign substances.
3. Tissue Repair:
Initiate the healing process by promoting tissue repair and regeneration.
4. Restoration of Homeostasis:
Restore normal tissue function and maintain homeostasis.
5. Defense Against Infection:
Prevent the spread of infection and defend the body against pathogen [13]

Inflammatory Mediators:

Chemical / Biological causes known as inflammation mediators encourage and control the inflammatory response. Key mediators of inflammation include the following :

1.Cytokines: Small proteins that facilitate communication between immune cells, including IL-6, TNF-α, IL-1β, and IL-12. They play crucial roles in initiating inflammation, inducing fever, and activating immune responses.
2.Chemokines: Small proteins that attract immune cells to sites of inflammation. Examples include CCL2, CCL5, and CXCL8. Their roles involve enhancing inflammation and gathering immune cells.
3.Prostaglandins: Lipid molecules that exacerbate pain and inflammation. PGE2 and PGD2 are two examples. Its functions include causing pain, inflammation, and fever.
4.Leukotrienes: Lipid compounds that encourage bronchoconstriction and inflammation. For instance, LTB4 and LTC4. Its functions include causing bronchoconstriction, promoting inflammation, and recruiting immune cells.
5.Histamine: Described as a biogenic amine that stimulates allergic reactions and inflammation. Its functions include causing smooth muscle contraction, promoting inflammation, and increasing vascular permeability.
6.Bradykinin: A peptide that increases pain and inflammation.
Its functions include causing pain, promoting inflammation, and increasing vascular permeability.
7. Nitric Oxide (NO): This gas molecule stimulates immunological responses and inflammation.
Its functions include vasodilation, inflammatory promotion, and immune cell activation.
8. ROS, or (Reactive oxygen species): Extremely reactive substances that encourage tissue damage and inflammation.
Its functions include causing tissue damage, promoting inflammation, and activating immune cells [6][8][10]

Mechanism of inflammation:

Pattern recognition receptors (PRRs) are specific transmembrane proteins that allow host cells to identify inflammation-inducing factors for the first time. Immune system cells, both innate and adaptive, produce these. Essential to the genetic code, PRRs play a critical role in identifying dangerous pathogens and assessing the extent of tissue damage. Known as pathogen-associated molecular patterns (PAMPs), these receptors identify common characteristics of pathogens and danger-associated molecular patterns (DAMPs), which are internal components released from injured tissues. Various kinds of pattern recognition receptors (PRRs) that are capable of specifically identifying PAMPs, DAMPs, or both have been found to date. Included in this category are Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-1-like receptors (RLRs), and C-type lectin receptors (CLRs). These receptors send messages to the nucleus when they detect the right signals, which causes transcriptional and post-transcriptional processes to activate specific genes.

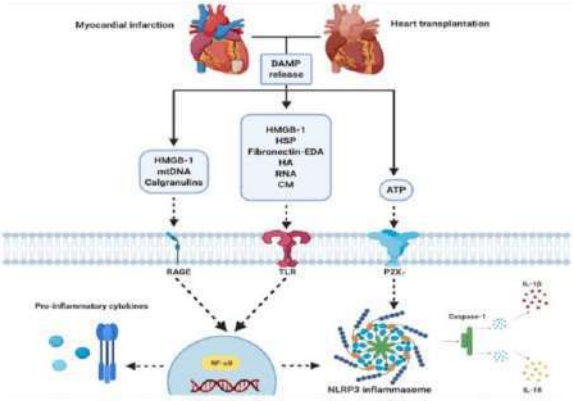

Figure: Mechanism of Inflammation

Anti-Inflammation Action

Classification:

A. Non-specific COX inhibitors (conventional NSAIDs)

1. Salicylates: Acetylsalicylic Acid.
2. Derivatives of propionic acid: Naproxen, Ibuprofen.
3. Fenamate: Mephenamic acid.
4. Derivatives of enolic acid: Piroxicam, Tenoxicam.
5. Derivatives of acetic acid: Indomethacin, Ketorolac.
6. Pyrazolone compounds: Phenylbutazone, Oxyphenbutazone.

B. Selective COX-2 inhibitors

Nimesulide, Diclofenac.

C. Selective COX-2 inhibitors

Celecoxib, Etoricoxib.

