

COMPARATIVE ANTI-INFLAMMATORY STUDIES ON TIMOLOL MALEATE AND DICLOFENAC SODIUM IN, *IN VITRO* AND *IN VIVO* MODELS BY ORAL ROUTES

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ABSTRACT

The present study was conducted on timolol maleate (a non-selective beta-blocker) using *in vitro* assays and *in vivo* models of rats. It was tested for its anti-inflammatory activity at three dose levels (1.028 mg kg⁻¹, 5.14 mg kg⁻¹ and 10.28 mg kg⁻¹). Furthermore, the investigation was supported by the estimation of tumor necrosis factor-alpha (TNF- α), interleukin-1 (IL-1) and histopathological examination. It was observed that at 1.028 mg kg⁻¹, 5.14 mg kg⁻¹ and 10.28 mg kg⁻¹ the drug showed anti-inflammatory activities. Moreover, plasma levels of TNF- α and IL-1 showed inhibition. Histopathological examination confirmed the highest anti-inflammatory activity at 5.14 mg kg⁻¹. Thus, the conducted studies revealed that timolol maleate, when given orally does possess an anti-inflammatory potential.

Keywords: Inflammation, beta-blocker, timolol maleate, carrageenan, paw-edema, diclofenac sodium, Interleukins

INTRODUCTION

Due to diapedesis, inflammation causes mast cells to become activated and release chemical mediators like free radicals, serotonin, prostacyclin, prostaglandin, neutrophils, nitric oxide (NO) and histamine at the site of action¹. Furthermore, due to the release of mast cells and histamine, vasodilation occurs simultaneously, after which macrophages actively take charge of the removal of damaged tissue and cells at the site of action². Further, inflammation gets promoted due to the release of thymus cells and bone marrow cells³. Inflammation causes signs of redness because of the dilation of blood vessels and swelling due to the release of white blood cells (WBCs). Whereas, due to the release of chemical mediators and compression of nerves at the site of injury the movement of fluids and pain sensation occurs. It is a highly controlled process, and depending on how severe it is, dysregulation may result in illnesses including acute, subacute, or chronic inflammation^{2,4}.

The first response of the immune system is toward pathogens and tissue injury, termed as acute inflammation. It is activated by vasoactive amines and eicosanoids, which increases the movement of plasma and leukocytes infected sites, which further leads to heat, redness, pain edema

and loss of function. Acute inflammation helps to defend the body from infections, and it lasts for a short period and is most of the time regarded as a therapeutic approach. Prostaglandins and leukotriene, which are known as pro-inflammatory mediators, play a very essential role in the early inflammatory response^{4,5}. In contrast, growth factors and cytokines are produced during chronic inflammation, which attracts fibroblasts, leukocytes, and lymphocytes. In addition to these illnesses, chronic inflammation can also cause cancer, atherosclerosis, diabetes, obesity, lung, neurological, cardiovascular illnesses and rheumatoid arthritis. Inflammatory mediators induce an inflammatory response and tissue damage. These mediators are generated through the up-regulation of inducible nitric oxide synthase (iNOS) and inducible pro-inflammatory genes cyclooxygenase 2 (COX-2). During the process of inflammation, many NO and prostaglandins are generated by the inducible iNOS and COX-2. They have been measured with the pathophysiology of inflammatory disorders as well as certain types of human cancers^{6,7}.

To achieve a therapeutic anti-inflammatory response, a drug should be able to have an inhibition effect against inflammatory and pro-inflammatory mediators⁸. The most utilized medicines are anti-inflammatory drugs (steroidal and non-steroidal)⁹. Corticosteroids are utilized for RA and asthma but show side effects of hypertension, high blood sugar levels, glaucoma, cataracts and osteoporosis¹⁰. Whereas, NSAIDs are known for their side effects such

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as stomach ulcers and gastric bleeding when used for chronic conditions over a long period^{10,11}.

