EFFECT OF SELECTIVE SODIUM GLUCOSE TRANSPORTER (SGLT -2) INHIBITORS - DAPAGLIFLOZIN AND CANAGLIFLOZIN ON ACUTE & SUBACUTE MODELS OF INFLAMMATION IN MALE WISTAR RATS – AN EXPERIMENTAL STUDY.

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ABBREVIATIONS

AA	Arachidonic acid
AGE	Advanced glycation end products
ANOVA	One way analysis of Variance
CNS	Central Nervous System
CV	Cardiovascular
COX	Cyclo-oxygenase
CPCSEA	Committee for the Purpose of Control and Supervision of Experiments on Animals
CRP	C-reactive protein
ELISA	Enzyme Linked Immuno Sorbent Assay
DM	Diabetes Mellitus
GM-CSF	Granulocyte macrophage colony stimulating factor
GLUT	Glucose transporter
H & E	Haematoxylin and eosin
HDL-C	High Density Lipoprotein Cholesterol
H_2O_2	Hydrogen peroxide
HOCl	Hypochlorite
5-HT	5-Hydroxytryptamine
Hsp	Heat shock protein
IAEC	Institutional Animal Ethics Committee

ICAM-1	Interstitial Cellular Adhesion Molecules-1
IFN – γ	Interferon – gamma
IgE	Immunoglobulin E
IL	Interleukin
IRS-1	Insulin receptor substrate 1
JNK	C-jun N-terminal kinase
LT	Leukotriene
MCP	Monocyte Chemoattractant Protein
MIP	Macrophage Inflammatory Protein
MIF	Macrophage Migration Inhibition Factor
NF-Kb	Nuclear factor kappa B
NO	Nitric Oxide
NOS	Nitric oxide synthase
NOX-4	NADPH oxidase 4
NSAID	Non Steroidal Anti-inflammatory Drug
OH	Hydroxyl radicals
PAF	Platelet activating factor
PCT	Proximal Convoluted Tubule
PG	Prostaglandins
PGE2	Prostaglandin-E2
PDGF	Platelet Derived Growth Factor

PGF-2α	Prostaglandin F – 2 alpha
PGI_2	Prostacyclin
RANTES	Regulated on activation Normal T Cell Expressed and Secreted
ROS	Reactive oxygen species
SGLT	Sodium glucose cotransporter
SEM	Standard Error of Mean
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
TGF	Transforming growth factor
TNF-α	Tumour necrosis factor alpha
TXA_2	Thromboxane A_2
TZD	Thiazolidinediones
UV	Ultraviolet

ABSTRACT

INTRODUCTION

The primary objective of the presentstudy was to determine the effect of selective sodium glucose transporter (SGLT -2) inhibitors - dapagliflozin and canagliflozin on acute as well as subacute models of inflammation in male Wistar rats. The secondary objective was to study the effect of these drugs on inflammatory markers like TNF – α, IL-6.

Materials and Methods:

Two models of inflammation were used:

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

Animals were divided into various, (n=6 in each) that received orally either vehicle, aspirin or subcutaneously any one of the test drug in clinically equivalent doses. Aspirin was given half an hour before, dapagliflozin and canagliflozin were given 1 hour before carrageenan injection respectively. The volume of edema was measured in millilitres (ml) with the help of digital plythesmometer at different hours and the change in the volume of edema in different treatment groups was estimated.

In subacute model of inflammation, two sterile cotton pellets each of l0mg weight and grass piths measuring 25x2mm were randomly implanted subcutaneously, in the axilla and groin, through a small incision under thiopentone anaesthesia. The treatment was started on the day of implantation and was given every twenty-four hours for ten days in control, aspirin, dapagliflozin and canagliflozin group .

On day eleven, 5ml blood was obtained through cardiac puncture for estimation of inflammatory cytokines like TNF-α and IL-6. The rats were sacrificed to obtain cotton pellets and grass piths. Mean granuloma dry weight of cotton pellets for different treatment groups was measured and percentage inhibition of granuloma dry weight was calculated. The sections of grass piths were stained with haematoxylin and eosin (H & E) for histopathological studies.

Results:

Dapagliflozin and canagliflozin did not reduce significantly the carrageenan induced inflammation in acute model. In subacute model, significant reduction in granuloma dry weight and inflammatory markers was not observed in the treatment groups. Also the treatment with dapagliflozin and canagliflozin did not decrease the granulation tissue formation, fibroblasts and collagen as revealed by Haematoxylin and Eosin stain.

Conclusion:

The present study exhibited dapagliflozin and canagliflozin did not exert significant anti-inflammatory effect in acute and subacute models of inflammation. The anti-inflammatory effect seen in previous studies could be attributed to their hypoglycemic effect and not direct anti-inflammatory effect. However, these findings need to be confirmed in inflammatory models in diabetic animals.

Key words: dapagliflozin, canagliflozin, aspirin, inflammation.

CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF GRAPHS

INTRODUCTION

Inflammation is a protective response, to get the body rid of microbes and toxins. The process of inflammation is brought about by vascular and cellular events, the former contributing maximum for the pathogenesis of acute inflammation.[1]However, in some situations inflammation is harmful and if inadequately controlled or directed against self tissues, can become the main cause of injury and disease.[1] In this regard, chronic inflammation is a characteristic of various conditions like metabolic syndrome, cardiovascular disease, obesity and diabetes mellitus (DM).[2] Prevalence of Diabetes Mellitus is increasing all over the world with a greater incidence of Type2 Diabetes Mellitus (T2DM). Factors such as increasing obesity, reduced activity with more industrialization, and aging of the population plays a very important role.[3] T2DM is characterized by different degrees of insulin resistance and the relative deficiency in its secretion. The causes ranges from reduced insulin-receptor binding, number of insulin receptors and even the failure of glucose reabsorption by a specific protein carrier like the glucose transporter 2 (GLUT2). Insulin resistance has also been attributed to activation of adipose tissue and associated with release of more inflammatory cytokines such as Tumour Necrosis Factor-α (TNF-α), Interleukin-6 (IL-6) and reduced production of anti-inflammatory Interleukin-10 (IL-10) by lymphocytes and macrophages.[4]

The growing body of evidence is emerging to show that Metabolic Syndrome, T2DM, Cardiovascular Disease and Microvascular diabetic complications (retinopathy, nephropathy) are mainly related to chronic inflammation. These observations have made the way for development of new pharmacological strategies which aim at reducing the silent inflammation. Interestingly, besides the specific anti-

inflammatory agents, the glucose-lowering drugs like Thiazolidinediones (Tzds) and Metformin also exerted anti-inflammatory effects that could contribute to the better outcomes in diabetic patients.[5]

Persistent hyperglycaemia is known to accelerate the formation of Advanced Glycation End products (AGEs) that induces the production of reactive oxygen species (ROS) and pro-inflammatory markers like TNF-α and IL-6. These in turn are responsible for release of potent inflammatory markers like C - reactive protein (CRP).[6] In addition, ROS and inflammation by themselves play an important role in initiating DM by virtue of mitochondrial dysfunction leading to insulin resistance.[6]Therefore, it could be hypothesised that therapeutic agents that can target hyperglycaemia and inflammation might be beneficial in the management of diabetes and its complications. Interestingly, a novel group of antidiabetic drugs, Sodium Glucose Co-transporter -2 (SGLT-2) inhibitors are reported to exert dual benefit of controlling hyperglycaemia and hyperglycaemia induced inflammation. SGLT-2 is responsible for approximately 90% of the glucose reabsorption in the proximal convoluted tubules of the kidney. SGLT2 inhibitors like Dapagliflozin, Ipragliflozin, Canagliflozin, Empaglifozin etc improve glycemic control independent of insulin secretion or action, by reducing the reabsorption of filtered glucose and increasing glucose excretion in the urine[7]. In addition, they are known to augment the functioning of pancreatic, liver and other cells of the body by down-regulating AGEs-mediated pathways[8] In addition, Canagliflozin, a drug from the same class has been reported to decrease inflammatory cytokine levels and macrophage accumulation in obesity related inflammation.

Dapagliflozin, the drug selected for the present study has been reported to exert dose-dependent glucosuria in healthy volunteers and in short-term (2- to 12-week) studies in patients with T2DM. It has been reported to exert renoprotective effects by lowering the glucose and also by reducing the oxidative stress in diabetic kidney.Dapagliflozin reduces oxidative stress by reducing the Nox4-derived ROS generation and hyperglycaemia induced ROS generation in db/db mice.[9]

Similarly, Canagliflozin has been reported to reduce hyperglycemia as well as blood pressure, body weight, albuminuria in diabetic people. Canagliflozin demonstrated suppression of inflammatory responses in skeletal muscle of high fat diet induced inflammation in mice. This action was attributed to increased glucose excretion and decreased hyperglycemia by canagliflozin.[10] However, there are no reports of intrinsic anti-inflammatory effects of these group of drugs.Therefore, in view of support that SGLT2 inhibitors suppress hyperglycaemia induced inflammation and scarcity of information regarding their actual effect on inflammation, the present study has been planned to evaluate anti inflammatory effects of selective SGLT2 inhibitors viz, Dapagliflozin and Canagliflozin on inflammation in male Wistar rats.

OBJECTIVES

Primary Objective

- To assess the effect of Dapagliflozin and Canagliflozin on-
- Carrageenan induced rat paw edema model of acute inflammation and
- Foreign body induced granuloma model of subacute inflammation in male Wistar rats.

Secondary Objective

- -To assess the effect of Dapagliflozin and Canagliflozin on the levels of inflammatory parameters -TNF- α, IL-6.

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REVIEW OF LITERATURE

A. INFLAMMATION:

History of inflammation

The word inflammation is derived from Latin word inflammare (i.eto set on fire).[11]A Roman writer, Aulus Celsus first documented (in 1st century AD) the fourcardinal signs of inflammation i.erubor (redness), tumor (swelling), calore (heat), dolore (pain).[12]Rudolf Virchow added a fifth sign called **functiolaesa** (i.e loss of function) to the four cardinal signs of Celsus. This brief description about inflammation made the researchers avid for more information leading to the present status of knowledge regarding inflammation.[13]A Scottish surgeon by name John Hunter, in 1793 has promulgated that inflammation is anonspecific response having beneficial effect on its host and not on the disease.[1] Thereafter, Julius Conheim postulated the association of inflammation with the emigration of leucocytes through microvasculature walls.[13] In 19th century, a Russian zoologist Elie Metchnikoff discovered the process of phagocytosis. Ultimately, he concluded that the purpose of inflammation is to bring the phagocytic cells to the area of injury for engulfing the bacteria which invades and prevailing theory was contradicted that the purpose of inflammation was to bring the factors in serum form that neutralizes the infectious agents.[1]Later, it became clear that both, the serum factors (antibodies) and the cells (phagocytes) were critical for defence against the microorganisms, and in the recognition of this, sharing the Noble Prize in 1908 by Paul Ehrlich (one who developed the humoral theory of immunity) and Metchnikoff.[14]Finally, Sir Thomas Lewis, in 1927, revealed that histamine was produced locally in response to injury, which lead to leukocyte migration into the extra vascular spaces by increasing the

vascular permeability.[1] This principal concept formed the basis of significant discoveries of different chemical mediators of inflammation and application of anti-inflammatory drugs in clinical medicine.[1]

Pathogenesis of inflammation

Inflammation is a local response of living mammalian tissues to injury due to any agent. It is a body defense mechanism in order to eliminate or to limit the spread of injurious agent, followed by removal of the necrosed tissues and cells.[15]

The agents of inflammation are: [15]

1. Immunological agents like antigen- antibody reactions and cell-mediated reactions.
2. Infective agents like viruses, bacteria and their toxins, fungi, parasites.
3. Chemical agents like organic, inorganic poisons.
4. Physical agents like heat, radiation, cold and mechanical trauma.
5. Inert materials like foreign bodies.[15]

Types of Inflammation – Based on the mode of onset and duration, inflammatory reaction are classified as acute, sub-acute or chronic.

Acute inflammation

It is characterized by sudden, rapid onset and it a short duration of action (few minutes to several hours). The signs of inflammation are usually accompanied with constitutional symptoms. The exudation of plasma, fluid and leukocytic emigration constitute the microscopic picture of acute inflammation.[16]

Sub-acute inflammation

It is the period between acute and chronic inflammation. It usually persists for two to six weeks. Vascular exudative changes of acute inflammation and proliferative changes of chronic inflammation are its main characteristics. Microscopically it is characterized by the exudate which consists mainly of lymphocytes, eosinophils, histiocytes, plasma cells and fibroblasts.[16]

Chronic inflammation

It is also called as insidious, long-term inflammation which lasts for several months to years. It consists of proliferation of blood vessels and connective tissue, lymphocytes, histiocytes and plasma cells, but absence of polymorphs.[16]Regardless of the type of injury, pathogenesis of acute inflammation comprises of:

I. Vascular changes

II. Cellular events

I. Vascular changes:

It is the alteration in the microvasculature (arterioles, capillaries and venules) and is the earliest response to tissue injury. They include haemodynamic changes and changes in vascular permeability.

Haemodynamic changes: Regardless of the type of injury, the immediate and an instant response is transient vasoconstriction, followed by sustained progressive vasodilation. The progressive vasodilation, may elevate the local hydrostatic pressure which results in transudation of fluid into the extracellular space. This results in swelling at the site of acute inflammation. Stasis or slowing is ensued by peripheral

orientation of leucocytes or leucocytic margination along the vascular endothelium. Leucocytic adherence to the vascular endothelium occurs briefly, which is followed by their migration between the endothelial cells into the extravascular space. This is called as emigration.[15]

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

II. Cellular events:

The cellular inflammatory phase consists of two processes:[15,16]

1. Exudation of the leucocytes
2. Phagocytosis.

1.Exudation of the leucocytes:

The sequence of events that takes place in the recruitment of leukocytes from vascular lumen to the extravascular space are

a) Margination, adhesion to endothelium, and rolling along the vessel wall;
b) Firm adhesion to the endothelium;
c) Transmigration between endothelial cells; and
d) Migration in interstitial tissues towards a chemotactic stimulus".[1]

Rolling, adhesion, and transmigration are mediated by binding of complementary adhesion molecules on endothelial surfaces and leukocytes.

Modulation of surface expression or avidity of the adhesion molecules affected by chemical mediators (chemoattractants) and some cytokines stimulates the directional movement of the leukocytes.[1,17,18]

2. Phagocytosis

Phagocytosis of particles is a beginning step in the elimination of harmful substances. It includes engulfment of invading organism, production of substances which destroy the phagocytosed microbes and remove the dead tissues; the leukocyte products include lysosomal enzymes, reactive oxygen and nitrogen species. "Phagocytosis consists of three steps:

a) Recognition and attachment of the particle to the ingesting leukocyte;
b) Engulfment, with subsequent formation of a phagocytic vacuole; and
c) Killing and degradation of the ingested material".[19]

Termination of acute inflammation

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

A. Resolution.
B. Healing by formation of scar with or without regeneration of lost parenchymal cells.
C. Suppuration.
D. Acute course may get converted to a chronic one.[15]

Molecular basis of inflammatory events

Chemical mediators:

Inflammation involving the cytokines and other mediators, are derived locally by cells at the site of inflammation, or may be circulating in the plasma as inactive precursors. These are activated in response to an etiological factor at the site of inflammation. Though their duration of action is short, due to an efficient disposal by catabolic enzymes, they may be produced continuously due to the persistence of causative factors.[1]These mediators are grouped into various categories, namely:

1. Vasoactive amines
    a. Histamine
    b. Serotonin
2. Membrane derived lipid substances
    a. Eicosanoids
    b. Prostaglandins
    c. Leukotrienes
3. Kinins
    a. Bradykinin and related kinins
    b. Tachykinins
4. Clotting system
5. Complement system
6. Lysosomal proteases
7. Cytokines
8. Biologically derived oxidants
    a. Hydroxyl radicals (OH^-)

b. Hydrogen peroxide (H_2O_2)

c. Hypochlorite (HOCl)

d. Superoxide anion radical (O_2^-)

9. Nitric Oxide (NO)

10. Others

Histamine:

Histamine, a widely distributed endogenous vasoactive amine is found in most of the tissues and in the physiological fluids, the richest sources are the mast cells adjacent to blood vessels, platelets, basophils and cerebrospinal fluid. It is one of the first mediators to be released when there is injurious stimuli, which is responsible for immediate phase of increased vascular permeability and it acts on microcirculation via H_1receptors. It causes arteriolar dilation and increase in vascular permeability of venules, endothelial contraction and increases the spaces of inter-endothelial junctions. Thus, histamine contributes to edema formation and appears to be an important mediator in acute inflammatory reaction.[1, 20, 21]

5-Hydroxytryptamine (Serotonin, 5HT):

5-Hydroxytryptamine is an endogenous biogenic amine having potent effects on smooth muscles and blood vessels. It is widely distributed in many plants and tissues and is highly concentrated in mammalian platelets, pineal gland and enterochromaffin cells. Serotonin release from the platelets are stimulated by platelet activating factor (PAF) which leads to vasodilation mainly through $5HT_1$ receptors by

1. Inhibiting the noradrenaline release from sympathetic nerve terminals.
2. A direct smooth muscle relaxant action and
3. Acting on endothelial cells, they release nitric oxide which relaxes smooth muscles.

