Review Article
Available online at www.ijpras.com

ISSN 2277-3657

Volume 1, issue2 (2012), 01-05

International Journal of Pharmaceutical Research & Allied Sciences

In Vivo Animal Models in Preclinical Evaluation of Anti-Inflammatory Activity- A Review

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Abstract:

Inflammation is defined as the local response of living mammalian tissues to injury due to any agent. Inflammation involves 2 basic processes early inflammatory response later followed by healing. There are two types of inflammation, Acute and Chronic. Acute inflammation is of short duration and represents the early body reaction, resolves quickly and is usually followed by healing. Chronic inflammation is defined as prolonged process in which tissue destruction and inflammation occur at same time. The anti-inflammatory activity of new substances can be evaluated by using various pre clinical screening method. Here by use of various phlogestic agent like carragenan, brewer's yeast, dextran, egg albumin, kaolin, aerosil, croton oil, cotton wool inflammation is induced and the amount of decrease in its inducing characteristics is measured.

Key words: *Inflammation, brewer's yeast, egg albumin, dextran*

Introduction:

Inflammation is defined as the local response of living mammalian tissues to injury due to any agent. It is a body defence reaction to prevent the spread of injurious agent and to remove the necrosed cells and tissues. Inflammation may be caused by like bacteria, viruses, fungi, parasites, antigen-antibody reaction. Mechanical trauma, organic and inorganic poisons and foreign bodies. Inflammation involves 2 basic processes early inflammatory response later followed by healing. Signs of inflammation includes redness, swelling, heat, pain, loss of function. There are two types of inflammation, Acute and Chronic. Acute inflammation is of short duration and represents the early body reaction, resolves quickly and is usually followed by healing. Chronic inflammation is defined as prolonged process in which tissue destruction and inflammation occur at same time.[1]

***In vivo* animal models:** [2] the inflammatory response is accompanied by clinical signs of erythema, edema, hyperalgesia and pain. Inflammatory responses occur in three different phases, each apparently mediated by different mechanisms:

1. An acute transient phase characterised by local vasodilation and increased capillary permeability.

2. A subacute phase, characterised by infilteration of leukocytes and phagocytic cells.

3. A chronic proliferative phase, in which tissue degeneration and fibrosis occurs.

Methods for testing acute and sub acute inflammation are:

• UV-erythema in guinea pigs
• Vascular permeability
• Oxazolone-induced ear edema in mice
• Croton-oil ear edema in rats and mice
• Paw edema in rats (various modifications and various irritants)
• Pleurisy tests
• Granuloma pouch technique (various modifications and various irritants)

The proliferative phase is measured by methods for testing granuloma formation, such as:

• Cotton wool granuloma
• Glass rod granuloma

Methods for testing acute and sub acute inflammation:

1. UV-erythema in guinea pigs:
Prostaglandin E (PGE) levels in the skin have been shown to be elevated during the 24 h period following exposure of guinea pig skin to ultraviolet radiation from 280-320 nm. The development of increased PGE levels paralleled the development of the delayed phase of erythema. Delay the development of ultraviolet erythema on albino guinea pig skin by systemic pretreatment with clinically equivalent doses of phenylbutazone and other nonsteroidal anti-inflammatory agents. Erythema (redness) is the earliest sign of inflammation, not yet accompanied by plasma exudation and edema. This model depicts the delay in development of UV erythema on albino guinea pig skin by systemic pretreatment with clinically equivalent doses of phenylbutazone and other NSAIDs.

Procedure: Albino guinea pigs of both sexes with an average weight of 350g are used. Four animals are used each for treatment and control group.18 hr prior testing, the animals are shaved on both the flanks and on the back. Then they are chemically depilated by a commercial depilation product or by a suspension of barium sulphide. 20 min later, the depilation paste and the fur are rinsed off in running warm water. On the next day the test compound is dissolved in the vehicle and half of the test compound is administered by gavage (at 10 ml/kg) 30 min before UV exposure. Control animas are treated with the vehicle alone. The guinea pigs are placed in a leather cuff with a hole of 1.5×2.5 cm size punched in it, allowing the UV radiation to reach only this area. Then animals are exposed to UV radiation. After 2 min of expose the remaining half of the test compound is administered. The erythema is scored 2 and 4 hr after exposure.