Moreover, in past decades, the sympathetic nervous system has been involved in inflammatory cascade. It has been explored and findings have revealed that both sympathetic agonists and sympathetic antagonists show potency as anti-inflammatory drugs¹². These results led to the conclusion that the adrenoceptor does play a significant role in the process of inflammation but further, also the question arises about its exact mechanism of action, e.g., propranolol has shown significantly reduced levels of Interleukin-13 and tumor necrosis factor-alpha (TNF- α) in a dose-dependent manner¹³. Whereas, metoprolol has shown a significant decrease in the serum levels of TNF- α specifically in patients suffering from dilated cardiomyopathy¹⁴. Earlier studies concluded that beta-blockers play an important immunoregulatory role and thereby participate in modifying the dysregulated cytokine network¹⁴. Techniques for assessing *in vitro* cell adhesion have been investigated for studying pharmacological action¹⁵. For the current research study, timolol maleate (test drug) *in vitro* and *in vivo* models anti-inflammatory potential was undertaken. It inhibits the levels of interleukins 6, 8, 1 β , and TNF- α in aqueous humor¹⁶.

MATERIALS AND METHODS

Chemicals

Carrageenan was obtained from Sigma Aldrich Chemicals Pvt. Ltd., Bangalore. Diclofenac sodium, timolol maleate and bovine serum albumin were procured as gift samples from Neon Laboratories Limited, Mumbai, FDC Pvt. Ltd., Mumbai and Himedia Pvt. Ltd., Mumbai, respectively.

Preparation of drug solutions

100 mg of carrageenan powder (1 %) was prepared in phosphate buffer saline (PBS, pH 7.4) of 10 mL. 2 g (2 %) was dissolved in 100 mL saline (0.9% w/v). 100 mg of diclofenac sodium (standard) and 10.28 mg, 51.4 mg and 102.8 mg of timolol maleate (test drug) were weighed and dissolved in 10 mL distilled water. All the solutions were freshly prepared and used within one week.

In vitro protein denaturation assay

Inhibition of protein denaturation study was done using bovine serum albumin BSA. 2 mL of 1% BSA solution was added, to 2 mL of the test drug (100, 200, 500, and 1000ppm), and the standard (100, 200, 500, and 1000 ppm) solution. In the case of control, 2 mL of BSA was

added to 2 mL of PBS (pH 7.4). After incubation for 15 mins at 37 \pm 2 $^{\circ}$ C, the mixture was heated for 5-10 mins at 70 $^{\circ}$ C for its denaturation process. After the liquid, had cooled for 15 min, a spectrophotometer set to 660 nm was used to measure absorbance. The assay was done in triplicates, and the formula used was^{17,18,19}.

$$\% \text{ Inhibition of denaturation} = \frac{[(\text{Mean abs. sample} - \text{Mean abs. of control}) - 1]}{\text{Mean abs. of control} - 1} \times 100$$

In vitro membrane lysis assay

Whole blood was collected from a healthy albino Wistar rat. The rat was not administered with NSAIDs for the last 2 weeks before the experiment and blood was collected in an anticoagulant-containing tube. The blood was washed 3 times using the equivalent amount of 0.9% w/v saline and centrifuged for 10 min at 3000 rpm. Later, a 10% V/V erythrocytes/RBC suspension was made using PBS (pH 7.4). Then 0.05 mL of RBC suspension and 0.05 mL of test drug and standard (at different conc. 100, 200, 500, 1000 ppm) were mixed with 2.95 mL of PBS (pH 7.4). Later the mixture was incubated in an orbital shaking incubator for 20 mins at 54 \pm 1 $^{\circ}$ C. After incubation, the mixture was cooled down and centrifuged at 2500 rpm for 3 mins. The absorbance was measured at 540 nm after the supernatant was collected. The assay was performed in triplicates and the levels of haemolysis were calculated by the formula^{20,21}.

$$\% \text{ Inhibition of haemolysis} = \frac{[(\text{Abs. of sample} - \text{Abs. of control}) - 1]}{\text{Abs. of control} - 1} \times 100$$

Study groups

Male albino Wistar rats weighing 140-180 g were obtained from India's National Institute of Biosciences, Pune. Rats were housed in a plastic perspex cage at a temperature of 25 \pm 10 $^{\circ}$ C and relative humidity of 45-55 % under light and dark cycle (12h:12h). Chow pellets and purified water were easily accessible to rats (*ad libitum*). Rats were acclimatized for one week before the commencement of experiments. The protocol was approved (CPCSEA/IAEC/P-010 Dt 23/1/2021) by the ethics committee. Rats were divided (n=36) and treated orally.