Being an acute inflammatory mediator, it is known to increase capillary permeability.[1]

Kinins:

These are very much potent polypeptides that are released from the cleavage of α_2 globulin, fraction of plasma kininogen. This normally, an inactive system, which is triggered by the contact activation of Hageman factor and releases bradykinin.[22] It is a potent vasodilator which induces the formation of edema, evokes pain and reflexes by acting on nerve endings.[23,24]

Clotting System:

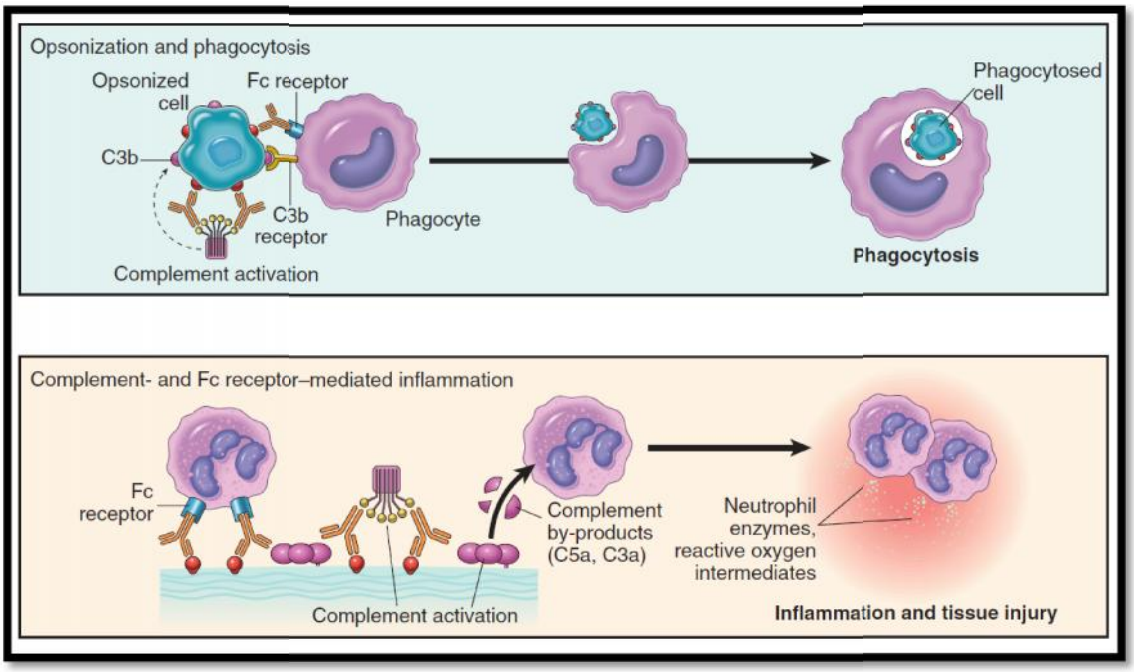
The clotting system is mainly activated by the Hageman factor. Although the primary function of the clotting system is to maintain vascular integrity, it has also a very important role in inflammation and microbial resistance. Fibrinopeptides that are released during conversion of fibrinogen to fibrin contribute significantly to the inflammatory reaction by their chemotactic property.[25]

Complement system:

The complement system is an essential part of the humoral defense mechanism of the body. It is also a primary mediator of the inflammatory process. It designates a major complex of plasma proteins, which consists of a group of proteases that circulate in the form of zymogens. They interact sequentially to affect different inflammatory events, either directly or through interaction with other factors.

The of components Complement system (C1-C9)are basically present in an inactive form in the plasma and advancement of the biologic function of complement components leads to an increased vascular permeability, chemotaxis and opsonization. C5a is generated in extra-vascular tissue fluid in response to inflammatory stimulus (Figure-1).[26,27] C3a, C5aand also C4aare known to increase the vascular permeability and causes vasodilation by releasing histamine from mast cells.

FIGURE : 1



Schematic presentation of the complement pathways and some of the biological activities mediated by the complement.[15]

Lipid derived mediators (The arachidonic acid metabolites) (Eicosanoids)

The arachidonic acid metabolites, the most studied lipid mediators are mainly involved in the inflammatory response which has capacity to generate different forms of Arachidonic Acid (AA) derivatives like thromboxane A_2 (TXA_2),prostaglandins

(PG), Leukotrienes and prostacyclin (PGI_2). The AA is metabolized by two pathways- the cyclooxygenase pathway and lipoxygenase pathway. The cyclooxygenase pathway give rise to prostaglandins and the lipoxygenase pathway gives rise to leukotrienes (LTs). While the cyclooxygenase enzyme which is found in many tissues, the lipoxygenase enzyme is mainly found in leucocytes and their derivatives.[22]

Prostaglandins:

During the inflammatory process, prostaglandins (PGs) which are generated at the site of inflammation and causes hyperalgesia. PGE_2, which is a potent pyrogenic substance that is known to produce vasodilation, increases the blood flow and synergizes with the other factors to increase vascular permeability and causes hyperalgesia. Also, it potentiate the action of kinins and increases the vascular permeability. Similarly, PGI_2 also causes vasodilation and pain. It potentiates the carrageenan induced edema and hyperalgesia in the rats, while in the rabbit skin it induces hyperaemia and augments the plasma exudation in response to permeability inducing stimuli like bradykinin. Thromboxane A_2 that causes platelet aggregation and vasoconstriction is a highly unstable product and gets rapidly converted to thromboxane B_2. It is mainly derived from the platelets.[22]

Leukotrienes:

The products of lipoxygenase pathway, mediates virtually almost each step of inflammation. Rather than being preformed, leucotriens are produced in the mast cells. Hence, considered as secondary mediators. LTB4, LTC_4, LTD_4 and LTE_4 are different types of leukotrienes, derived from the common precursor, LTA_4, LTB_4 is a potent inflammatory mediator.[28,29]

Platelet activating factor (PAF):

A mediator derived from phospholipid, PAFis generated and released from the sensitized mast cells by immunoglobulin E (IgE) action. It is synthesized by monocytes, platelets, neutrophils, renal mesangial cells, eosinophils, medullary cells and vascular endothelial cells.[29]Cytosolic calcium increases with PAF due to the influx of calcium through the membrane bound calcium channels and also due to mobilization of intracellular calcium secondary to inositol 1,4,5, triphosphate. Chemotactic peptides, thrombin, collagen, autacoids stimulate its release. It is known to induce vasoconstriction. However, in low dose, it is known to cause vasodilatation leading to increase in venule permeability. It helps in leucocytic rolling, adhesion and migration through endothelial monolayer and it is chemotactic to monocytes, neutrophils and eosinophils.[1,30]

Cytokines:

Cytokines like TNF-α and β, interleukin-1 (IL-1) and interferon-γ are polypeptides and are soluble immunoglobulins which are produced by activated T-lymphocytes (lymphokines) and macrophages (monokines). They are the important cytokines that are involved in inflammation.[15]

Chemokines:

The chemokines are structurally related proteins which act as chemoattractants for various leukocytes, resulting in recruitment to the inflammation site. Chemokines binds to a specific G-protein-coupled receptors (eg: CXCR4 andCCR5) on their target cells. CC and CXC are two major groups of chemokines that play an important role in inflammation.[1]CXC chemokines have one amino acid that separates conserved

cysteines and primarily act on neutrophils. IL-8 which belongs to the same group is produced by activated mast cells, macrophages, endothelial cells and fibroblasts, in response to cytokines such as TNF and IL-1 and microbial products. CC chemokines have adjacent cysteine residues and it includes "monocyte chemoattractant protein 1 (MCP-1) , macrophage inflammatory protein 1α (MIP-1α) (both chemotactic predominantly for monocytes), RANTES (regulated on activation normal T cell expressed and secreted) which are chemotactic for monocytes, basoplhils, eosinophils, memory CD4+ T cells and monocytes".[1]

Nitric oxide (NO):

It is a highly reactive mediator, synthesized endogenously by L-arginine dependent enzyme, nitric oxide synthase (NOS).[31,32] Recently, some studies suggested that increase in NO release by activated macrophages may contribute to tissue injury and inflammation.[33,34]

Oxygen free radicals in biological systems:

These are not only toxic because of their intrinsic activity as inflammatory mediators and their ability to modulate inflammatory processes but, also due to their synergistic activity with serum proteases.[35, 36]

Neuropeptides:

Neuropeptides are a class of compounds which have capacity to modulate various events of inflammation. These are produced from neural and neuroendocrinal tissue. The mammalian tachykinins comprise three related peptides-neurokinin A, neurokinin B and substance P. Substance-P has been incriminated in nociception in rats and mice, and also in the early phase of carrageenan induced edema.[37] The

inflammatory response of substance-P suggests to be mediated by cholinergic neuronactivation or by histamine release.[38]

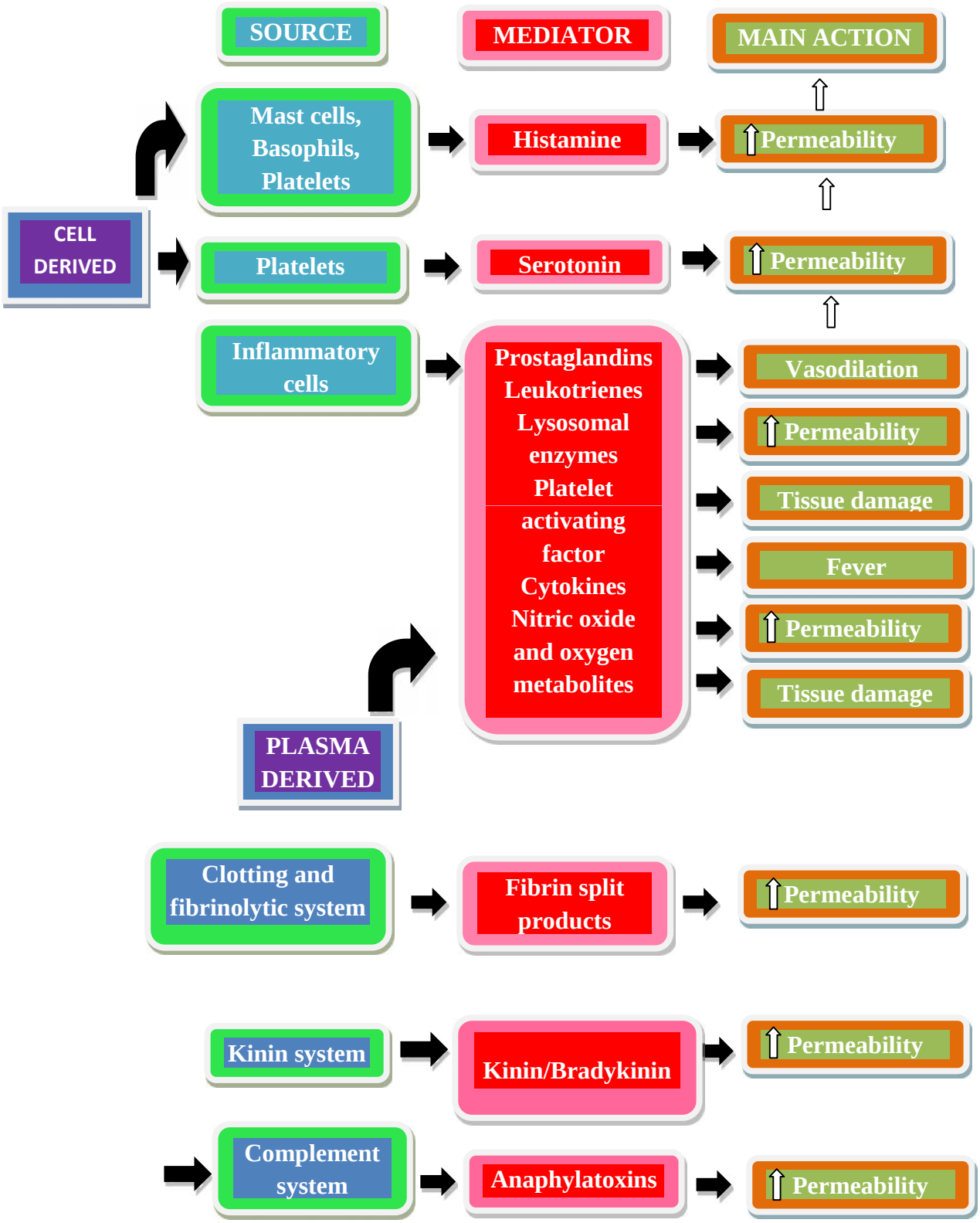
Lysosomal enzymes:

Lysosomes are known to release so many agents during inflammation like neutral proteases and acid proteases.[1]

Others:

There are various other exogenous and endogenous substances that are involved in the pathogenesis of inflammation. Growth factors like transforming growth factor (TGF) and platelet derived growth factor (PDGF) are chemotactic and resembles the cytokines in their functions.[39]

MEDIATORS OF INFLAMMATION[38]



B. SCREENING OF ANTI-INFLAMMATORY AGENTS

The widespread use of anti-inflammatory agents treating common clinical inflammatory conditions has evoked interest in their laboratory evaluation of laboratory. The various methods of evaluation has been described in text books.[40] A variety of models have been introduced to assess the anti-inflammatory activity of different drugs. They measures cardinal signs of inflammation viz. dolor (pain), rubor (redness), tumor (swelling),calor (heat) and functiolaesa (loss of function). Some methods work by modifying events in inflammation.

I. SCREENING METHODS BASED ON MODIFICATION OF SIGNS OF INFLAMMATION.

1. ERYTHEMA (Redness):

It is an early stage of an acute process of inflammation and is reproduced in guinea pigs by inducing cutaneous erythema using by Ultraviolet (UV) rays. The technique was first described by Adams in the year 1960.[40]

Albino guinea pigs are pretreated with test drug 30 min before exposing to UV rays for 2 minute to depilated skin. After 2 hours, the degree of erythema is visually estimated on a scale of 0-4, preferably by a blind observer. The main draw-back is the inability to get the accurate measurement of reaction and variability of response in animals. Indomethacin, Phenylbutazone and aspirin were found to be effective.[41]

2. EDEMA (Swelling):

This is one of the popular methods for anti-inflammatory agents screening. It is easily performed as a routine test, and is cheap and reproducible.

Pedal inflammation (edema) can be produced by various phlogistic (irritating) agents injected into the hind paw of the rat. Various irritants like mustard, dextran, carrageenan, egg-white, histamine, formalin, kaolin, compound 48/80, yeast, serotonin, substance-P, nystatin, cobra venom, glass powder, naphthoylheparamine etc have been used to induce edema. Carrageenan was used for the first time by E.A Risley, Winter C.A, and G.W.Nuss and is still the most commonly used irritanti.e 1% carrageenan in NS.[39] It is injected in the volume of 0.05 ml into one of the hind paws. The test drugs are given prior to carrageenan injection and paw edema is measured by using plethysmometer at regular intervals of time. Plethysmometer remains the most popular method because of its reliability and reproducibility of results. Though rats are commonly used, mice can be used for this experiment.[41]

Mouse ear edema test:

This method was described by Robson and Brown in 1964.Ear edema is produced by xylol application to mouse ear. Animals are sacrificed 30 minutes later and 6mm section of ear disc is obtained by ear punching and weighed. The edema index is calculated by difference in the weight between xylol treated and untreated ear and the edema is quantified.[42]

3. HEAT (Fever):

Brewer's yeast suspension is known to produce fever in rats. Administration of compounds having antipyretic activity reduces the temperature. Fever is produced in the animals by subcutaneous injection of 10ml/kg of Brewer's yeast on the back, below nape of the neck.The temperature is recorded by inserting thermocouple to 2 cm deep into the rectum. This method is called as classical method in pharmacology.[41]

4. PAIN:

Pain is a symptom of various diseases neccesitating treatment with analgesics. Pain can also be elicited by inflammation. Suppressing inflammatory pain indirectly indicates their anti-inflammatory activity. Various tests are employed to evaluate analgesic activity of the drug. The most commonly used method is Randall Selitto technique.[40]Inflammation is produced by injection of Brewer's yeast locally, mechanical pressure is applied over the rat paw. The study of response in healthy paw permits the direct comparison of the drug effect on inflammatory and non-inflammatory pain. The test appears to be more sensitive with carrageenan than brewer's yeast.[41]

Pain can also be induced by various stimuli like wet heat, dry heat, radiant heat, mechanical pressure, chemical irritants and by electrical stimulus. Various equipments like Eddy's hot plate, analgesiometer and Janssen's warm water bath[39] are used where direct heat, radiant heat, and wet heat are used as noxious stimulus respectively. Chemical irritants like benzoquinone, oxytocin, and hydrochloric acid are injected intraperitoneally which induces pain. Acetic acid is used to induce writhing.[40]

Use of mechanical pressure by putting bull dog clamp at the base of tail of rats and mice as described by Francheschni and Bianchi C, in 1954 is also used for rapid screening of analgesic drugs. Charlin used the pododolorimeter where electrical stimulus is applied to the foot pad of rats/mice to elicit the pain. The rectodolorimeter is used to apply stimulus to rectum. Electrical stimulus can also be applied to the incisor tooth pulp of guinea pig.[40]

Tail immersion method is used to assess analgesic activity. A cup of fresh water is maintained at 55°C. The lower part of the tail (5cm) is marked and is immersed in hot water and the the time taken from introducing the tail into the bath to the time taken for the withdrawal of tail i.e. reaction time is noted. An increase in the reaction time indicates analgesic activity. This method differentiates between centrally acting analgesics (opioids) and peripheral acting analgesics.[41]

In humans, analgesics are evaluated by different pain models like radiant heat, cold water stress, BP cuff inflation, electrically induced pain model,hand dynamometer, laser-induced pain model etc.[43]

5. LOSS OF FUNCTION:

In 1964, Weisinger D. employed rats with Mycoplasma L4 induced arthritis and measured the grip function of the inflammed articulation. Aminopyrine, Phenylbutazone, indomethacin and gold preparations were found to improve joint motility.