Evaluation: The degree of erythema is evaluated visually by 2 different investigators in a double-blinded manner. The followings scores are given:

0 = no erythema,
1 = weak erythema,
2 = strong erythema,
4 = very strong erythema.

Animals with a score of 0 or 1 are considered to be protected. The scoring after 2 and after 4 h gives some indication of the duration of the effect. *ED*50 values can be calculated.[3][2]

2. Vascular permeability: During inflammation, vascular permeability increases to allow plasma constituents such as antibodies and complement to access injured or infected tissues. The test is used to evaluate the inhibitory activity of drugs against increased vascular permeability which is induced by phlogistic substances. Mediators of inflammation, such as histamine, prostaglandins and leucotrienes are released following stimulation e.g. of mast cells. This leads to a dilation of arterioles and venules and to an increased vascular permeability. As a consequence, fluid and plasma proteins are extravasated and edemas are formed. The increase of permeability can be recognized by the infiltration of the injected sites of the skin with the vital dye Evan's blue.

Procedure: Albino Wistar are used each group containing 4 rats. Control group will receive distilled water 1%w/v 1ml/100g by oral route and other group will receive test compound by oral route and standard group will receive diclofenac 10ml/kg by intraperitoneal route. After 1h of these administration rats are injected with 0.25ml of 0.6% v/v solution of acetic acid intraperitoneally. Immediately, 10 ml/kg of 10%w/v Evans blue is injected intravenously via tail vain. After 30 min , the animals are anesthetized with ether anaesthesia and sacrificed. The abdomen is cut open and exposed viscera. The animals are held by a flap of abdominal wall over a Petri dish. The peritoneal fluid (exudates) collected, filtered and made up the volume to 10 ml using normal saline solution and centrifuged at 3000 rpm for 15 min. The absorbance (A) of the supernatant is measured at 590 nm using spectrophotometer.

Evaluation: Decreased concentration of dye with respected to absorbance indicates reduction in permeability. The result of test is compared with that of standard. ED_{50} values can also be calculated.[4][2]

3. Oxazolone-induced ear edema in mice: The oxazolone-induced ear edema model in mice is a model of delayed contact hypersensitivity that permits the quantitative evaluation of the topical and systemic anti-inflammatory activity of a compound following topical administration. The oxazolone-repeated challenge increased the level of Th2 cytokines and decreased that of a Th1 cytokine in the lesioned skin. The Th2 cytokines, especially IL-4, play major roles in the development of dermatitis in the present mouse model.

Procedure: Using 12mice in each group, the same skin site of the right ear was sensitized by a single application of 10 µl (each 5 µl for inner and outer of ear) of 0.5% oxazolone in acetone 7 days before the first challenge (day 0), and 10 µl of 0.5% oxazolone in acetone was repeatedly applied to the sensitized right ear 3 times per week. In the non-sensitized animals, acetone alone was applied to the right ear. The mice are challenged 8 days later again under anesthesia by applying 0.01 ml 2% oxazolone solution to the inside of the right ear (control) or 0.01 ml of oxazolone solution, in which the test compound or the standard is solved. Groups of 10 to 15 animals are treated with the irritant alone or with the solution of the test compound. The left ear remains untreated. The maximum of inflammation occurs 24 h later. At

this time the animals are sacrificed under anesthesia and a disc of 8 mm diameter is punched from both sides. The discs are immediately weighed on a balance. The weight difference is an indicator of the inflammatory edema.