Group 1- Vehicle (0.9 w/v saline, sterile).

Group 2- Disease control (1mg/0.1 mL/paw Carrageenan).

Group 3 -Standard (10 mg kg⁻¹ diclofenac).

Groups 4, 5, and 6 were given 1.028 mg kg⁻¹, 5.14 mg kg⁻¹, and 10.28 mg kg⁻¹ of timolol maleate (test dose), respectively.

Carrageenan-induced hind paw edema model

In the model (paw edema), the test drug and standard were administered via the oral route of administration. 30 mins later, 0.1 mL (1% carrageenan solution) was injected into the rat right hind paw plantar side and the paw volume was measured (Vernier calliper) before and after the injection of carrageenan at the time intervals of 0, 90, 180, 270, and 360 mins^{22,23}.

Carrageenan-induced air pouch model

In the air pouch model, 6 days before the induction of inflammation, an air pouch was generated under the effect of anesthesia. 20 mL sterile air was injected subcutaneously (s.c) at the shaved dorsal cervical region of the rats to create the air pouch. 3 days later, the rats were anesthetized again, and 10 mL of sterile air was injected into the pouch. The rats were given another round of anaesthesia six days following the initial air injection, and 2 mL (2% carrageenan solution) was injected into the pouch to cause inflammation. The injection for the test drug and standard were administered 30 mins before carrageenan treatment. At different time points (4 h, 8h and 24h) after carrageenan injection, the rats were sacrificed and the entire exudate volume of the air pouch was collected and WBC estimation was done^{24,25}.

TNF- α and IL-1 estimation

Blood samples were collected in an EDTA-containing tube at the endpoint in both *in vivo* models. The samples were then centrifuged for 10 mins at 1000 x g within 30 mins of collection. TNF- α and IL-1 levels were then assayed using a kit that uses an enzyme-linked immunosorbent assay based on the biotin double antibody sandwich technology (Krishgen Biosystems, India). The assay was carried out using an ELISA microplate reader (Epoch, India). Samples and standards were pipetted into microwells and IL-1 and TNF- α present in the sample were bound by the antibodies. Further, the sample was incubated to form a complex. The substrate solution was

added to the microwells after washing them to get rid of any non-specific binding. As a result, colour changes are based on the concentrations of IL-1 and TNF- α in the sample. Then, using the standard concentration and standard absorbance, the standard curve was plotted to determine the concentration of TNF- α and IL-1. The optical density of each well was measured with a microplate reader at 450 nm within 30 mins of delivering the stop solution.

Histopathological examination

Rats were sacrificed, and the exudate was collected from the air pouch, s.c skin of the air pouch was fixed with neutral-buffered 10% formalin, and then further tissues were trimmed transitionally and routinely processed. Processing of the tissue involved dehydrating it, in progressively stronger alcohols, making it clear in xylene, and embedding it in paraffin wax. The Microtome cut tissue blocks with paraffin wax embedded into pieces that were 4-5 μ m thick. The slides of vital organs were stained with hematoxylin and eosin and examined under a microscope (at Low Power 100X and High Power 400X). The discovered lesions were classified as having the following severity levels of scores with focal, multifocal, and diffuse distributions, the severity levels are very severe (++++; 76–100%), severe (51–75%; +++), moderate (++; 26–50%), minimal (0; 1%) and mild (+; 1-25%).

Statistical analysis

The GraphPad Prism 5 (64-bit Windows version) was employed. A one-way analysis of variance (ANOVA) and Dunnett (multiple comparisons) test were conducted after statistical analysis. The results were expressed using Mean + SD.