II. SCREENING METHODS BASED ON MODIFICATION OF EVENTS IN INFLAMMATION.

Burns of standard duration (27 seconds) and standard intensity (55°C) are produced in anaesthetized rats. The Shaved abdomen of the rat is placed in contact with end of a hollow brass cylinder which is closed, through which the thermostatically controlled water is circulated. Resultant edema is measured by weighing and excising a standard area of the skin.[44]

Leakage of circulating protein bound dye can be used to measure the drugs ability to suppress the endogenous mediators effect on vascular permeability. The

permeability enhancing substance, like histamine or bradykinin is injected intradermally into the flank or abdomen and the intensity or area of dye-staining is estimated. Quantitative estimation are done by excising the skin area and extracting the dye.

Arthusreaction is another test that helps to study the increased vascular permeability. The animals are actively sensitized by injecting egg albumin or horse serum or passively sensitized by giving corresponding antibodies. An intradermal injection of antigen is made and rapidly developing inflammation is assessed by estimating the edema or dye leakage. The technique was first described in 1960 by Marks V and Smith M.J.H.[44]

INFLAMMATORY EXUDATE FLUID

Pleurisy and peritonitis induced by irritant substances in rats permits the experimental reproduction of phenomenon which is more typically exudative. It is followed by second phase of granulation. Pleurisy and Peritonitis are produced by injecting 1 ml of 1.5% formalin, intraperitoneally, as described by Teotino et al. or by injecting Evans blue into the pleural cavity according to Weisbach et al. method of 1963. The animals are sacrificed after 4-8 hours and ascitic fluid /pleural fluid is collected and measured. Phenylbutazone, a dose of 100mg/kg reduces ascites fluid of 37%, 4 hours later and its administration 1 hour before the Evans blue dye administration reduces the pleurisy fluid volume compared to control animals.[40]

EXPERIMENTAL ARTHRITIS

Adjuvant arthritis has described by Wood and Pearson (1959), exhibits numerous similarities to human rheumatoid arthritis.[r-31]Formalin, aqueous suspension

of kaolin, mustard powder and mycobacterium suspended in heavy paraffin oil (Freund's adjuvant) etc. have also been tried. Freund's adjuvant method is the commonest technique used.[40]0.05 ml of Freund's adjuvant, when injected intradermally into the foot pad, beneath the plantar surface, produces local inflammatory lesions (primary lesions) after 3 to 5 days. Secondary lesions appear after 11 to 12 days in non-injected sites like hindleg, forepaws, nose, ears and tail.[41]Before giving the Freund's adjuvant, the foot pad thickness is measured by using calipers and compared with the thickness at the end of 13 days. The test drug is given everyday for 13 days.[40]

Drugs like phenylbutazone, indomethacin shows effect against primary lesions while it is not effective on secondary lesions. Immunosuppressants like cyclophosphamide inhibits secondary lesions.[41]

EMIGRATION OF LEUKOCYTES

Some of the models that have been described earlier appears to be less popular and include:-

a. An irritant/antigen is injected subcutaneously or intradermally into the sensitized animal and that area is to be excised six hours later for histological examination for quantifying the particular type of leukocyte.
b. Clark-Sandison rabbit ear chamber is used to study leukocytic adhesion to vascular endothelium and their subsequent emigration.
c. Boyden's method devised by M.R Smith, Alison F and W.B Wood, is used to study the rate of leukocytic movements.[44]

GRANULATION TISSUE FORMATION:

This method is used for testing the proliferative phase which was first described in 1950 by Meier et al. In male albino rats, under anaesthesia the prior sterilized cotton pellets of 7-10mg are implanted subcutaneously. Treatment is given everyday throughout the study. The granulomas are dissected on day five for the purpose of quantification. The cotton pellets are dried overnight at 60°C and weighed.[40]

However this technique was modified by using grass piths, plastic rods as foreign bodies and prolonging the study for 10 days.[45]Then the grass piths are stored in 10% formalin for subsequent microscopic studies.

GRANULOMA POUCH METHOD:

Granuloma are produced by injecting 25 ml air into the subcutaneous tissue of the rat dorsally, followed by chemical irritant like croton oil, given to the same site, by the Fisher J.W technique, 1961.On day 2 the air is removed by vacuum from the pouch. On the day 4, the pouch is opened and the exudative fluid is aspirated and the volume measured. Glucocorticoids inhibits the exudation of fluid. Inhibition of hyaluronidase described by Fabinyi-Szebehely et al. in 1953, is another method used but is very uncommon.[40]

C. ANTI-INFLAMMATORY DRUGS

Anti-inflammatory drugs can be classified as follows:

I. THE PROSTAGLANDIN INHIBITORS (NSAIDs)[46]

A. Non selective COX inhibitors (Conventional NSAIDs)

1. Propionic acid derivatives: Ibuprofen, Naproxen, Ketoprofen, Flurbirofen
2. Salicylates: Aspirin.
3. Fenamates: Mefenamic acid.
4. Acetic acid derivatives: Ketorolac, Indomethacin, Nabumetone.
5. Enolic acid derivatives: Piroxicam, Tenoxicam.
6. Pyrazolone derivatives: Phenylbutazone, Oxyphenbutazone.

B. Preferential COX-2 inhibitors

Aceclofenac, Diclofenac, Meloxicam, Nimesulide, Etodolac.

C. Selective COX-2 inhibitors

Etoricoxib, Celecoxib, Parecoxib

D. Analgesic-antipyretics with poor anti-inflammatory action

1. *Paraaminophenolderivative:*Paracetamol (Acetaminophen)
2. *Bezoxazocine derivative:* Nefopam
3. *Pyrazolone derivative:* Metamizol (Dipyrone), Propiphenazone

NSAID's exerts their anti-inflammatory effect by inhibiting cycloxygenase(COX) enzyme and blocking prostaglandins (PG) synthesis that lead to inflammation. Aspirin inhibits COX enzyme irreversibly by acetylating its serine residues; while other NSAIDs are competitive reversible inhibitors.

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

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

- Closure of ductusarteriosus.

These are the most common anti-inflammatory drugs used clinically and share certain common toxicities[47] like

- Bleeding-due to inhibition of platelet function.
- Asthma and anaphylactoid reactions in susceptible individuals.
- Gastric mucosal damage (more common with aspirin).
- Delay / prolongation of labor.
- Limitation of the renal blood flow, sodium and water retention.[46]

They act on both COX-1 and COX-2. Newer agents that have been developed are COX-2 selective.[46]They are Rofecoxib, Celecoxib etc. effective as analgesic and anti-arthritic agents but, are not devoid of specific side effects like raise in blood pressure when used in hypertensives and increased risk of cardiovascular problems.[22]

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

1. Steroids:[22,30,46]

- Decrease the adhesion by inducing the changes in inflammatory cells of membranes.
- Decrease the migration of neutrophils into inflammatory site.
- Modulation of adhesion molecule receptors.
- Decrease the response to chemotactic factor.
- Block the function of CD-4, IL-2 and helper T cells.
- However in chronic conditions, they cause various adverse reactions, that includes[22,46]
- Affect production of lymphokines.

- ❖ Blocks the release of IL-1, IL-6, TNF-α cytokines by inhibiting T-lymphocyte interactions and macrophage.
- ❖ Increase in susceptibility to infections.
- ❖ Delayed healing of surgical incisionsand wounds.
- ❖ Iatrogenic Cushings syndrome.
- ❖ Peptic ulceration.
- ❖ Osteoporosis.
- ❖ Suppression of hypothalamo-pitutary axis

2. Hydroxychloroquine:

Inhibits lysosomal enzymes,cytokine secretion and macrophage functions. But causes corneal opacityand reversible retinal damage.[46]

3. Gold Salts - Oral-Auranofin, Parenteral- Gold sodium thiomalate:

- ❖ Inhibits IL-2productionand T-cells proliferation. [46,48,49]
- ❖ Inhibition of protein kinase-C.[48]

But, these are known to cause [46]

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

4. Penicillamine:

- ❖ Inhibit helper T-cells and angiogenesis.
- ❖ Decreases the immune complex concentration in plasma synovial fluid.[22]

It is associated with various toxicities like:

- ❖ Bone marrow depression.
- ❖ Proteinuria, kidney damage.
- ❖ About half the patients develop anti nuclear antibodies.
- ❖ It also precipitates myasthenia gravis and systemic lupus erythematosus.[46]

5. Sulfasalazine:[22,46]

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

Some adverse effects reported are

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

6. Cytotoxic drugs:

a. Cyclophosphamide:[46]

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

However, it causes alopecia and cystitis.

b. Colchicine:

- ❖ Binds to microtubules that is necessary for cellular migration and mitosis.[22] Higher doses causes CNS depression, kidney damage, intestinal bleeding and death due to respiratory failure and muscular paralysis. Chronic

therapy is associated with agranulocytosis, aplastic anaemia, myopathy and loss of hair.[46]

c. Methotrexate:

- ❖ Inhibition of enzyme dihydrofolate reductase.
- ❖ Inhibits chemotaxis.

However, not recommended in patients with renal insufficiency, and also reported to cause nodulosis.[46]

1. Cyclosporine:

- ❖ Suppresses activation of T-cell by blocking the cytokine genes transcription such as IL-2.[50]
- ❖ Inhibits the transcription of TNF-α, IL-3, IL-4,Interferon (IFN) - γ, Granulocyte macrophage colony stimulating factor (GM-CSF) by activated T-cells.[46]

It is known to impair the renal function.

As most of the drugs having anti-inflammatory activity are used in the clinical practice that causes mild to severe adverse effects, the discovery for newer and safer anti-inflammatory drugs continues.

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

I. Xenobiotic immune suppressants:

Tacrolimus**:** Inhibits the transcription of IL-2, IL-4, IFN-γ, IL-3, TNF- α and GM-CSF.[51]

Rapamycin:Blocks the response that is induced by IL-2.[51]

Gusperimus:Binds to the regulatory C-terminal motif of heat shock 90 kDa protein (Hsp90) and heat shock 70 kDa protein(Hsp70) that reduces the nuclear trans localization of nuclear factor-κB (NF-κB) transcription factor, inhibiting the activation of T cells, B cells, dendritic cells and monocytes and affects the antigen presentation.[52]

II. Cytokine modulators:

1. Monoclonal antibodies to Cytokines, especially to IL-6 and TNF-α.[53]
2. Blockade of cytokine receptors**:** interleukin-1β antagonist, eg : Anakinra and Interleukin-1α antagonist.[46]
3. Lisofylline: Promotes mitochondrial metabolism and forms proinflammatory cytokines.[54]
4. Bicyclic imidazoles: It Inhibits biosynthesis of cytokines by acting at
5. transcriptional level.[55]
6. IL-13:Causes inhibition of activated monocytes functions and inhibits the inflammatory cytokines production.[56]

IV. Others:

a. Calcium channel blockers : Verapamil,Nifedipine.[57]

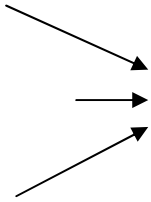
b. Adrenergic agonists :

   i. Clonidine.[58]

   ii. Adrenaline :

   iii. Nor-Adrenaline :

   iv. Isoprenaline



Decreases the vascular permeability and causes vasoconstriction.[59]

   v. Salmeterol : Inhibits the release of inflammatory mediator.[60]

vi. Salbutamol :possesses anti-inflammatory activity in acute (carrageenan induced rat paw edema) and chronic (formalin induced arthritis) models of inflammation.[61]

d) Adrenergic antagonists:Phenoxybenzamine.[62]

e) Tetracycline derivatives: Minocycline and Doxycycline[63]

D. INFLAMMATION AND DIABETES MELLITUS.

Inflammation is defined as local response of the living mammalian tissues to an injury due to any agent. It is the body defence mechanism in order to limit or eliminate the spread of injurious agent, followed by the removal of the tissues and necrosed cells.[15]

Chronic inflammation is characteristic of certain chronic diseases like metabolic syndrome, hypertension, cardiovascular diseases, obesity non-alcoholic fatty liver disease, and diabetes mellitus.[2] T1DM is due to near-total or complete insulin deficiency while, T2DM is characterised by variable degrees of impaired insulin secretion, insulin resistance, and increased glucose production.[3] Recent developments indicate that pathophysiological mechanisms leading to cell damage, insulin resistance, and vascular complications of diabetes include activation of the inflammatory cascade.[64]Although T2DM is based on hyperglycaemia, but the underlying pathophysiology is that of prothrombotic milieu where a raft of risk factors, including insulin resistance, high triglycerides (TG),increased visceral fat, low high-density lipoprotein cholesterol (HDL-C) and obesity which exacerbates deleterious hyperglycaemic effects.[65]

The recent rise in obesity and its impervious association with T2DM and insulin resistance lead to the development of interest to reveal the underlying

mechanisms of these pathologies.[66]The link between insulin resistance and obesity is determined by molecular signaling pathways which involve the activation of kinases namely- the C- jun N-terminal kinase (JNK), protein kinase R and K kinase β inhibitors which leads to release and nuclear translocation of nuclear factor kappa B (NF-KB) that is a transcription factor known to stimulate production of several inflammatory mediators production including IL-6, IL-1, TNF-α. These kinases have shown to play a very important inhibitory action in insulin cell signalling by targeting insulin receptor substrate 1 (IRS-1) for serine phosphorylation, that inhibits the signalling of insulin cascade.[67]

Therefore, there is much need to use pharmacological agents that is not only to treat hyperglycaemia but also associated obesity and related inflammation. One of such pharmacological agents are SGLT2 inhibitors that control hyperglycaemia by virtue of inhibition of glucose reabsorption. Several compounds like Canagliflozin, Dapagliflozin, Empagliflozin and Ipragliflozin are already available in many countries for clinical use[68]. The inhibition involves competitive binding of the glucose moiety of the SGLT2 inhibitor to the glucose moiety binding site on the transport protein.[69] Inhibition of SGLT2 increases excretion of urinary glucose and calorie, hereby reducing plasma glucose levels and body weight.