D. Analgesic - antipyretics featuring limited anti-inflammatory properties

1. Para amino phenol derivatives: Acetaminophen (Paracetamol).
2. Derivatives of pyrazolone: Metamizole (Dipyrone), Propiphenazone.
3. Derivatives of Benzoxazocine: Nefopam [15]

➢ **Mode of action**

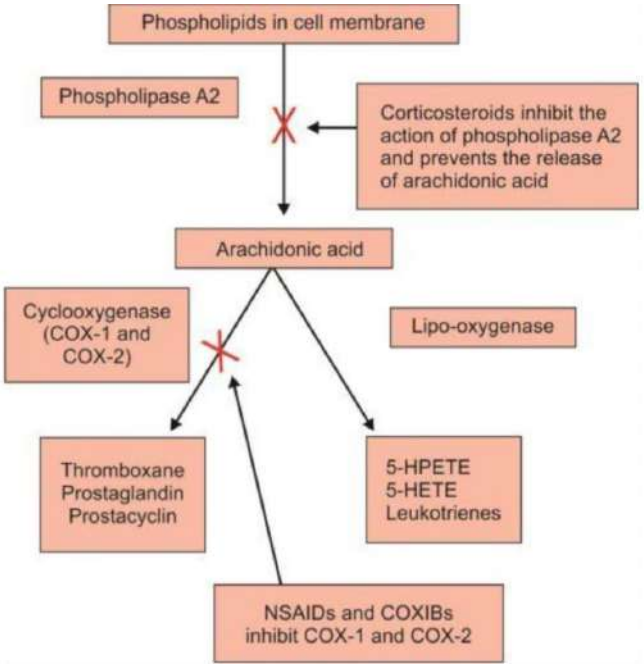

Figure: Mode of action on Anti-Inflammatory Drugs

The main way NSAIDs function is by blocking the cyclooxygenase (COX) enzyme. Cyclooxygenase is necessary to convert arachidonic acid for the production of prostacyclins, prostaglandins, and thromboxanes. The absence of these eicosanoids is thought to contribute to the advantageous effects of NSAIDs.

COX-1

The body always contains the constitutive isoform of COX. It supports renal function, platelet aggregation, and the lining of the gastrointestinal system.

COX-2

the COX isoform that can be induced, which is generated in reaction to inflammatory stimuli. It exacerbates inflammation-related discomfort and oedema.

In-vitro modules of Anti- Inflammatory Activity:

To Perform and Evaluate Anti Inflammatory Study by Using In Vitro Anti-Oxidant Model For Some Herbal Extract

1. Egg Albumin Denaturation Assay
2. Anti-proteinase action
3. Heat Induced Hemolysis
4. Anti-lipoxygenase activity
5. Hypotonicity-induced hemolysis

1. Egg albumin denaturation assay:

The primary objective of the egg albumin denaturation test is to determine whether specific compounds or materials can stop or retard the denaturation process of egg albumin under specific circumstances. When a protein undergoes denaturation, its structure is altered and its biological activity is lost. When exposed to high temperatures, pH extremes, or different denaturing agents, the experiment's model protein, egg albumin, undergoes denaturation. During denaturation, the original structure of egg albumin is altered, affecting its physical properties and lowering its functional activity. To determine a drug's anti-inflammatory properties, the egg albumin denaturation test assesses the substance's ability to stop or lessen egg albumin denaturation.

Based on the theory that compounds with anti-inflammatory properties may stabilize protein structures and stop denaturation, which is frequently connected to inflammation and tissue damage, the egg albumin denaturation test was developed. Chemicals or agents that considerably reduce the denaturation of egg albumin in this test may therefore have anti-inflammatory qualities. Denaturation of proteins is a possible cause of inflammation. NSAIDs prevent denaturation of proteins while blocking the COX enzyme. Under controlled experimental conditions, the various test sample concentrations can be incubated with the egg albumin solution to allow for the reactions to occur. The absorbance can then be measured to determine the percentage inhibition.

Procedure:

The anti-inflammatory qualities of the uncharacterized crude extracts allow for an in vitro evaluation of their capacity to stop the denaturation of egg albumin (a protein). 2 mL of the sample extract or standard (Diclofenac sodium) at different concentrations, 2.8 mL of phosphate-buffered saline (pH 7.4), and 0.2 mL of a 1-2% egg albumin solution (obtained from a fresh hen's egg or commercially available egg albumin powder) are combined to create a reaction mixture that has a volume of 5 mL. For the control, mix 2 mL of triple-distilled water, 0.2 mL of a 1-2% egg albumin solution, and 2.8 mL of phosphate-buffered saline to make a final volume of 5 mL. After that, heat the reaction mixtures in a water bath at 70±2°C for 15 minutes after maintaining them at 37±2°C for 30 minutes. After cooling, triple-distilled water was used as a blank and the absorbance was measured at 280 nm using an appropriate UV/Vis spectrophotometer [16]

$$\%\mathbf{inhibition} = [\frac{\{\mathbf{Abs\ control} - \mathbf{Abs\ sample}\}}{\mathbf{Abs\ Control}}] \times \mathbf{100}$$