RESULTS

In vitro protein denaturation assay

It was observed (Table I) that the inhibition of bovine serum albumin was in a concentration-dependent

Table I: *In vitro* assays of diclofenac sodium and timolol maleate

Concentration (ppm)	Diclofenac sodium (% inhibition)		Timolol maleate (% inhibition)	
	Protein denaturation assay	Membrane lysis assay	Protein denaturation assay	Membrane lysis assay
10	4.74	57.07	-1.96	40.44
20	9.48	63.35	0.98	54.67
40	12.5	54.98	5.88	60.89
60	13.79	63.35	1.98	44.89
80	15	68.58	4.9	65.33
100	15.9	68.58	5.39	65.33

manner. As the concentration increases, the percentage inhibition of the protein denaturation increases. It can be concluded that, the standard had shown much greater inhibition activity as compared to the test drug.

In vitro membrane lysis assay

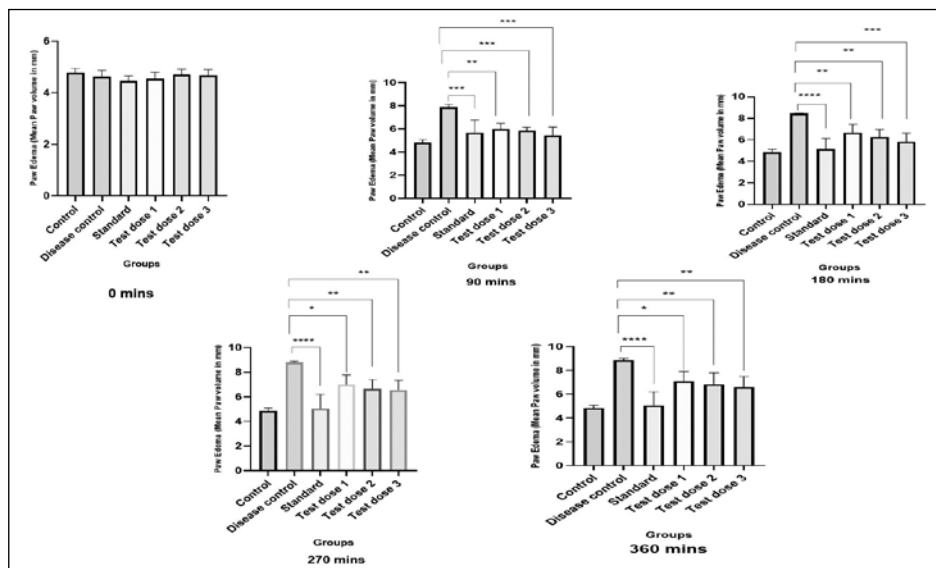
The inhibition of the membrane lysis was observed to be concentration-dependent by both the test drug and standard (Table I). It was observed that at 10 ppm, the inhibition was 57.07 % and 40.44 % for standard and test drugs, respectively. At 100 ppm it was 68.58 % and 65.33 % for standard and test drugs, respectively.

Carrageenan-induced hind paw edema model

Standard showed the highest % inhibition at 360 mins to be 83.4 ± 0.54 and its lowest effect was observed to be 59.94 ± 0.54 at 90 mins. The highest inhibition of the test drug was observed for 10.28 mg kg^{-1} at 90 mins as 82.19 ± 0.36 and the lowest inhibition was observed for 1.028 mg kg^{-1} at 46.42 ± 0.39 at 360 mins (Fig. 1).