The kidneys plays an important role in the regulation of plasma glucose levels. Glucose levels in blood circulation is filtered continuously by the glomeruli of the kidneys and it is reabsorbed in the renal proximal convoluted tubules by active transport. The first step is to reabsorb the glucose from the urine which involves transportation of glucose into the epithelial cells of proximal tubule from the

glomerular filtrate, a process which is accomplished by the two sodium-glucose cotransporters i.e SGLT-1 and SGLT-2.[70]

SGLT-1 is the main transporter in the gastrointestinal tract for glucose absorption, but in kidney it is accounting for only 10% of glucose reabsorption. SGLT-2 is exclusively expressed in the S1 segment of proximal tubule and it accounts for 90% of renal reabsorption of glucose.[70]

TABLE 1: DRUGS USED IN THE TREATMENT OF DIABETES MELLITUS[46]

A. Enhance Insulin secretion

1. Sulfonylureas (KATP Channel blockers)

First generation: Tolbutamide

Second generation: Glibenclamide (Glyburide),Glipizide, Gliclazide,

2. Meglitinide/phenylalanine analogues

Repaglinide, Nateglinide

3. *Glucagon-like peptide-1 (GLP-1) receptor agonists* (Injectable drugs)

Exenatide, Liraglutide

4. Dipeptidyl peptidase-4 (DPP-4) inhibitors

Sitagliptin, Vildagliptin, Saxagliptin, Alogliptin, Linagliptin

B. Overcome Insulin resistance

1. Biguanide (AMPK activator)

Metformin

2. Thiazolidinediones (PPARαactivator)

Pioglitazone

C. Miscellaneous antidiabetic drugs

1. α-Glucosidase inhibitors

Acarbose, Miglitol, Voglibose

2. Amylin analogue

Pramlintide

3. Dopamine-D2 receptor agonist

Bromocriptin

4. Sodium-glucose cotransport-2 (SGLT-2) inhibitor

Dapagliflozin, Canagliflozin

DRUGS USED IN THE PRESENT STUDY:

SGLT-2 Inhibitors – Dapagliflozin and Canagliflozin.

HISTORY

In 1800s, Phlorizin, a natural compound was isolated and for decades it played an important role in diabetes and renal physiology research.

In 1835, the French chemists isolated the phlorizin, from the bark of the apple trees. The compound was bitter in flavor and reminded them willow tree and cinchona having similar extracts was referred as the "glycoside from the bark of apple trees."

By 1970s, phlorizin research revealed that it had a higher affinity for active-transport system located in proximal tubule brush border of the kidney which, is responsible for the glucose reabsorption.[71]

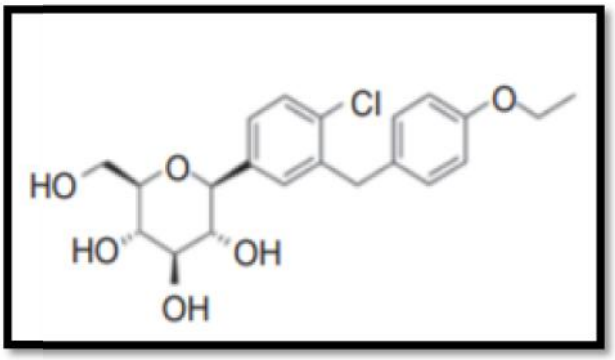
Table 1: Sodium glucose co-transporters inhibitors in advanced development or already approved[72]

Molecule	Approval/development status
Canagliflozin	40 countries including EU, USA, China, Russia
Dapagliflozin	40 countries including EU, USA, Japan,
Empagliflozin	Phase 3 [37]
Ipragliflozin	Japan
Luseogliflozin	Under review for approval in Japan
Tofogliflozin	Phase 3 [43, 44]

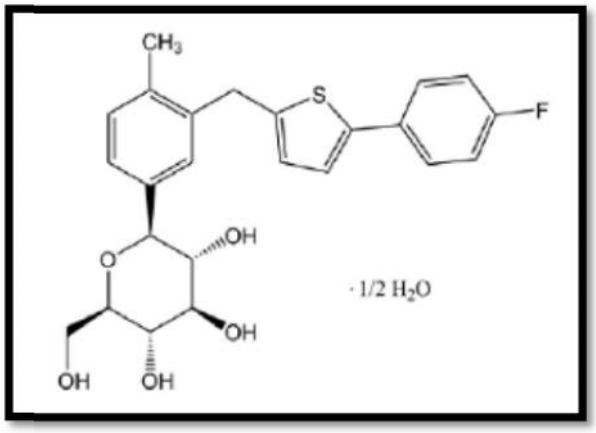
CHEMISTRY

Phlorizin, a β-Dglucoside, the first non-selective SGLT inhibitor was isolated from the root bark of the apple tree. It is made up of glucose moiety and an aglycone in which the two aromatic carbocycles are joined by an alkyl spacer.

Dapagliflozin is an aromatic C-glycoside containing aromatic and heteroaromatic C-glycoside, in which the glucose moiety binds to aglycone directly through a carbon–carbon bond.



CHEMICAL STUCUTRE OF DAPAGLIFLOZIN[73]



CHEMICAL STUCUTRE OF CANAGLIFLOZIN

MECHANISM OF ACTION

The kidneys plays an important role in the regulation of plasma glucose levels. Glucose levels in the blood circulation is filtered continuously by the glomeruli of the kidneys and it is reabsorbed in the renal proximal convoluted tubules by active transport. The first step is reabsorption of glucose from the urine, involves the transportation of glucose into the proximal tubule epithelial cells from the glomerular filtrate, a process which is accomplished by the two sodium-glucose cotransporters i.e SGLT-1 and SGLT-2.

SGLT-1 is the main transporter in the gastrointestinal tract for glucose absorption, but the kidney accounting for only 10% of glucose reabsorption. SGLT-2 is exclusively expressed in S1 segment of the proximal tubule and it accounting for 90% of renal glucose reabsorption.[70]

Sodium-glucose co-transporter-2 inhibitors inhibits SGLT2 in PCT, prevents the glucose reabsorption and facilitates its excretion in urine. As the glucose is

excreted, its plasma levels fall leading to an improvement in all glycaemic parameters.[72]

PHARMACOKINETICS

The SGLT2 inhibitors have good oral bioavailability of (60%–80%).They reach peak levels in 1–2 hrs after ingestion. They are 90% protein bound with half-lives of about 12h. They are suitable for once-daily dosing. They are metabolized by glucuronidation. The inactive metabolites are excreted through kidney. All three drugs are available in two doses, dapagliflozin 5 and 10 mg, canagliflozin 100 and 300 mg, and empagliflozin 10 and 25 mg.[74]

ADVERSE EFFECTS :

- Lower urinary tract infections
- Genital mycotic infections
- Hypotension
- Increase risk of fractures.

CONTRAINDICATIONS:

- Patients with high CV risk patients.
- Patients prone to hypovolaemia.
- Patients with renal disease.

USES: As an adjunct to exercise, diet, insulin or any oral hypoglycemic drugs for diabetes for improvement in glycemic control in adults with T2DM and also T1DM.[74]

REVIEW OF STUDIES

SGLT2 inhibitors are the novel group of pharmacological agents that control hyperglycaemia by virtue of inhibition of glucose reabsorption.Several compounds like Canagliflozin, Dapagliflozin, Empagliflozin and Ipragliflozin are already available in many countries for clinical use.[68] The inhibition involves competitive binding of the glucose moiety of the SGLT2 inhibitors to the glucose moiety binding site on the transport protein.[69] Hence, reducing glucose levels in plasma and body weight.

Dapagliflozin, has been reported to exert dose-dependent glucosuria in healthy volunteers and in short-term (2- to 12-week) studies in patients with T2DM. It has been reported to exert the renoprotective effects through their glucose lowering effect and reducing oxidative stress in the diabetic kidney. Dapagliflozin reduces the oxidative stress by suppressing Nox4-derived ROS generation and hyperglycaemia induced ROS generation.[75]

In animal models, dapagliflozin treatment decreased the mRNA levels of acute phase MI. cardiac inflammatory cytokines like IL-1β and IL-6 while, it increased anti-inflammatory cytokines like IL-10.It also increased the M2/M1 phenotype macrophage ratio. SGLT-2inhibitors are known to possess antioxidant property by reducing cardiac oxidative stress independent of glucose lowering effects.[76] Dapagliflozin reported to reduce the macrophage infiltration and the gene expression of inflammation like OPN, MCP-1 and TGF-b in the kidney of diabetic db/db mice.[77]

Similarly, Canagliflozin has been reported to reduce hyperglycemia as well as blood pressure, body weight, and albuminuria in people with diabetes. Canagliflozin demonstrated suppression of inflammatory responses in skeletal muscle of high fat

diet induced inflammation in mice. This action was attributed to increased glucose excretion and decreased hyperglycemia by canagliflozin.[77]Several recent studies have reported that canagliflozin administration in rodent models of diabetes decreased the pro-Inflammatory parameters like MCP-1, IL-6 and ICAM-1 gene expression in blood vessels.[r-70]

However, there are no reports regarding the effect of dapagliflozin and canagliflozin in inflammatory models.Therefore, in view of paucity of literature regarding effects of dapagliflozin and canagliflozin on animals models of inflammation, the present study was planned to study the effect of these drugs on acute and sub acute models of inflammation by comparing them with control and aspirin (standard anti-inflammatory drug) in male Wistar rats.

MATERIALS AND METHODS

Healthy, adult, male Wistar rats weighing 180±20 gm were obtained from the central animal house, J. N. Medical College, Belagavi. Under the standard laboratory conditions animals were housed and acclimatized for 10 days, prior to the day of experimentat ionto 12 : 12 h light - dark cycle and they were maintained on standard rat chow pellet (Amrut brand) with water *ad libitum*.

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

Animals were randomly as signed to various groups. To eliminate the observer bias blinding was done. Before starting the experiments coding and masking was done to all the drugs used in the experiment by the guide.

Acute inflammation was induced by injecting carrageenan in left hind paw and subacute inflammation was produced by subcutaneous implantation of foreign body in male wistar rats as described below.

ACUTE MODEL

1. CARAGEENAN INDUCED RAT PAW EDEMA[40]

Rats were divided into four groups with six animals in each. Prior to the day of experiment they were fasted overnight with water *ad libitum*. Control group received 0.5 ml of one percentgum acacia suspension orally whereas, other groups received calculated clinical equivalent doses orally of either aspirin in 1% gum acacia, Dapagliflozin or Canagliflozin. Aspirin was used as a standard anti-inflammatory drug.

According to the technique of Winter et al. 1962,[40] thirty minutes after aspirin, one hour thirty minutes after Dapagliflozin and Canagliflozin administration, 0.05ml of 1% carrageenan in normal saline was injected into the subplantar region of the left hind paw. A mark was made at the malleolus of the leg to facilitate uniform dipping at every readings. The paw edema volume was measured in millilitres with the help of digital plythesmometer by water displacement method at zero hour (immediately after injecting carrageenan) (Figure-2). The same procedure was repeated at 30 minutes and 1,2,3,4 and 5 hours. The difference between zero hours and subsequent readings was considered as actual oedema volume and the same was used for analysis.

SUBACUTE MODEL

2. FOREIGN BODY INDUCED GRANULOMA METHOD[45]

Rats were divided into four groups with six animals in each. Under sodium thiopentone anaesthesia, hair was clipped in the axillae and groin and two sterile cotton pellets of 10mg each and two sterile grass-piths (25 x 2 mm) were implanted

randomly, subcutaneously, through a small incision (Figure-3,4). Then wounds were sutured and animals were caged separately after recovery from anaesthesia. Aseptic precautions were maintained throughout the experiment. The treatment was started on the day of implantation and it was repeated every 24 hours in control, aspirin and Dapagliflozin and Canagliflozin group regularly for 10 days.

On the day eleven, 5ml blood was collected through cardiac puncture for estimation of inflammatory cytokines. Rats were sacrificed by using overdose of thiopentone anaesthesia and cotton pellets and grass piths were obtained (Figure-5,6). The pellets, free from extraneous tissue were dried overnight in the incubator at 60 degree Celsius and noted for their dry weight. Net granuloma formation was calculated by subtracting initial weight of cotton pellet (10mg), calculated and were expressed as mg/100gm of body weight.

The percentage inhibition of granuloma dry weight was calculated as follows:

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

The grass piths were preserved in 10% formalin and were processed in the Department of Pathology, J.N. Medical College, Belagavi, and sections were staining with haematoxylin and eosin were studied microscopically for granulation tissue.

The blood which was obtained before sacrificing the rats was centrifuged and the serum obtained was used to estimate the levels of inflammatory markers viz. TNF – α and IL -6using ELISA kits.

Drugs used and their dosages

1. **Dapagliflozin**– was obtained as tablet from Astrazaneca Pharmaceuticals Ltd. The clinical dose of 10mg was converted to rat equivalent dose that was 0.9mg/kg of rat. It was administered orally.[74]
2. **Canagliflozin** - was obtained as tablet from Janssen Pharmaceutical Company. The clinical dose of 100mgwas converted to rat equivalent dose that was 27mg/kg of rat. It was administered orally.[74]
3. **Aspirin** – was obtained as a tablet from Reckitt Benckiser India Ltd. The clinical dose of 2222 mg was converted to rat equivalent dose that was 200 mg/kg of rat.[74]It was administered orally, as a suspension of 1% gum acacia. Clinical doses[81]for Dapagliflozin, Canagliflozin and Aspirin were converted into rat equivalent doses with the help of the table devised by Paget and Barnes.[44] Calculated doses of various drugs are shown in Table 2.

Table2: Drugs and dosages used in the study

S.No	Groups	Drugs Administered	Dose
ACUTE MODEL			
1	control (Vehicle only)	1% gum acacia in distilled water	10ml/kg p/o
2	Standard Control	Aspirin	200mg/kg p/o
3	Treatment 1	Dapagliflozin	0.9mg/kg p/o
4	Treatment 2	Canagliflozin	27mg/kg p/o
SUBACUTE MODEL			
1	control (Vehicle only)	1% gum acacia in distilled water	10ml/kg p/o
2	Standard Control	Aspirin	200mg/kg p/o
3	Treatment 1	Dapagliflozin	0.9mg/kg p/o
4	Treatment 2	Canagliflozin	27mg/kg p/o

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

Statistical analysis: The data was expressed as Mean ± SEM for all the groups. Data was analyzed by one way ANOVA (Analysis of variance). Post hoc Dunnett's test was used to compare treatment groups with control group. Comparison between aspirin and treatment groups (Dapagliflozin and Canagliflozin) was done by one way ANOVA followed by Bonferroni's test. Analysis was done by using Graph pad prism software and $p \leq 0.05$ was considered as statistically significant.

FIGURE2:PLETHYSMOGRAPHIC MEASUREMENT OF THE RAT PAW EDEMA IN THE ACUTE INFLAMMATION STUDY

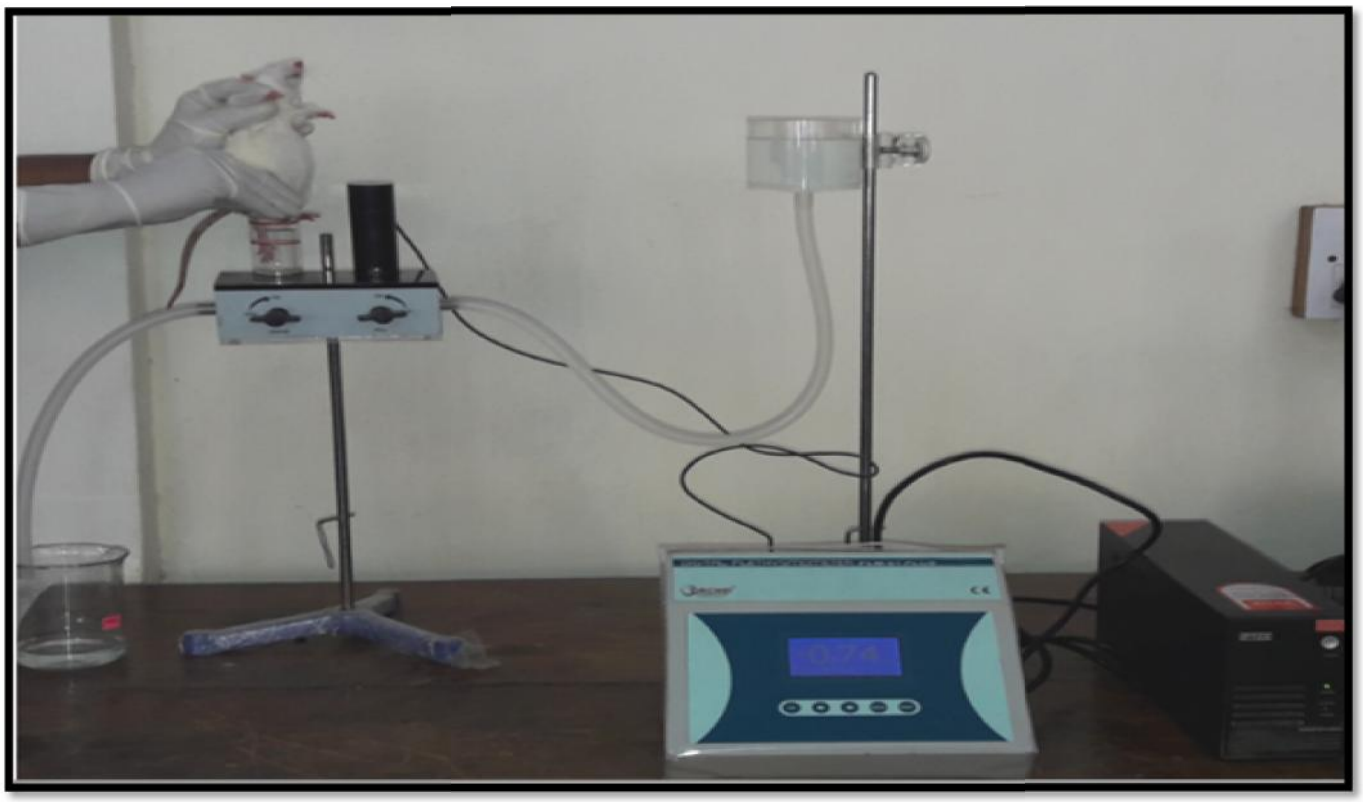
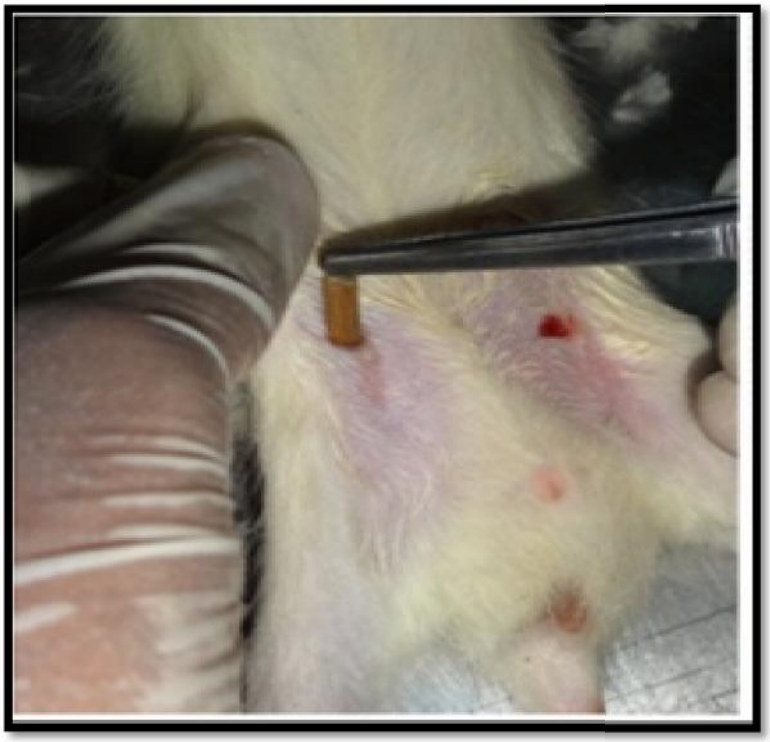