2. Antiproteinase action:

The process was performed with specific modifications in accordance with the recommendations of Sakat et al. and Oyedepo et al. [9,10]. 2 milliliters of a solution were made by mixing 1 milliliter of a 1 mM Tris HCl buffer (pH 7.4), 0.001% trypsin, and 1 milliliter of a test sample at different concentrations (100–500 µg/ml). After five minutes of being held at 37°C, 1 milliliter of 0.02% (w/v) casein was added. At the same temperature, the mixture was then left to incubate for a further twenty minutes. The reaction was stopped by adding 2 milliliters of 2% perchloric acid.
After centrifuging the collected cloudy solution, the absorbance of the clear liquid—using the buffer as a reference blank—was measured at 210 nm. Next, the proteinase inhibitory activity proportion was evaluated [17]

$$\%\textbf{inhibition} = [\frac{\{\textbf{Abs control} - \textbf{Abs sample}\}}{\textbf{Abs Control}}] \times \textbf{100}$$

3. Heat Induced Hemolysis

Preparation of Erythrocytes (RBCs)
The centrifuge tubes were filled with heparin and a fresh 10 ml sample of whole human blood. The tubes were rinsed three times with the same volume of regular saline after centrifuging them for ten minutes at 3000 rpm. Normal saline was used to make a 10% v/v suspension after the blood volume was measured and
adjusted.

Procedure:

The 2 ml reaction mixture contained 1 ml of 10% RBC suspension and 1 ml of test extract at various concentrations; saline was used in place of the medication in the control test tube. Sodium diclofenac was the standard medication. The reaction mixture-filled tubes were all submerged in a water bath set at 56 degrees Celsius for 30 minutes. Following the incubation period, the tubes were left to cool under running water from the faucet. After centrifuging the mixture for five minutes at 2500 rpm, the absorbance of the resultant supernatant was measured at 560 nm. There were three runs of the experiment. Using the formula below, the percentage of membrane stabilization activity was calculated [16] [17]

$$\%\textbf{inhibition} = [\frac{\{\textbf{Abs control} - \textbf{Abs sample}\}}{\textbf{Abs Control}}] \times \textbf{100}$$

4. Anti - lipoxygenase activity:

To investigate the effectiveness of anti-lipoxygenase, linoleic acid was used as the reactive component and lipoxidase as the catalyst. Samples were prepared in 0.25 ml of a 2M borate buffer at pH 9.0 and mixed with a solution of lipoxidase enzyme (20,000 U/ml). The mixtures were then kept at 25°C for five minutes. After 1.0 ml of a 0.6 mM linoleic acid solution was thoroughly mixed, the absorbance was measured at 234 nm. The standard used as a benchmark was indomethacin [18].

$$\%\textbf{inhibition} = \left[\frac{\{\textbf{Abs control} - \textbf{Abs sample}\}}{\textbf{Abs Control}}\right] \times \textbf{100}$$

5. Hypotonicity - induced hemolysis:

Phosphate buffer at pH 7.0 (1 ml), diluted saline (2 ml), a red blood cell suspension (0.5 ml), extracts at varying concentrations (100–500 µg/ml), a reference sample (diclofenac sodium at 100 µg/ml), and a control were all added separately to each component. At 37°C, the reaction mixtures were incubated for 30 minutes. They were centrifuged at 3000 rpm after that. The absorbance at 560 nm was measured following the meticulous isolation of the supernatant. It was assumed that the control was 100% when calculating the hemolysis percentage. [19].

$$\%\textbf{Protection} = \textbf{100} - \frac{\textbf{OD sample}}{\textbf{OD control}} \times \textbf{100}$$

CONCULSION:

Inflammation acts as a crucial biological response to injuries, infections, or harmful stimuli; however, chronic or excessive inflammation is a significant characteristic of several conditions, such as rheumatoid arthritis, heart diseases, and cancer. Laboratory-based anti-inflammatory models are essential for investigating the molecular mechanisms linked to inflammation and for assessing possible therapies. These models include various approaches like molecular, biochemical, and cellular assays that illuminate inflammatory pathways, such as cytokine signaling, COX enzyme inhibition, and reactive oxygen species activity. They also enable the evaluation of anti-inflammatory properties in both synthetic and natural compounds. Widely utilized methods such as the egg albumin denaturation assay, antiproteinase activity assay, and heat-induced hemolysis assay are acknowledged techniques for assessing anti-inflammatory effects. Each of these approaches highlights

specific inflammation mechanisms, such as stabilizing proteins, inhibiting proteolytic enzymes, and protecting cellular health during stressful conditions. Employing in vitro methods provides benefits like lower expenses, ethical factors, and the capacity to control experimental conditions accurately. However, these models have limitations, especially their failure to fully replicate the intricacies present in living organisms, requiring the incorporation of various in vitro methods for a comprehensive understanding of anti-inflammatory mechanisms. Future research might include novel techniques like three-dimensional cultures, organoids, and CRISPR gene editing to enhance these models. This will enhance their accuracy and significance in drug development and the formulation of customized therapies for inflammatory conditions.

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