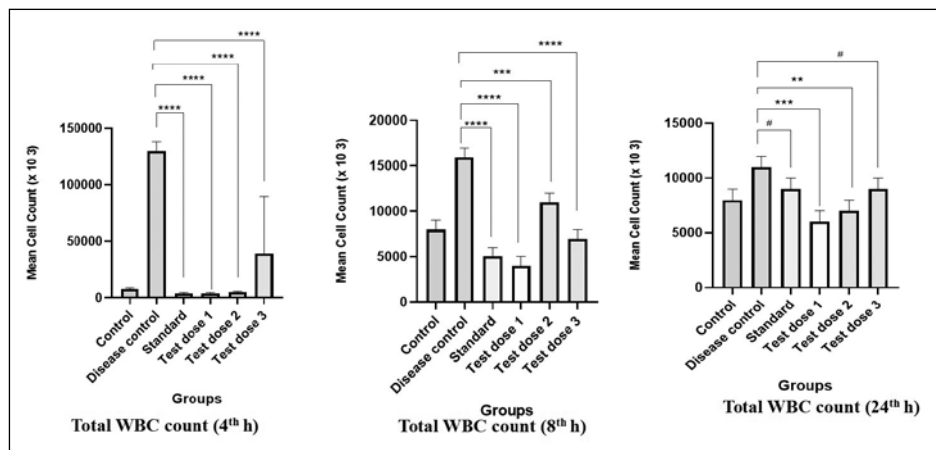
Carrageenan-induced air pouch model

There was a significant inhibition observed between all the groups at the time point 4th h and 8th h of sacrifice. For the time, point 24th h there was no significant difference observed between the standard and 10.28 mg kg^{-1} (Fig. 2).



A one-way analysis of variance (ANOVA), then multiple comparisons using Dunnett's formula. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$)

Fig. 1: Carrageenan-induced hind paw edema model of different groups



A one-way analysis of variance (ANOVA), then multiple comparisons using Dunnett's formula. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$)

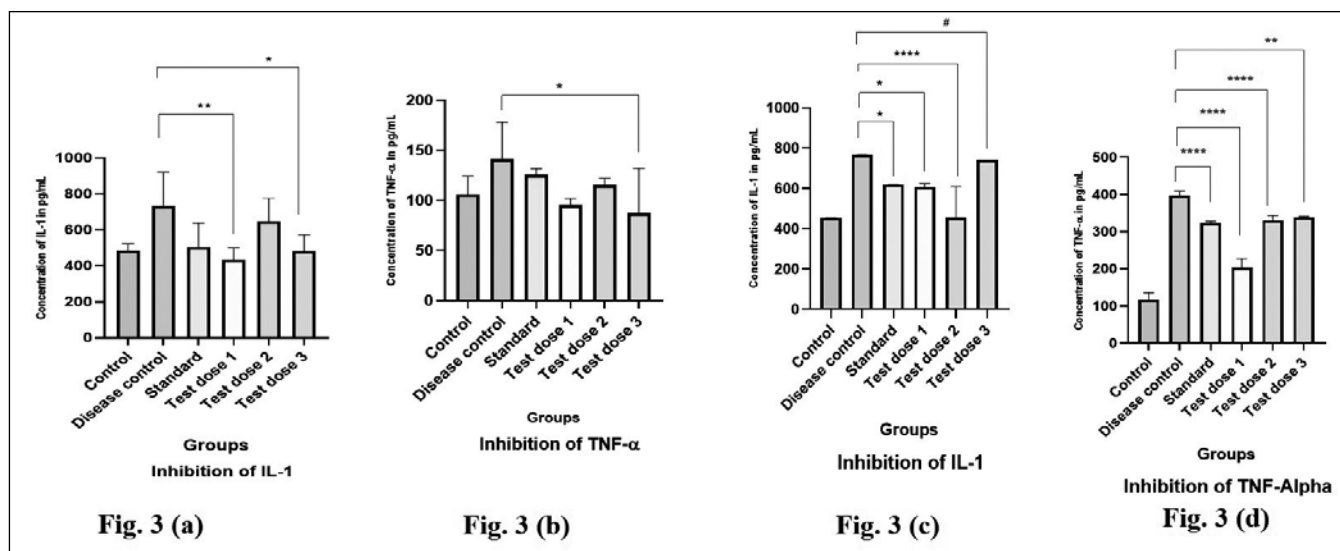
Fig. 2: Carrageenan induced air pouch model at different time intervals

Measurement of TNF- α and IL-1

In carrageenan induced paw edema, there was a significant inhibition observed between disease control and treated groups (10.28 mg kg^{-1}) in TNF- α , whereas, for IL-1, there was a significant inhibition observed between the disease control and treated groups (1.028 mg kg^{-1} and 10.28 mg kg^{-1}). Whereas in carrageenan induced air pouch there was a significant inhibition observed between all the groups in TNF- α , but no significant difference was observed between disease control and 10.28 mg kg^{-1} for IL-1 (Fig.3 a, b,c,d).