FIGURE 3:IMPLANTATION OF COTTON PELLET AS A FOREIGN BODY IN THE SUBACUTE INFLAMMATORY STUDY

FIGURE4:IMPLANTATION OF GRASS PITH AS A FOREIGN BODY IN THE SUBACUTE INFLAMMATORY STUDY



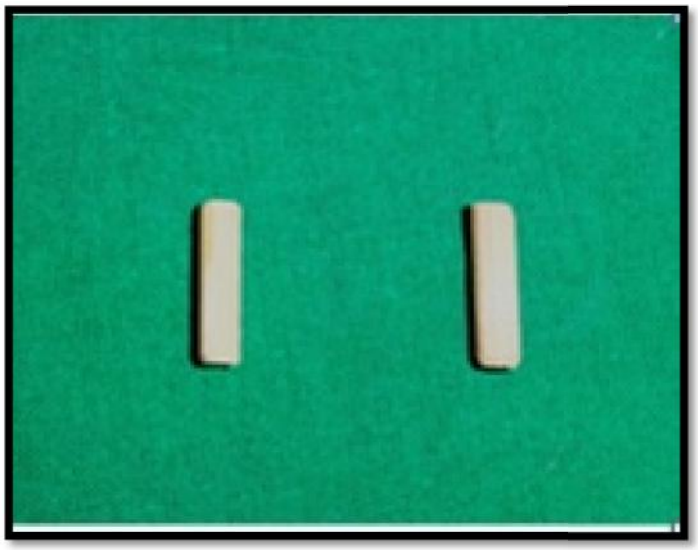


FIGURE5:DISSECTION OF RAT TO OBTAIN GRASS PITH ON 11TH DAY

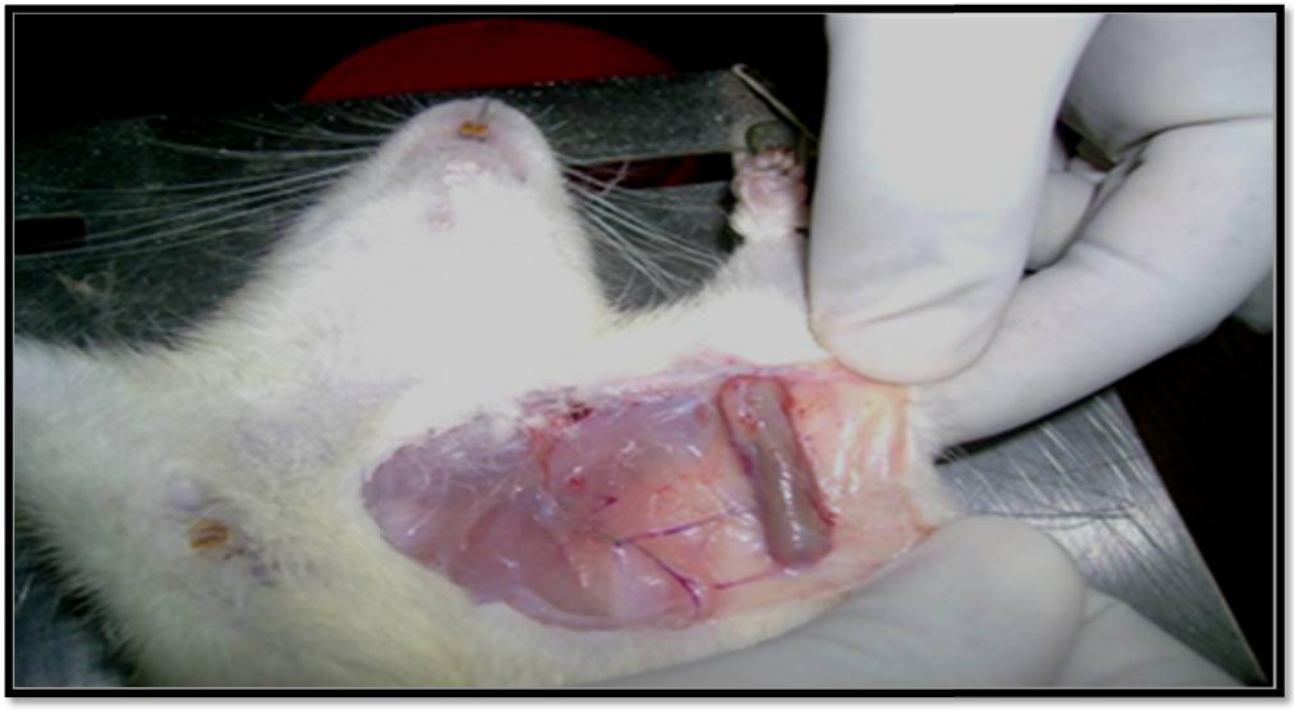
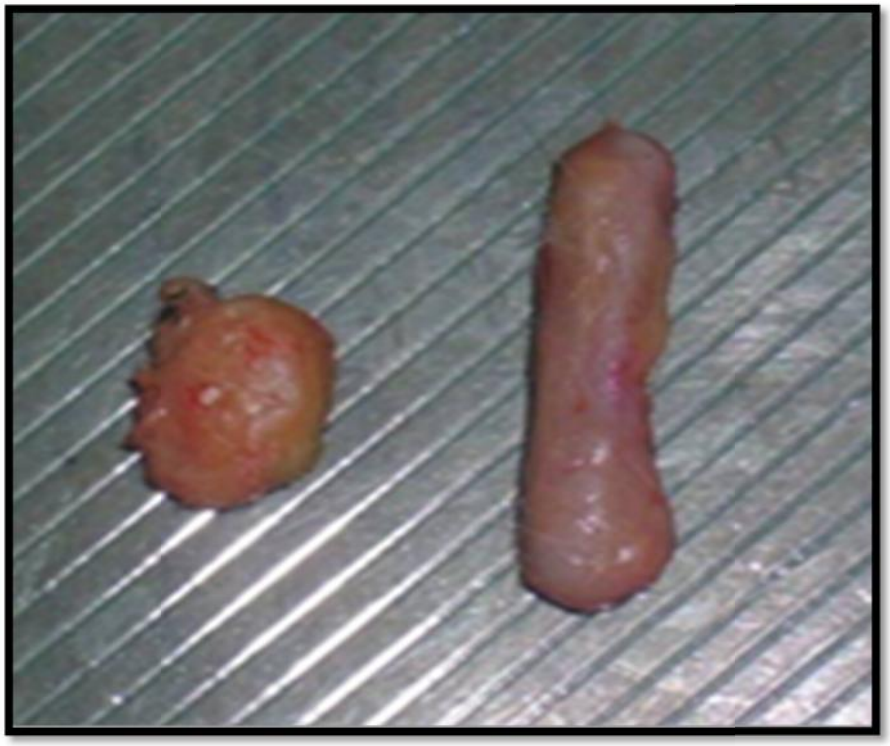


FIGURE 6: COTTON PELLET AND GRASS PITH COVERED WITH GRANULATION TISSUE



RESULTS

In the present study, anti-diabetic drugs Dapagliflozin and Canagliflozin in their therapeutic equivalent doses, were investigated for their possible anti-inflammatory activity, in acute and subacute models of inflammation, in male Wistar rats.

CARRAGEENAN INDUCED ACUTE INFLAMMATION:

The mean edema volume in millilitres (ml), as measured by water displacement using a digital plethysmometer , for control group at ½ h, 1h, 2h, 3h, 4h, and 5h, was 0.07±0.01, 0.12±0.005, 0.12±0.01, 0.13±0.02, 0.05±0.02, 0.02±0.02 (Table-3) respectively, while the corresponding mean volumes in aspirin (200 mg/kg) treated group were 0.06±0.01, 0.12±0.01, 0.07±0.01, 0.02±0.01, 0.01±0.005, 0.01±0.003 respectively (Table-3, Graph-1).

The oedema volume in ml in Dapagliflozin treated group (0.9mg/kg) at ½h, 1h, 2h, 3h, 4h, and 5h was 0.06±0.01, 0.11±0.01, 0.08±0.01, 0.11±0.04, 0.045±0.01, 0.005±0.008 respectively (Table-3, Graph-1).The oedema volume in ml in Canagliflozin treated group (0.15mg/kg) at ½h, 1h, 2h, 3h, 4h, and 5h was 0.08±0.01,0.12±0.01,0.11±0.009,0.07±0.01,0.04±0.008,0.006±0.004 respectively (Table-3, Graph-1). According to post-hoc analysis by Dunnett's test, there was no significant reduction in the paw oedema volume in the Dapagliflozin and Canagliflozin treated groups compared to the control group (Table-3, Graph-1). While aspirin treated group showed significant reduction ($p < 0.05$) in paw oedema volume at 3hr when compared to control group.(Table-3, Graph-1).According to the post-hoc Bonferroni's analysis there was no significant difference in the edema observed in dapagliflozin and canagliflozin group as compared to aspirin group (Table-4, Graph-

2). Above results indicate that these SGLT-2 inhibitors Dapaglifozin and Canagliflozin have not shown statistically significant anti-inflammatory activity in acute model of inflammation.

SUBACUTE INFLAMMATION (FOREIGN BODY INDUCED GRANULOMA METHOD):

The mean dry weight of ten days old granuloma, expressed as mg per 100g body weights, in control group was 41.50±4.69, while in aspirin (200mg/kg) treated group it was 28.5 ± 0.67 with percentage inhibition of 31.3% which was statistically significant($p<0.05$) as compared to control (Table-5,Graph-3).The mean granuloma weight with Dapagliflozin (0.9mg/kg) was 41.17±2.28 and that of Canagliflozin (27mg/kg) was found to be 39.42±3.75 with percentage inhibition of 0.79% and 5.01% respectively. This did not exhibit statistically significant difference in granuloma weight of the treatment groups as compared to control or aspirin treated group (Table- 5 and 6).

The effect of Dapagliflozin and Canagliflozin was also studied on inflammatory markers like TNF –α and IL-6. The level of TNF-α, expressed as picograms per milliliter (pg/ml) in control group was 0.02±0.04, while that in aspirin, dapagliflozin and canagliflozin was 0.03±0.02, -0.003±0.01 and 0.03±0.009 respectively. There was no statistical significant difference among the control, aspirin and treatment groups (Table-7graph-4).

Similarly IL-6 levels expressed as pg/ml in control was -0.08±0.005 and that in aspirin, dapagliflozin and canagliflozin groups were -0.09±0.006, -0.09±0.008 and -0.09±0.004 respectively. These values did not show statistically significant difference as compared to control (Table-7, Graph-5).

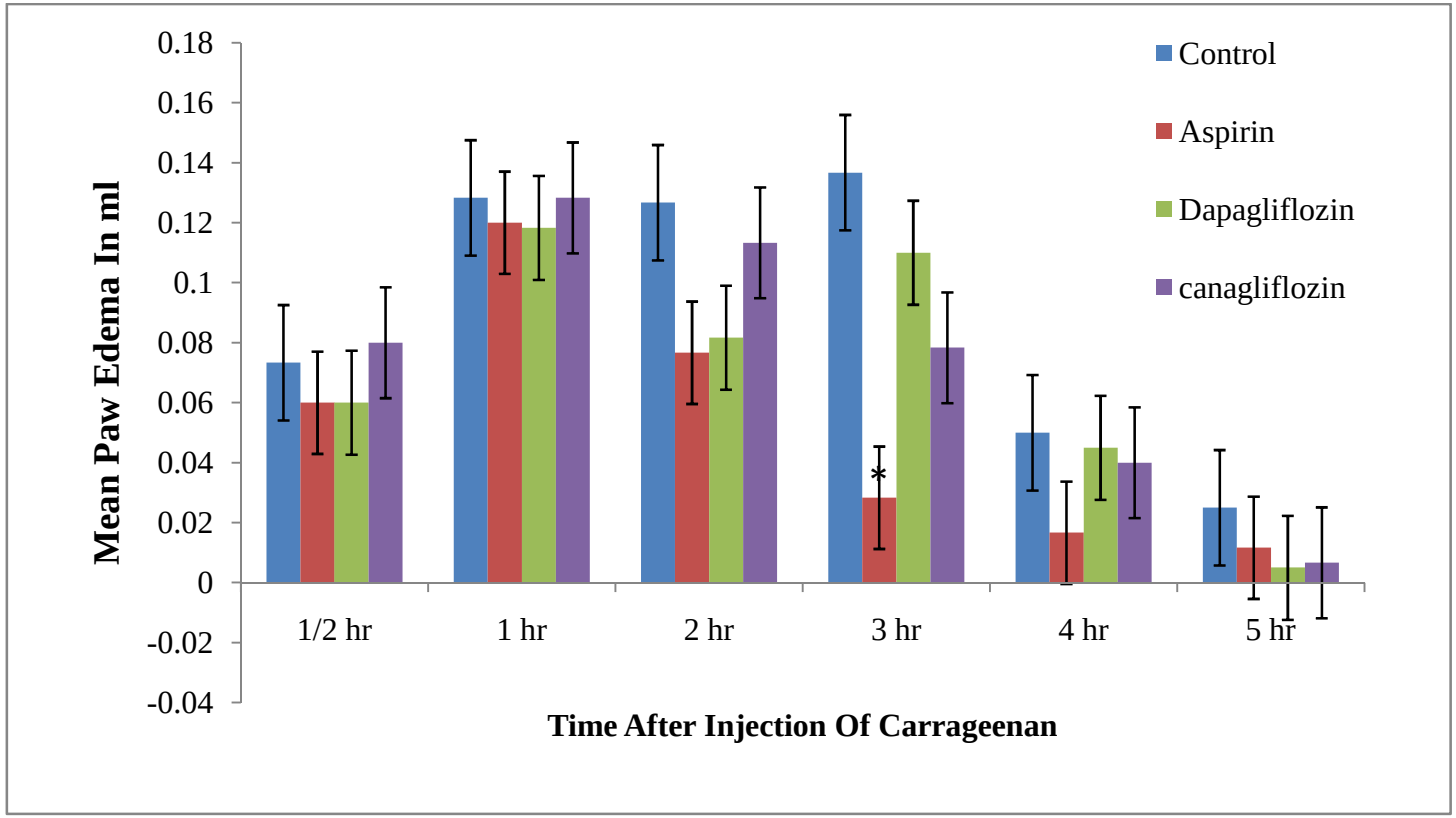
The effect of Dapagliflozin and Canagliflozin on inflammation was further evaluated by histopathological studies. The sections of grass pith when stained with haematoxylin and eosin showed dense area of inflammation, abundant granulation tissue and dense band of fibrous tissue in control animals, while revealed markedly sparse inflammation and thin band of fibrous tissue in aspirin group. Both dapaliflozin and canagliflozin group revealed dense inflammation, thick band of fibrosis almost similar to control group (Fig-7). The results indicate that dapagliflozin and canagliflozin did not exhibit anti-inflammatory activity in subacute model of inflammation.