Histopathological examination

Histopathological examination at different intervals revealed that at the 4th h: Severity from lowest score to highest: control < TM dose 2 < standard < disease control < TM dose 1 < TM dose 3; at 8th h: control < TM dose 2 < standard < TM dose 1 < disease control < TM dose 3, and at 24th h: control < TM dose 1 < standard < TM dose 2 < disease control < TM dose 3. It was concluded that the standard significantly reduces inflammation at the 4th and 8th h of the endpoint. The test



A one-way analysis of variance (ANOVA), then multiple comparisons using Dunnett's formula. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$)

Fig. 3: TNF- α and IL-1 levels in carrageenan induced paw edema (Fig. 3a and 3b) and carrageenan induced air pouch models (Fig. 3c and 3d)

Table II: Histopathological examination of groups

Group	4 th h	8 th h	24 th h
Vehicle control	No lesion of pathological significance was observed	No lesion of pathological significance was observed	No lesion of pathological significance was observed
Disease control	Mild inflammatory cell infiltration was observed (+); there was mild (+) accumulation of oedematous fluid in subcutis	Moderate infiltration of inflammatory cells was observed (++); there was mild (+) accumulation of oedematous fluid in subcutis. Mild congestion (+)	Mild infiltration of inflammatory cells was observed (+); there was mild (+) accumulation of oedematous fluid in subcutis
Standard (Diclofenac 10 mg kg ⁻¹)	Mild infiltration of inflammatory cells was observed (+)	Moderate infiltration of inflammatory cells was observed (++)	There was mild (+) congestion
Test drug (Timolol maleate 1.028 mg kg ⁻¹)	Mild infiltration of inflammatory cells was observed (+); there was mild (+) accumulation of oedematous fluid in subcutis	Mild infiltration of inflammatory cells was observed (+); there was mild (+) accumulation of oedematous fluid in subcutis	No lesion of pathological significance was observed
Test drug (Timolol maleate 5.14 mg kg ⁻¹)	There was mild (+) accumulation of oedematous fluid in subcutis	There was mild (+) accumulation of oedematous fluid in subcutis	There was mild (+) accumulation of oedematous fluid in subcutis
Test drug (Timolol maleate 10.28 mg kg ⁻¹)	Mild infiltration of inflammatory cells was observed (+); there was moderate (++) accumulation of oedematous fluid in subcutis and mild congestion	Mild infiltration of inflammatory cells was observed (+); there was mild (+) accumulation of oedematous fluid in subcutis	Severe infiltration of inflammatory cells was observed (+++); there was moderate (++) accumulation of oedematous fluid in subcutis and moderate congestion (++)

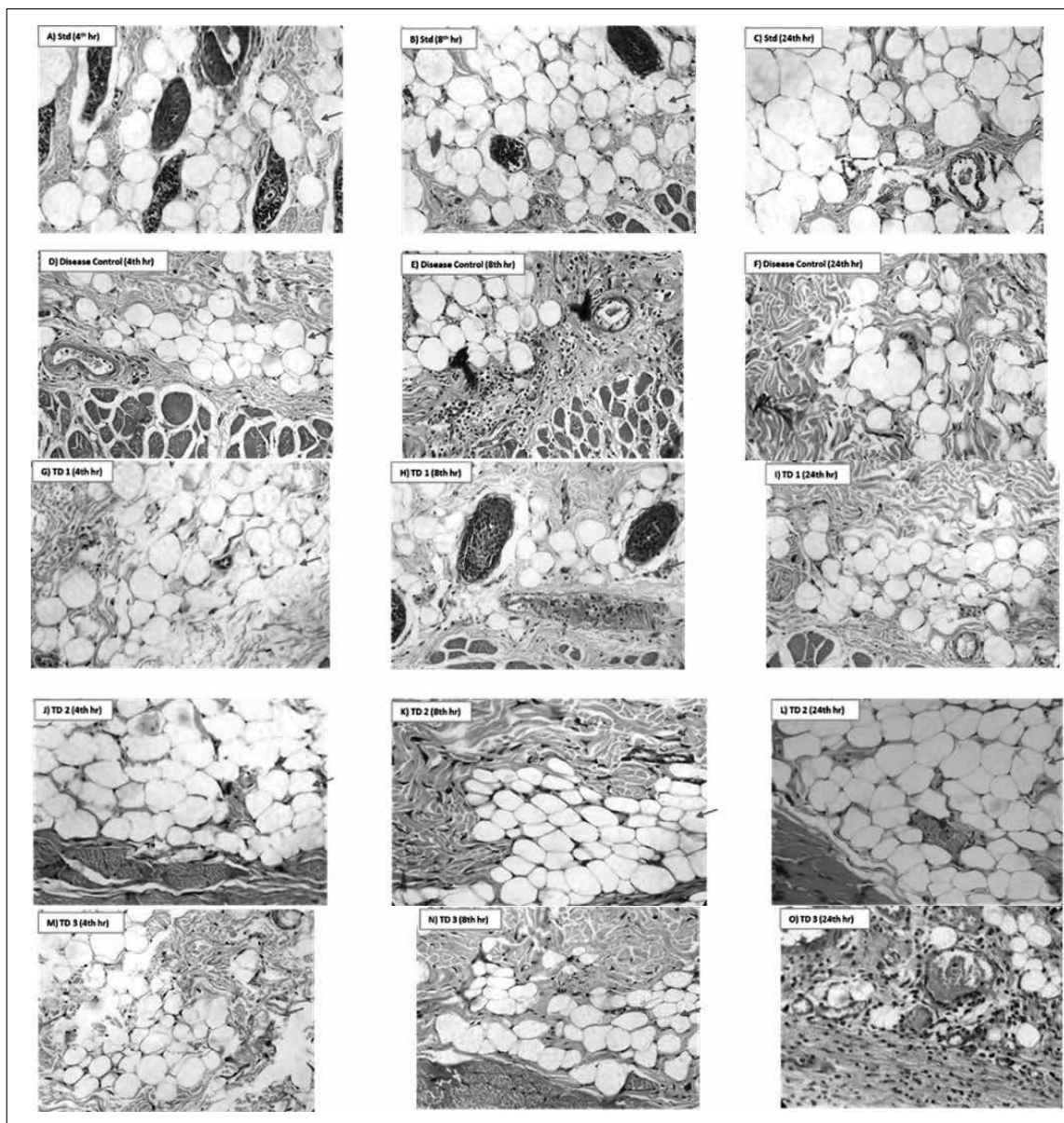


Fig. 4: Histopathological examination of different groups

drug (5.14 mg kg^{-1}) was observed to be higher than dose levels (1.028 mg kg^{-1} and 10.28 mg kg^{-1}), as depicted in Fig. 4 and discussed with observation intervals in Table II. Also, no toxicity or adverse effects were reported with the treatment approach.

DISCUSSION

There has been observed an advancement in treatment approaches that are used in the treatment of inflammatory conditions and further studies have been conducted to identify newer and novel treatment strategies. Likewise, the sympathetic nervous system has also been explored and findings have revealed that

it does collide with the inflammatory cascade and has the potential to become a potent target for the treatment of many inflammatory conditions in the future. All the experimental models selected for the research study have been designed in such a way that it includes acute and chronic phases of the inflammation¹².

Inhibition of denaturation of protein assay was conducted as it is known to be a well-documented cause of inflammation. BSA becomes denaturated when heated, indicating the presence of an antigen linked to a type III hypersensitivity reaction, which is connected to conditions like RA, glomerulonephritis, and systemic

lupus erythematosus. As a result, the assay is frequently employed for medications that can prevent the protein from becoming denaturated. Anti-inflammatory medications have been found to exhibit a dose-dependent capacity for thermally induced protein denaturation activity^{17,19}. In this study, the standard showed better activity than the test drug, thus revealing the activity of protein denaturation^{18,19}.