TABLE 3 :EFFECT OF VARIOUS TREATMENTS IN ACUTE INFLAMMATION – CARRAGEENAN INDUCED RAT PAW EDEMA

Time after carrageenan injection	Paw edema in ml (Mean ±SEM)				ANOVA Result
	Control	Aspirin	Dapagliflozin	Canagliflozin	$F_{3,20}$
½ hr	0.07 ± 0.01	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.4235
1hr	0.12 ± 0.005	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.1958
2hr	0.12 ± 0.01	0.07 ± 0.01	0.08 ± 0.01	0.11 ± 0.009	2.383
3hr	0.13 ± 0.02	0.02 ± 0.01*	0.11 ± 0.04	0.07 ± 0.01	3.257
4hr	0.05 ± 0.02	0.01 ± 0.005	0.04 ± 0.01	0.04 ± 0.008	1.237
5hr	0.02 ± 0.02	0.01 ± 0.003	0.005 ± 0.008	0.006 ± 0.004	0.604

ANOVA followed by Post hoc Dunnet's test: *p<0.05.SEM : Standard error of mean. n=6 in each group.

GRAPH 1: EFFECT OF VARIOUS TREATMENTS IN ACUTE INFLAMMATION - CARRAGEENAN INDUCED RAT PAW EDEMA



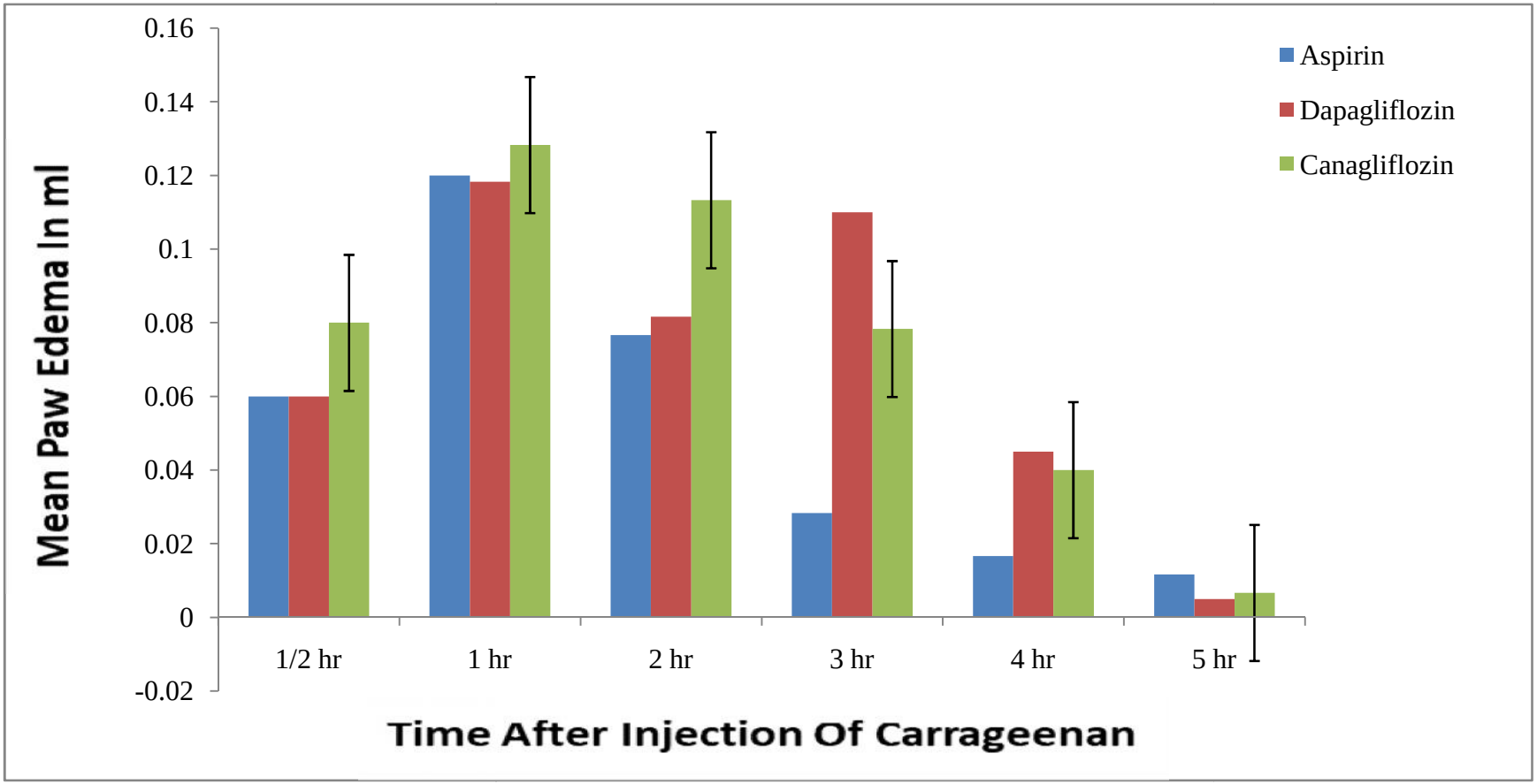
ANOVA followed by Post hoc Dunnet's test :*p<0.05.n=6 in each group.

TABLE 4: EFFECT OF DAPAGLIFLOZIN AND CANAGLIFLOZIN IN ACUTE INFLAMMATION- CARRAGEENAN INDUCED RAT PAWEDEMA WHEN COMPARED WITH ASPIRIN GROUP

Time after carrageenan injection	Paw edemain ml(Mean ± SEM)			ANOVA Result	
	Aspirin	Dapagliflozin	Canagliflozin	$F_{3,20}$	p Value
½ hr	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.01	0.4235	0.5074
1hr	0.12 ± 0.01	0.11 ± 0.01	0.12 ± 0.01	0.1958	0.8980
2hr	0.07 ± 0.01	0.08 ± 0.01	0.11 ± 0.009	2.383	0.5869
3hr	0.02 ± 0.01	0.11 ± 0.04	0.07 ± 0.01	3.257	0.0431
4hr	0.01 ± 0.005	0.04 ± 0.01	0.04 ± 0.008	1.237	0.3225
5hr	0.01 ± 0.003	0.005 ± 0.008	0.006 ± 0.004	0.604	0.6198

ANOVA followed by Post hoc Bonferroni's Test: SEM : Standard error mean. n=6 in each group

GRAPH 2:EFFECT OF VARIOUS TREATMENTS IN ACUTE INFLAMMATION- CARRAGEENAN INDUCED RAT PAW EDEMA VOLUME WHEN COMPARED WITH ASPIRIN.



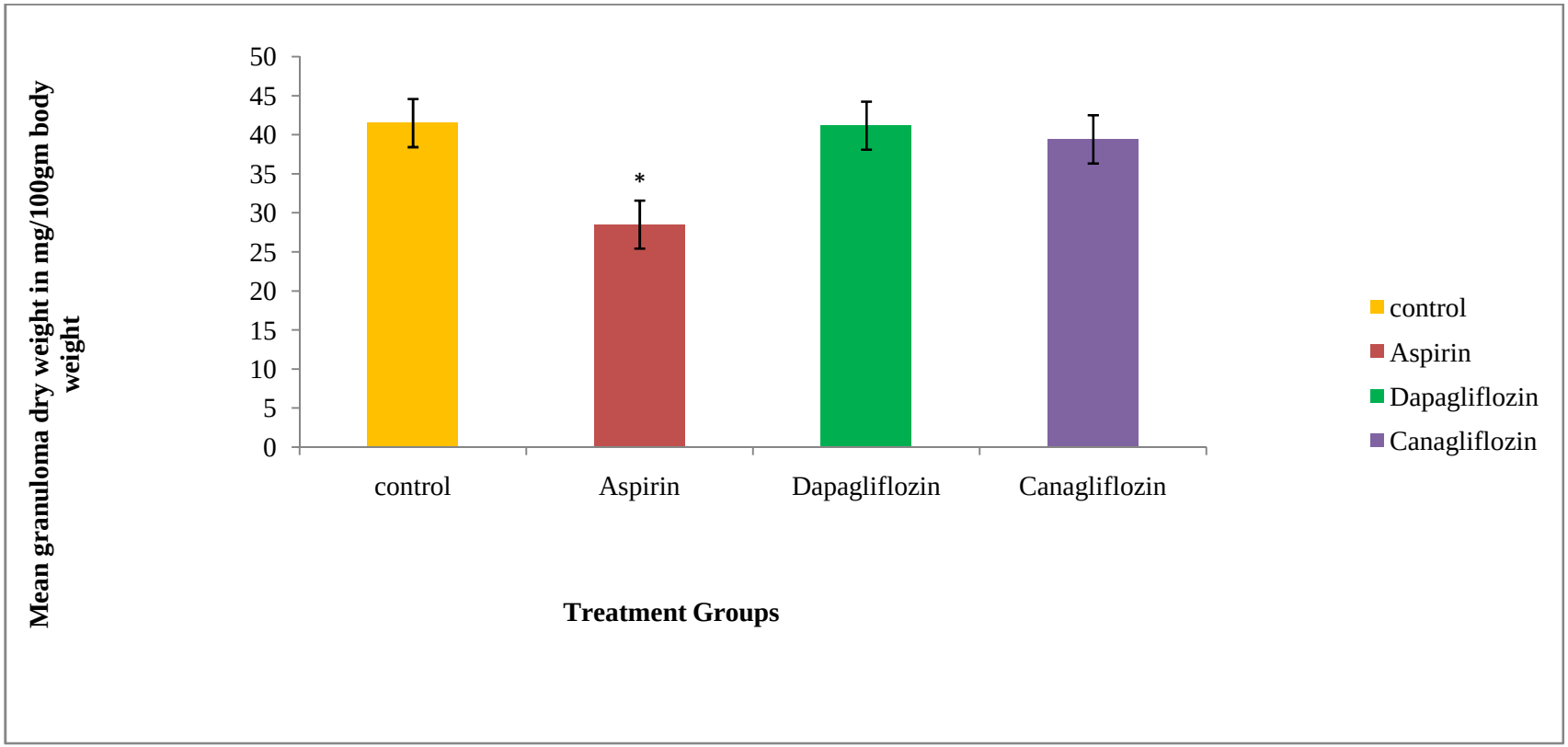
ANOVA followed by Post hoc Bonferroni's test :n=6 in each group.

TABLE 5: EFFECT OF VARIOUS TREATMENTS IN SUBACUTE INFLAMMATION - GRANULOMA DRY WEIGHT

Sl. No	Drug Treatment	Mean granuloma dry weight mg/100gm body weight (Mean ± SEM)	Percentage inhibition
1.	**Control**	41.50 ± 4.69	-
2.	**Aspirin**	28.5± 0.67*	31.3%
3.	**Dapagliflozin**	41.17± 2.28	0.79%
4.	**Canagliflozin**	39.42±3.75	5.01%

ONE WAY ANOVA: $F_{3,19}$= 3.031. *P<0.05. Post hoc Dunnet's test: SEM: Standard error of mean. n=6 in each group.

GRAPH 3 : EFFECT OF VARIOUS TREATMENTS IN SUBACUTE INFLAMMATION - MEAN GRANULOMA DRY WEIGHT



ANOVA followed by Post hoc Dunnet's test :*P<0.05.n=6 in each group.

TABLE NO. 6: EFFECT OF DAPAGLIFLOZIN AND CANAGLIFLOZIN IN SUBACUTE INFLAMMATION - GRANULOMA DRY WEIGHT WHEN COMPARED TO ASPIRIN GROUP

Sl. No	Drug Treatment	Mean granuloma dry weight mg/100gm body weight (Mean+/-SEM)
1.	**Aspirin**	28.5 ± 0.67
2.	**Dapagliflozin**	41.17± 2.28
3.	**Canagliflozin**	39.42±3.75

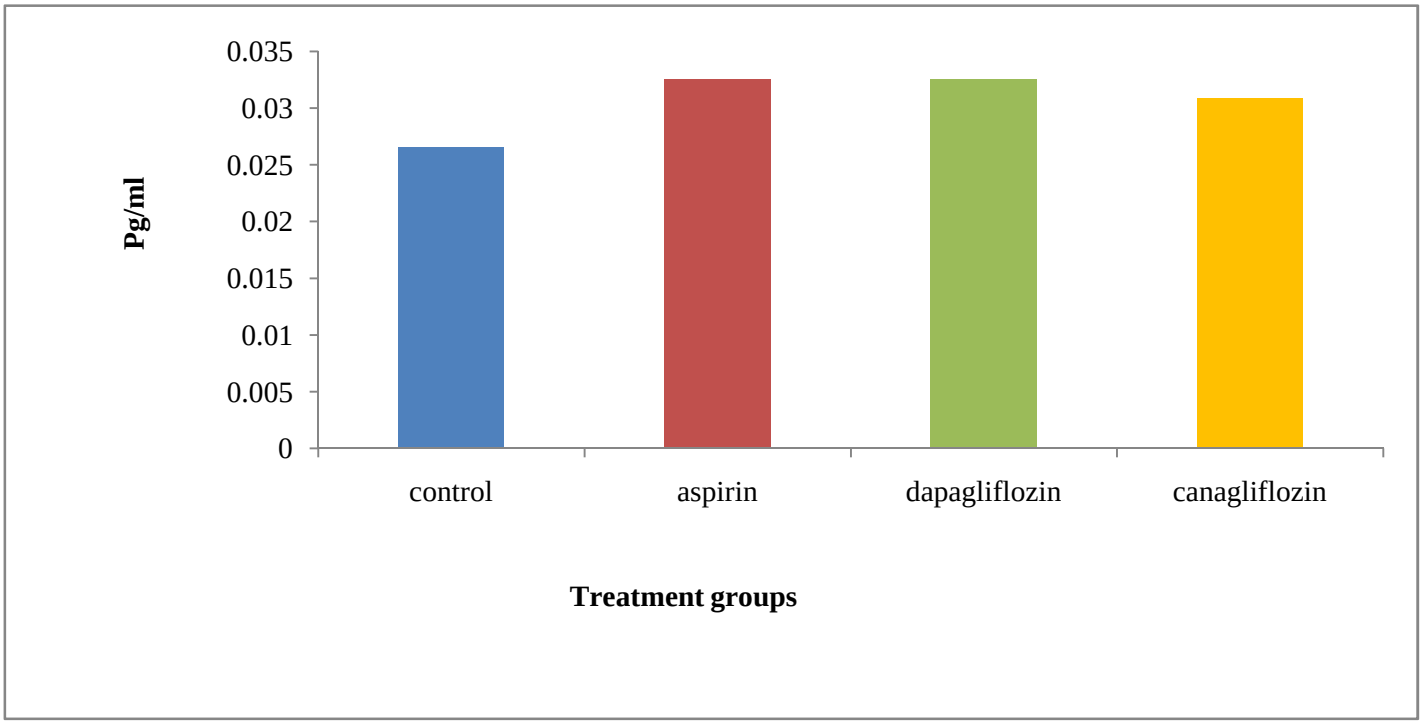
ANOVA: $F_{3,19}$=3.021. Post hoc Bonferroni's test :SEM : Standard error of mean. n=6 in each group.