Inhibition of membrane lysis assay was conducted because it is considered that the erythrocyte (RBC) membrane resembles a lysosomal membrane. The integrity of a cell's membrane determines whether it will survive. When RBCs are exposed to harmful substances like hypotonic media, their membranes lyse, causing hemolysis and the oxidation of haemoglobin. A lesion to the RBC membrane makes the cell much more vulnerable to subsequent damage due to lipid peroxidation caused by free radicals. According to studies, stabilizing the lysosomal membrane is essential for limiting the extracellular escape of lysosomal components of activated neutrophils, including as proteases and bactericidal enzymes, which result in tissue inflammation and injury^{18,19}. It was observed that the test drug has an effective potency in stabilizing the RCB membrane in comparison to the standard.

The most frequent and widely utilized method to evaluate an anti-inflammatory compound's potential is carrageenan-induced paw edema. Edema shown following carrageenan injection is a sign of acute inflammatory alterations, which may be determined by the values of volume differential in the paws. The inflammation caused by the s.c injection of carrageenan into the rat paw is distinguished by increased tissue water and plasma arachidonic acid metabolism by the pathways of cyclooxygenase and lipoxygenase enzymes. Carrageenan-induced edema involves two phases. The initial phase starts immediately after injection and fades out about an hour. Early hyperemia is caused by the release of histamine and serotonin during this phase. After 1 h, the second phase starts. Prostaglandin and cyclooxygenase enzymes are released during this delayed phase^{20,21,22}. It has been discovered that the anti-inflammatory patterns of test medications and standards varied.

The airpouch model is used to research granulomatous inflammation, oxidative stress assessment and acute inflammatory response resolution, in addition to acute and chronic inflammation. When air is s.c. injected into the thoracic region of the back of rats, the cellular lining of the pouch undergoes morphological change, resulting in a lining like that of the synovial cavity. Exudate quantities were measured because they vary amongst rats while

receiving the same amount of lavage solution from the injection. This exudate volume is used to evaluate a test drug's ability to reduce inflammation. Carrageenan injection into the air pouch causes a significant increase in the synthesis of biochemical mediators like cytokines and leukotrienes. An increase in these mediators was observed within 1 h of carrageenan injection and peaks at various time intervals^{22,24,25}. The anti-inflammatory activity of the test drug (10.28 mg kg⁻¹) was higher than its other two dose levels (5.14 mg kg⁻¹ and 1.028 mg kg⁻¹) and was comparable to the standard. Hence, in both *in vivo* models, the standard had shown time-dependent activity which means the activity increases as time increases. Test drug has shown a dose-dependent activity, and its potency decreases as time increases.

TNF- α and IL-1 initiate inflammatory responses causing changes in the immune response of the body. Studies suggest that plasma levels of TNF- α , and IL-1 are generally high, among patients suffering from any inflammatory conditions. In the case of the disease control, a higher concentration of TNF- α and IL-1 was observed. Conclusions from the current study include the observation that the treatment group has helped to decrease the levels of TNF- α and IL-1 in blood plasma as compared to the disease control. Histopathological findings were illustrated in the form of lesions and observed as very severe (++++; 76–100%), severe (51–75%; +++), moderate (++; 26–50%), minimal (0; 1%) and mild (+; 1-25%), they were recorded as focal, multifocal, and diffuse. It was concluded that the standard significantly reduces inflammation at the 4th and 8th h of the endpoint. Whereas, the test drug (5.14 mg kg⁻¹) was observed to be higher than at other dose levels (1.028mg kg⁻¹ and 10.28 mg kg⁻¹). Also, no toxicity or adverse effects were reported with the treatment approach. Thus, it can be inferred that timolol maleate exhibits an anti-inflammatory effect against classical models of inflammation at selected dose levels.

CONCLUSION

The studies undertaken suggest that timolol maleate can be a valuable investigational candidate that possesses an anti-inflammatory effect to counter various inflammatory disorders. Further preclinical studies are required to confirm its potency in comparison to marketed NSAID formulations.

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