TABLE 7: EFFECT OF VARIOUS TREATMENTS IN SUBACUTE INFLAMMATION- SERUM INFLAMMATORY MARKERS

	Serum level(Mean ± SEM)		
Sl. No	**Drug Treatment**	**TNF– α (pg/ml)**	**IL-6 (pg/ml)**
1	**Control**	0.02 ± 0.04	-0.08 ± 0.005
2	**Aspirin**	0.03 ± 0.02	-0.09 ± 0.006
3	**Dapagliflozin**	-0.003 ± 0.01	-0.09 ± 0.008
4	**Canagliflozin**	0.03 ± 0.009	-0.09 ± 0.004

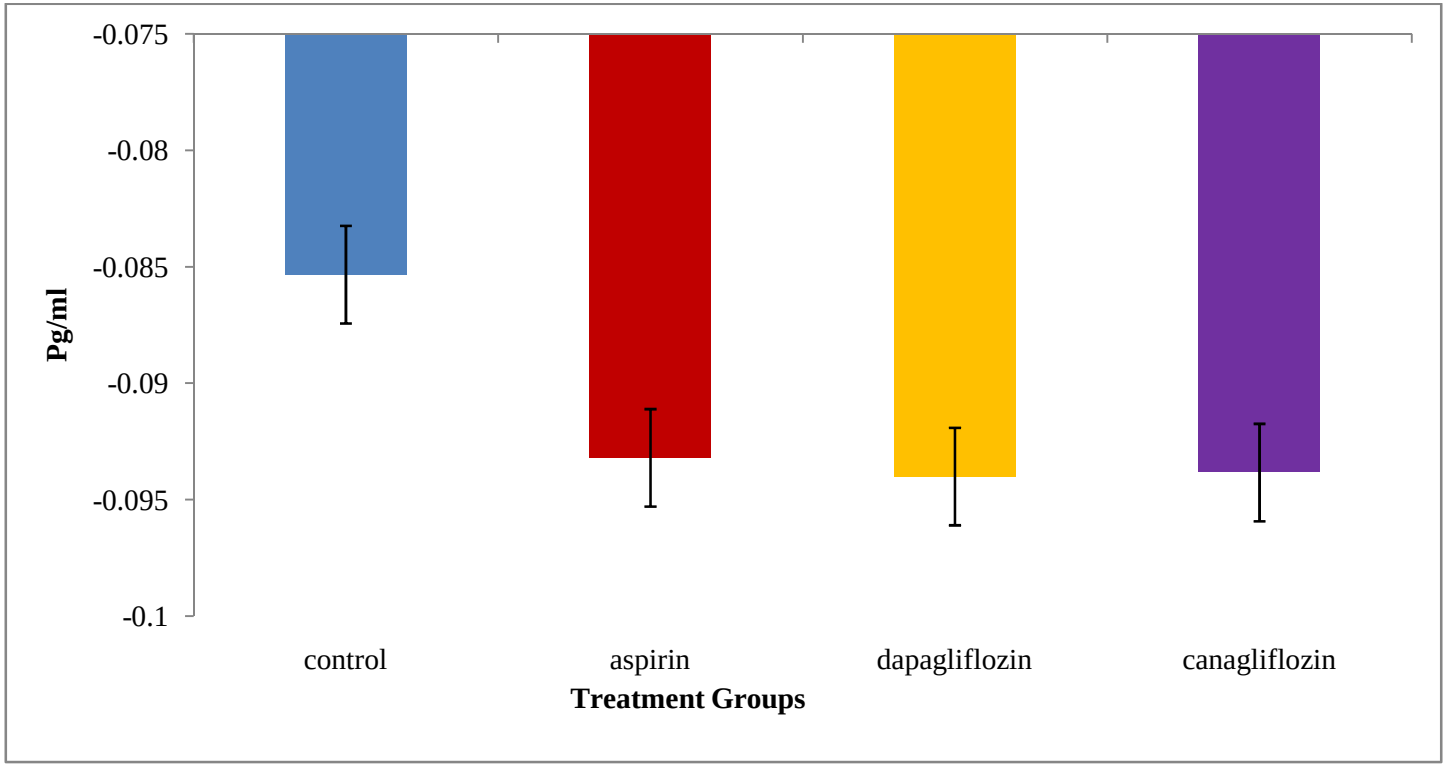
Post hoc Dunnet's test. n=6 in each group. ANOVA: $F_{3,20}$ = 0.3967 for TNF-α; $F_{3,19}$ = 0.4257 for IL-6.

GRAPH 4 :EFFECT OF VARIOUS TREATMENTS IN SUBACUTE INFLAMMATION - SERUM TNF – α



ANOVA followed by Posthoc Dunnet's test. TNF-α – Tumour necrosis factor, pg/ml – picogram per milliliter. n=6 in each group.

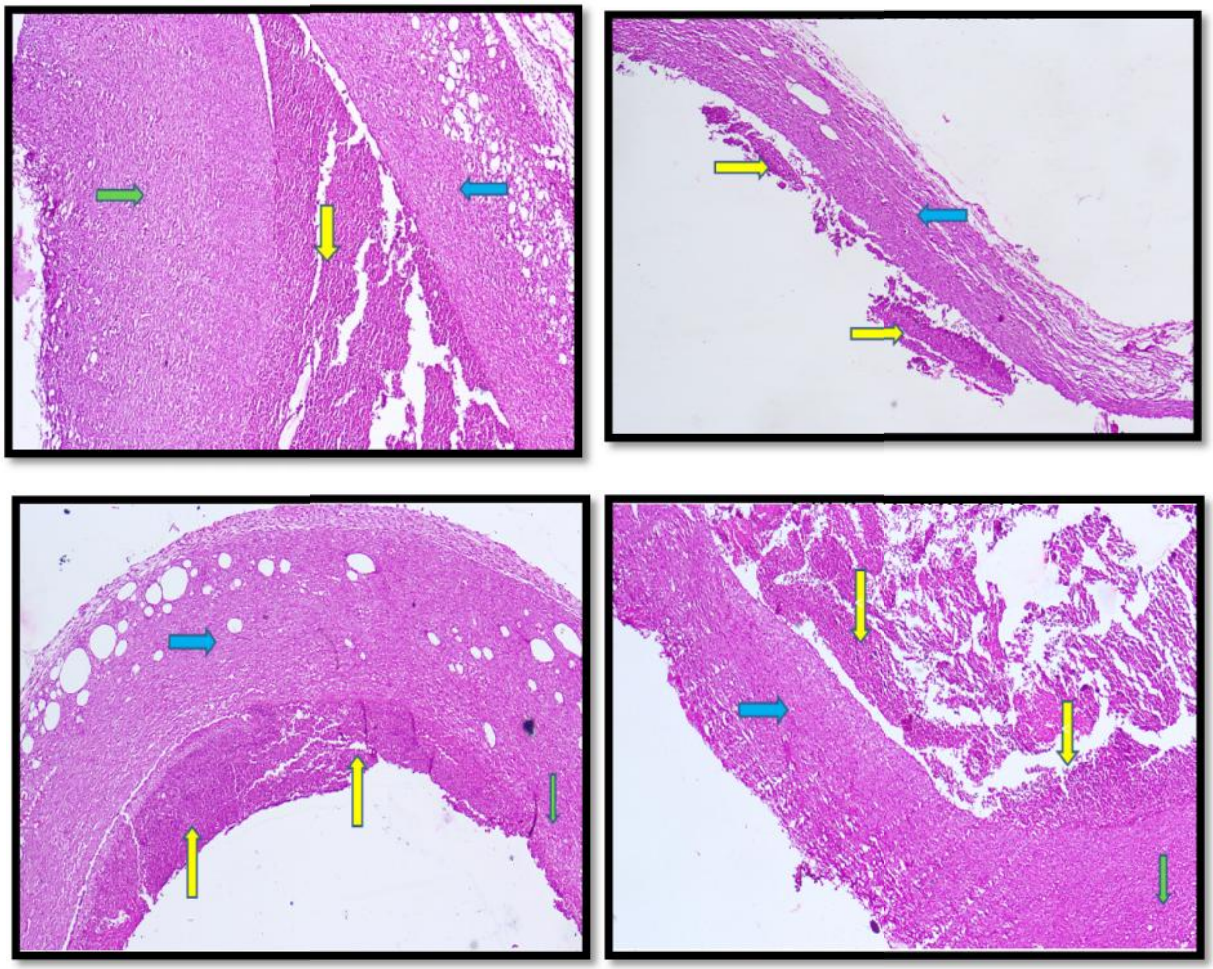
GRAPH 5: EFFECT OF VARIOUS TREATMENTS IN SUBACUTE INFLAMMATION - SERUM IL-6



ANOVA followed by Post hoc Dunnet's test IL-6– Interleukin-6, pg/ml – picogram per milliliter. n=6 in each group.

FIGURE 7: PHOTOMICROGRAPHS OF GRASS PITH WITH GRANULATION TISSUE (H & E Stain - 100X)

CONTROL **ASPIRIN**



DAPAGLIFLOZIN CANAGLIFLOZIN

Granulation Tissue, inflammation, fibrosis.

NOTE:

- **Abundant granulation tissue, inflammatory cells and fibrous tissue in Control group.**
- **Markedly reduced granulation tissue and fibrosis in Aspirin group.**
- **Abundant granulation tissue, inflammatory cells and fibrous tissue in Control, dapagliflozin and canagliflozin treated groups.**

DISCUSSION

In the present study, SGLT-2 inhibitors, namely dapagliflozin and canagliflozin were investigated for their anti-inflammatory effects using acute and subacute models of inflammation in male Wistar rats. Dapagliflozin and Canagliflozin are recently approved drugs for the treatment of diabetes mellitus (DM) and have been reported to exert anti-inflammatory effect in diabetes. However, their anti-inflammatory potential in acute and subacute models of inflammation has not been explored which has been the objective of the present study.

Acute carrageenan-induced rat paw edema and subacute cotton pellet-induced granuloma models were selected, as they are simple, basic, and time-tested anti-inflammatory screening methods. To assess the acute inflammatory activity carrageenan was used to induce rat hind paw edema. Carrageenan is a polysaccharide used for inducing acute inflammation, which releases various inflammatory and proinflammatory mediators like leukotrienes, histamine, bradykinin, TNF-α, prostaglandins etc. Acute inflammation shows a biphasic course. First phase begins with the release of serotonin, histamine and kinins after the injection of phlogistic agent in the first few hours. However, in the second phase prostaglandin like substances are released 2 to 3 hours after carrageenan injection.[42] Accordingly, in the present study aspirin (the standard anti-inflammatory agent) reduced the edema at 2,4 and 5 hours of carrageenan injection that coincides with its onset of action (0.5 to 1 hour), duration of action and its mechanism of action i.e. inhibition of prostaglandin synthesis. However, though carrageenan was injected during peak plasma concentration of single administration of dapagliflozin (1hr) and canagliflozin (1hr), they did not reduced the edema suggesting that they do not possess anti-inflammatory

effect. A single dose of dapagliflozin and canagliflozin each was studied in the acute model, that probably could have led to their variable effects on inflammation owing to pharmacokinetic properties.

Similarly, in cotton pellet granuloma model, assessing subacute anti-inflammatory effects, dapagliflozin and canagliflozin did not significantly reduce granuloma dry weight and serum inflammatory markers – TNF- α, IL-6. Also, these drugs did not decrease the granulation tissue as reported through the histopathological H & E stain of the grass piths. These observations indicate that the SGLT-2 inhibitors do not exert anti-inflammatory effect in both acute and subacute models of inflammation. While, aspirin, a NSAID markedly reduced the granuloma dry weight and granulation tissue, fibrous tissue and inflammatory cells but, demonstrated no effect on inflammatory markers (which can be explained on the basis of mechanism of action).

The observations of the present study disagree with some previous studies wherein SGLT2 inhibitors have been reported to possess anti-inflammatory activity. The anti-inflammatory effect of SGLT-2 inhibitors has been demonstrated in male diabetic db/db mice. The anti-inflammatory effect of dapagliflozin was observed by the virtue of significant reductionin the expression of genes involved in inflammation and macrophage infiltration and oxidative stress in the kidney.[9]Dapagliflozin treatment suppressed expression of genes of inflammatory cytokines and oxidative stress that were induced by high glucose in cultured m Prox 24 cells. The discrepancy in the effect of SGLT-2 inhibitors on inflammation can be explained on the basis that these anti-inflammatory effects could be due to the effect of SGLT-2 inhibitors on glucose levels. Sodium-glucose co-transporter-2 inhibitors inhibit SGLT2 in PCT,

there by prevents reabsorption of glucose and facilitate its urinary excretion. As glucose is excreted, its plasma levels reduces, leading to an improvement in all the glycaemic parameters.[72]

Hyperglycemia per se is known to provoke oxidative stress and endoplasmic reticulum stress that in turn can induce the generation of various proinflammatory mediators. These pro-inflammatory mediators may further lead to inflammation in pancreatic islets and peripheral tissues and thereby cause insulin resistance in the peripheral tissues.[80]Considering the role of hyperglycaemia in inflammation, it can be explained that anti-inflammatory effect of SGLT-2 inhibitors observed in the previous study could be attributed to their property to reduce blood glucose levels rather than direct anti-inflammatory effects. Similarly, many earlier clinical and experimental studies have documented SGLT-2 inhibitors to decrease various inflammatory markers locally and systemically, giving an indirect evidence of their anti-inflammatory activities.In short-term (2- to 12-week) studies in patients with T2DM Dapagliflozin, has been reported to exert dose-dependent glucosuria in healthy volunteers. It has also been reported to reduce the oxidative stress by virtue of suppressing ROS generation derived by Nox4 and high-glucose-induced ROS generation.[75] Dapagliflozin treatment has been reported to decrease the inflammatory cytokine mRNA levels like IL-1β and IL-6[76] in acute phase of cardiac inflammation of MI in Wistar rats.

Similarly, Canagliflozin demonstrated suppression of inflammatory responses in skeletal muscle of high fat diet induced inflammation in mice. This action was attributed to increased glucose excretion and decreased hyperglycemia by canagliflozin.[78]Several recent studies have also reported canagliflozin administration

to improve pro-inflammatory MCP-1,IL-6 and ICAM-1 gene expression in blood vessels of rodent models of diabetes.[79]Also, Canagliflozin inhibits IL-1β-stimulated secretion of the key pro-inflammatory cytokine IL-6 and chemokine MCP-1 in cultured human umbilical vein endothelial cells (HUVECs) and aortic endothelial cells (HAECs).[79]These discrepancies with the findings of the present study can also be explained by virtue of variation in species and experimental methodology.

However, Studies exhibiting direct evidences of cellular events and vascular changes with respect to inflammation are very much lacking in above mentioned studies.

Hence, based on the findings of present study and the literature reviewed, it can be concluded that SGLT2 inhibitors based therapy do not have anti-inflammatory effect independent of their glucose lowering potential. Thus, SGLT2 inhibitors based therapy might offer the advantage of reducing inflammation associated with diabetes. But they do not possess potential to be used as independent anti-inflammatory agents. However, this speculation needs to be confirmed by checking the effects of the drugs on inflammation in diabetic rats, which is the limitation of the present study. Moreover, a chronic model of inflammation with regular follow up would help to provide more meticulous information.

CONCLUSION

The results of present study exhibited that dapagliflozin and canagliflozin did not exert significant anti-inflammatory effect in acute and subacute models of inflammation. The anti-inflammatory effect seen in previous studies could be attributed to their hypoglycemic effect and not direct anti-inflammatory effect. However, these findings need to be confirmed in inflammatory models in diabetic animals.

SUMMARY

The present study was conducted to study the effect of SGLT-2 inhibitors i.e dapagliflozin and canagliflozin on acute (carrageenan induced rat paw edema) and subacute (foreign body induced granuloma) models of inflammation in male Wistar rats.

These drugs did not exert significant reduction in rat paw edema in acute model. Similarly the experimental drugs did not significantly reduce granuloma dry weight in sub acute model as compared to control and aspirin group. Histopathological H&E examination of grass piths revealed marked reduction in granulation tissue, fibroblasts, and collagen in aspirin group as compared to control. While in the drug treated groups, fibroblasts, granulation tissue and collagen were seen to be abundant with no marked reduction. The results indicate that dapagliflozin and canagliflozin do not exhibit independent anti-inflammatory activity as such.

Since inflammation forms the foundation for diabetes mellitus and its various macrovascular and microvascular complications like diabetic retinopathy, nephropathy, drugs that simultaneously decrease inflammation, may be of additional benefit in the management of cardiovascular disorders based on its anti-inflammatory property in addition to its glucose lowering action. Existing literature suggests that these drugs have been reported to exhibit anti-inflammatory activity in diabetes induced animals owing to its hypoglycemic action. Therefore it could be concluded that dapagliflozin and canagliflozin do not exhibit anti-inflammatory effect on their own but may do so by the virtue of their hypoglycemic action. However these speculations need to be confirmed in inflammatory models in diabetic animals. Clearly, the role of SGLT-2 inhibitors as anti-inflammatory agents is an area that warrants further investigation.

BIBLIOGRAPHY

1. Kumar V, Abbas AK, Fausto N, Aster JC. Robbins and Cotran Pathological basis of disease. 8th ed. Philadelphia : Saunders An imprint of Elsevier; 2010.p.83.
2. Hernandez HR, Mendia LE, Ramirez GR, Romero MA. Obesity and Inflammation: Epidemiology,risk factors and markers of inflammation. International Journal of Endocrinology. 2013; p.1-11.
3. Powers AC. Diabetes Mellitus: Diagnosis, Classification and Pathophysiology. In: Kasper DL, Hauser SL, Jameson JL, Fauci AS, Longo DL, Loscalzo J. Harrison's Principles of Internal Medicine. New York: McGraw-Hill; 2015.p.2399-400.
4. Cruz NG, Sousa LP, Sousa MO, Pietrani NT, Fernandes AP, Gomes KB. The Linkage between inflammation and Type 2 Diabetes Mellitus. Diabetes Research and Clinical Practice. 2013; 99 : 85-92.
5. Scheen AJ, Esser N, PaquotN.Antidiabetic agents: Potential anti-inflammatory activity beyond glucose control. Diabetes and Metabolism.2015;41:183-94.
6. Pillarisetti S, Saxena U. Role of oxidative stress and inflammation in the origin of type 2 diabetes- a paradigm shift. Ther. Targets 2004;(8(5): 401-408.
7. Schwartz SS, Katz A. Sodium–glucose cotransporter-2 inhibitor combination therapy to optimize glycemic control and tolerability in patients with type 2 diabetes: focus on dapagliflozin–metformin. Diabetes, Metabolic Syndrome and Obesity: Targets and Therapy. 2016;9: 71–82.
8. MatthaeiS, Bowering K, Rohwedder K,Grohl A, Parikh S. Dapagliflozin Improves Glycemic Control and Reduces Body Weight as Add-on Therapy to

Metformin Plus Sulfonylurea: A 24-WeekRandomized, Double-Blind Clinical Trial.Diabetes care.2015;1-5.

9. Terami N, Ogawa D, Tachibana H, Hatanaka T, Wada J, Nakatsuka A, EguchiJ, Horiguchi CS, Nishii N, Yamada H, Takei K, Makino H. Long-term treatment with the sodiumglucosecotransporter2inhibitor, dapagliflozin, amelioratesglucose homeostasis and diabetic nephropathy in db/db mice. PLoS One. 2014;9(6):e100777.
10. Naznin F, Sakoda H, Okada T, Tsubouchi H, WaiseT, Arakawa K et al. Canagliflozin, a sodium glucose cotransporter 2 inhibitor, attenuates obesity-induced inflammation in the nodose ganglion, hypothalamus and skeletal muscle of mice. European Journal of Pharmacology.2017;794:37- 44.
11. Scott A, Khan KM, Cook JL, Duronio V. What is ‘‘inflammation’’? Are we ready to move beyond Celsus? Br J Sports Med. 2004;38(3):248–49.
12. Benaroyo L. How do we define inflammation? SchweizRundsch Med Prax. 1994;83:1343–7.
13. Murphy HS. Inflammation. In Rubin E, Strayer DS. Rubin’s Pathology. Clinicopathologic foundations of Medicine. Philadelphia:Wolters and Kluwers Health. Lippincott Williams & Wilkins; 2015.
14. Vassileva V, Piquette Miller. Inflammation: Extinguishing the fire with in. ClinPharmacolTher. 2010;87(4):375-9.
15. Mohan H. The Text book of Pathology. 6th ed. New Delhi: JaypeeBrothers Medical Publishers Pvt Ltd; 2010.p.130-48.
16. Dey NC, Sinha D, Dey PK. Text book of Pathology. Calcutta: New Central Book Agency (Pvt) Limited; 1995.

17. Cronstein BN, Weissma G. Targets for anti-inflammatory drugs. Ann Rev PharmacolToxicol. 1995;35:449-62.
18. Muller WA. Migration of leukocytes across endothelial junctions: some concepts and controversies. Microcirculation. 2001;8:181.
19. Underhill DM, Ozinsky A. Phagocytosis of microbes: complexity in action. Annual Rev Immunol. 2002;20:825.
20. Kuna P. RANTES, a monocyte and T-lymphocyte chemotactic cytokine releases histamine from human basophils. J Immunol. 1992;149:636-42.
21. Ortiz BD, Aean K, Nelson JP. Kinetics of Transcription factors regulating the RANTES chemokine gene reveal a developmental switch in nuclear events during T-lymphocyte maturation. Mol Cell Biol. 1996;16:202-10.
22. Rang HP, Dale MM, Ritter JM, Flower RJ, Henderson G. Rang and Dale's Pharmacology. 7th ed. NewYork: Churchill Livingstone; 2012.p.130-49.
23. Morecau ME, Garbacki N, Molinaro G, Brown NJ, Marceau F, Adam A. The Kallikrien-Kinin system. Current and future of Pharmacological targets. J Pharmacol Sci. 2005;99:36-8.
24. Calixto JB, Medeiros R, Fernandes ES, Ferreira J, Cabrini DA, Campos MM. Kinin B1 receptor: Key G-Protein-coupled receptor and their role in inflammatory and painful processes. Br J Pharmacol. 2004;143:803-18.
25. David W, Thomas, Kun H, John J, Schauster R, Susan MM, et al. Contribution of individual peptide residues of human fibrinopeptide B to T-lymptocyte responses. J Exp Med. 1980;152:620-32.
26. Malmsten CL. Prostaglandins, thrombaxanes and leukotrienes in inflammation. Am J Med. 1986;80:11-7.

27. Robert D, Zipser T. Prostaglandins, thrombaxanes and leukotrienes in clinical medicine. West J Med. 1985;143:485-97.
28. Smith MJH, Ford H, Bray MA. Leukotriene B: A potential mediator of inflammation. J Pharm Pharmacol. 1980;32:517-18.
29. Maddox JF. Lipoxin B4 regulates human monocyte / neutrophil adherence and motility: design of stable lipoxin B4 analogs with increased biological activity. FASEB J. 1998;12:487-594.
30. Brunton LL, Chabner BA, Knollmann BC. Goodman & Gilman's The Pharmacological Basis Of Therapeutics. 12thed. New York: McGraw Hill Medical; 2011.
31. Roy P, Venkatramana G, Naidu MUR, Usharani P. Recent trends in nitrogen nervous system. Indian J Pharmacol. 2005;37(2):69-76.
32. Rubanyi GM. Vascular effects of oxygen derived free radicals. Free Radic Bio Med. 1998;4:107-20.
33. Laskin DL, Pendino KJ. Macrophages and inflammatory mediators in tissue injury. Annu Rev PharmacolToxicol. 1995;35:655-77.
34. Chandran S, Shridhar N, Veeranjaneyulu A. Nitric Oxide: Concepts, Current Perspectives and Future Therapeutic Implications. Ind J Pharmacol. 1998;30: 351-6.
35. Hemnani T, Parihar MS. Reactive oxygen species and oxidative DNA damage. Ind J Pharmacol. 1998;42:440-52.
36. Blake DR, Allen RE, Lunec J. Free Radicals in Biological systems. A review oriented to inflammatory processes. Br Med Bull. 1987;43:371-85.
37. Gilligan JP. Modulation of carrageenan induced hind paw edema by Substance-P. Inflammatory. 1994;18:285-92.

38. Thomas KL. Substance-P induced hydrolysis of inositol phospholipids in rat skin in an invivo model of inflammation. Neuropeptides. 1989;13:191.
39. Cronstein BN. Neutrophil adherence to endothelium is enhanced via Adenosine A1 receptor and inhibited via Adenosine A2 receptor. J Immunol. 1992;148:2201-6.
40. Turner RA. Screening Methods in Pharmacology. New York and London: Academic Press Inc;1965.
41. VogelHG.Drug discovery and evaluation: Pharmacological Assay. 3rd ed. New York: Springer-Verlag; 2008.
42. Mathew A, Taranalli AD, Torgal SS. Evaluation of Anti-inflammatory and Wound Healing Activity of Gentianalutea Rhizome Extracts in Animals. Pharm Biol. 2004;42(1):8–12.
43. Medhi B,Prakash A. Practical manual of experimental and clinical pharmacology. 1sted. New Delhi:Jaypee Brothers Medical Publishers (P) Ltd; 2010.
44. Laurence DR, Bacharach AL. Evaluation of Drug Activities: Pharmacometrics Vol. 2. New York and London: Academic Press Inc; 1964.
45. Patil PA, Kulkarni DR. Effect of anti proliferative agents on healing of dead space wounds in rats. Ind J Med Res. 1984;74:445-7.
46. Tripathi KD. Essentials of Medical Pharmacology. 7thed. New Delhi: JAYPEE Brothers Medical Publishers Limited; 2013.
47. Holsapple MP. Central and peripheral anti-inflammatory actions by clonidine and a structurally related imidazoline analogue. J PharmacolExpTher. 1984;229: 399-405.

48. Hashimoto K. Immunomodulatory effects of therapeutic gold compounds-Gold sodium thiomalate inhibits the activity of T-cell protein kinase-C. J Clin Invest. 1992;89:1839-48.
49. Lipsky PE, Ziff M. Inhibition of antigen and mitogen induced human lymphocyte proliferation by gold compounds. J ClinInvest. 1997;59:455-66.
50. Ruppert SM, Falk BA, Long SA, Bollyky PL. Regulatory T cells resist cyclosporine-induced death via CD44-mediated signalling pathways. Int J Cell Biol. 2015:1-10.
51. Quesniaux VFJ. Immunosuppressants: Tools to investigate the physiological role of cytokines. Bioassays. 1993;15:731-9.
52. Perenyei M, Jayne DRW, Flobmann O. Gusperimus: Immunological mechanism and clinical implications. Rheumatology. 2014;53:1732-41.
53. Tanaka T, Hishitani Y, Ogata A. Monoclonal antibodies in rheumatoid arthritis: comparative effectiveness of tocilizumab with tumor necrosis factor inhibitors. Biol. Targets Ther. 2014;8:141–53.
54. Chen M, Yang Z, Wu R, Nadler JL. Lisofylline, a novel anti-inflammatory agent, protects pancreatic beta-cells from proinflammatory cytokine damage by promoting mitochondrial metabolism. Endocrinology. 2002;143(6):2341-8.
55. Baldassare JJ, Bi Y, Bellone CJ. The Role of p38 mitogen-activated protein kinase in IL-1beta transcription. J Immunol. 1999;162:5367-73.
56. Dembic Z. Cytokines of the Immune System - Interleukins. In Dembic Z. The Cytokines of the Immune System. Elsevier Inc; 2015:143–239.
57. Khaksari M, Mahani SE, Mahmoodi M. Calcium channel blockers reduce inflammatory edema in the rat: Involvement of the hypothalamus-pituitary-adrenal axis. Indian J Pharmacol. 2004;36(6):351-54.

58. Holsapple MP. Central and peripheral anti-inflammatory actions by clonidine and a structurally related imidazoline analogue. J PharmacolExpTher. 1984;229: 399-405.

59. Green KL. The anti-inflammatory effect of catecholamines in peritoneal cavity and hind paw of mouse. Br J Pharmacol. 1972;45:322-32.

60. Perng DW, Tao CW, Su KC, Tsai CC, Liu LY, Lee YC. Anti-inflammatory effects of salmeterol/ fluticasone, tiotropium/fluticasone or tiotropium in COPD. EurRespir J. 2009;33:778-84.

61. Jadhav AD, Padwal SL, Jadhav RR, Jadhav SS, Pise HS, Choure MK. Anti-inflammatory property of Salbutamol on Acute and Chronic Models of Inflammation. Natl J Physiol Pharm Pharmacol. 2015;5(2):101-4.

62. Green KL. Role of endogenous catecholamines in the anti-inflammatory activity of α-adrenoceptor blocking agents. Br J Pharmacol. 1974;51:45-53.

63. Leite LM, Carvalho AG, Ferreira PF, Pessoa IX, Goncalves DO, Lopes AA, et al. Anti-inflammatory properties of Doxycycline and Minocycline in experimental models: an in vivo and in vitro comparative study. Inflammo pharmacology. 2011;19:99–110.

64. Goldberg RB. Cytokine and Cytokine like Inflammatory markers, endothelial dysfunction and imbalanced coagulation in development of diabetes and its complications. J Clin Endocrinol Metab. 2009;94:3171-82.

65. Katz P, Leiter LA, Mellbin L, Ryden L. The clinical burden of type 2 diabetes in acute coronary syndromes : Prognosis and Implications for short and long term management. DiabVasc Dis Res. 2014;11(6):395-409.

66. R-56(68)Gregor MF, Hotamisligil GS. Inflammatory mechanism in obesity. Annu Rev Immunol. 2011;29:415-45.

67. Akash MS, Rehaman K, Chen S. Role of Inflammatory mechanisms in pathogenesis of type 2 diabetes mellitus. J Cell Biochem. 2013;114:525-31.
68. Scott A, Khan KM, Cook JL, Duronio V. What is ‘‘inflammation’’? Are we ready to move beyond Celsus? Br J Sports Med. 2004;38(3):248–49.
69. Benaroyo L. How do we define inflammation? SchweizRundsch Med Prax. 1994;83:1343–7.
70. Dandona P, Chaudhuri A. Sodium-glucose co-transporter 2 inhibitors for type 2 diabetes mellitus: An overview for the primary care physician. International Journal of Clinical Practice. 2017;71(5):e12937.
71. White J. Apple Trees to Sodium Glucose Co-Transporter Inhibitors: A Review of SGLT2 Inhibition. Clinical Diabetes. 2010;28(1):5-10.
72. Kalra S. Sodium Glucose Co-Transporter-2 (SGLT2) Inhibitors: A Review of Their Basic and Clinical Pharmacology. Diabetes Therapy. 2014;5(2):355-366.
73. Isaji M. SGLT2 inhibitors: molecular design and potential differences in effect. Kidney International. 2011;79:S14-S19.
74. gillman g. the pharmacological basis of therapeutics. 13th ed. New York Chicago San Francisco Athens London Madrid Mexico City Milan New Delhi Singapore Sydney Toronto: mc graw hill; 2019.
75. Kagal UA, Angadi NB, Matule SM. Effect of dipeptidyl peptidase 4 inhibitors on acute and subacute models of inflammation in male Wistar rats: An experimental study. Int J Appl Basic Med Res. 2017;7(1):26–31.
76. Lahnwong S, Chattipakorn S, Chattipakorn N. Potential mechanisms responsible for cardioprotective effects of sodium–glucose co-transporter 2 inhibitors. Cardiovascular Diabetology. 2018;17(1).

77. Terami N, Ogawa D, Tachibana H, Hatanaka T, Wada J, Nakatsuka A et al. Long-Term Treatment with the Sodium Glucose Cotransporter 2 Inhibitor, Dapagliflozin, Ameliorates Glucose Homeostasis and Diabetic Nephropathy in db/db Mice. PLoS ONE. 2014;9(6):e10077

78. Xu C, Wang W, Zhong J, Lei F, Xu N, Zhang Y et al. Canagliflozin exerts anti-inflammatory effects by inhibiting intracellular glucose metabolism and promoting autophagy in immune cells. Biochemical Pharmacology. 2018; 152:45-59.

79. Mancini SJ, Boyd D, Katwan OJ, Strembitska A, Almabrouk TA, Kennedy S, Palmer TM, Salt IP. Canagliflozin inhibits interleukin-1β-stimulated cytokine and chemokine secretion in vascular endothelial cells by AMP-activated protein kinase-dependent and-independent mechanisms. Scientific reports. 2018 Mar 27;8(1):5276.

80. Akash M, Rehman K, Chen S. Role of Inflammatory Mechanisms in Pathogenesis of Type 2 Diabetes Mellitus. J Cell Biochem. 2013;114:525–31.

81. Mashrani U. Diabetes Mellitus and Hypoglycemia. In Papadakis MA, McPhee SJ. Current Medical Diagnosis and Treatment. 57thed. New York: McGraw Hill; 2018:1237-38.

ANNEXURE- I- ETHICAL CLEARANCE LETTER

KLE ACADEMY OF HIGHER EDUCATION AND RESEARCH
(Deemed to be University)
JAWAHARLAL NEHRU MEDICAL COLLEGE,
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Shri Sunil.R.Patil.
Non-scientific Social worker,
Nidasosi.

Dr. Sudha Devareddy.
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Dr. Vishwanatha Swamy AHM
Link Nominee CPCSEA.
Dept of Pharmacology &
Toxicology
KLE's Coll Of Pharmacy,
Hubballi

CERTIFICATE

This is to certify that the M.Sc/ M.D/ Ph.D/ Research project Entitled " Effect of Selective Sodium Glucose Transporter (SGLT-2)inhibitors- Dapagliflozin and Canagliflozin on Acute and subacute Models of Inflammation in Male Wistar Rats – an experimental study"

Submitted by Reg.No.BO0117001 Dept of Pharmacology

Has been approved by the Intitutional Animal Ethical Committee Meeting held on 27-1-2018 vide Resolution No. 9/2

For sanction of 24 Male Wistar Rats.

Signature and Name :
CPCSEA-Main Nominee

Signature and Name :
Chairman/Mem.Secretary