Evaluation: Average values of the increase of weight are calculated for each treated group and compared statistically with the control group.[5][2]

4. Croton-oil ear edema in rats and mice: Croton oil contains 12-o-tetracanoilphorbol-13-acetate (TPA) and other phorbol esters as main irritant agents. TPA is able to activate protein kinase C (PKC), which activates other enzymatic cascades in turn, such as mitogen activated protein kinases (MAPK), and phospholipase A2 (PLA2), leading to release of platelet activation factor (PAF) and AA. This cascade of events stimulates vascular permeability, vasodilation, polymorphonuclear leukocytes migration, release of histamine and serotonin and moderate synthesis of inflammatory eicosanoids by cyclooxygenase (COX) and 5-lipoxygenase (5-LOX) enzymes. COX and 5-LOX inhibitors, leukotriene B4 (LTB4) antagonists and corticosteroids show topical anti-inflammatory action in animal models of Croton oil or TPA-induced skin inflammation.

Procedure: A total of 15µl of an acetonic solution containing 75µg of croton oil is applied to the inner surface of right ear of each mouse. Left ear remains untreated. Control animals receives only the irritant while indomethacin (100µg/ear) serves as reference. Varying dose levels of test drug are applied to the inner surface of right ear of each mouse by dissolving them in inflammation inducing solution. Animals are sacrificed by cervical dislocation 6 hr later and a plug (6 mm in diameter)is removed from both the treated and untreated ear. The difference in weight between the two plugs is taken as measure of edematous response. Since tetradecanoyl porbol acetate (TPA) is the chief ingredient of croton oil, purified TPA has also been used to induce ear edema in mice.

Evaluation: The antiphlogistic effect can be determined by expressing the increase in weight of the treated ear as percentage of the weight of the contralateral control ear. The difference between both ears or excised discs is calculated as the average values for treated and control groups and the effect is evaluated by statistical methods.[6][7][2]

5. Paw edema in rats: The ability of anti-inflammatory drugs to inhibit the edema produced in the hind paw of the rat after injection of a phlogistic agent. Many phlogistic agents (irritants) have been used, such as brewer's yeast, formaldehyde, dextran, egg albumin, kaolin, Aerosil, sulfated polysaccharides like carrageenan or naphthoylheparamine. The volume of the injected paw is measured before and after application of the irritant and the paw volume of the treated animals is compared to the controls.[Plethysmograph]. Carrageenan-induced rat paw edema is associated with three distinct phases. The first phase is early mediated by mast cell degranulation and histamine and serotonin release (1 h), the second phase (60 to 150 min) is characterized by bradykinin release and pain, and further eicosanoid production in the late phase (3-4 h). So here the anti-inflammatory effect of the test compound is due to inhibition of which mediator can also be known.

Procedure: Male or female Sprague-Dawley rats with a body weight between 100 and 150 g are used. The animals are starved overnight. To insure uniform hydration, the rats receive 5 ml of water by stomach tube (controls) or the test drug dissolved or suspended in the same volume. Thirty minutes later, the rats are challenged by a subcutaneous injection of 0.05 ml of 1% solution of carrageenan into the plantar side of the left hindpaw. The paw is marked with ink at the level of the lateral malleolus and immersed in mercury up to this mark. The paw volume is measured plethysmographically immediately after injection, again 3 and 6 h, and eventually 24 h after challenge. Various devices have been developed for plethysmography of the paw, like mercury for immersion of the paw, more sophisticated apparatusbased on the principle of transforming the volume being increased by immersion of the paw into a proportional voltage using a pressure transducer, sensitive method of measuring mouse paw volume by interfacing a Mettler Delta Range top-loading balance with a microcomputer, commercially available plethysmometer.

Evaluation: The increase of paw volume after 3 or 6 h is calculated as percentage compared with the volume measured immediately after injection of the irritant for each animal. Effectively treated animals show much less edema. The difference of average values between treated animals and control groups is calculated for each time interval and statistically evaluated. The difference at the various time intervals gives some hints for the duration of the anti-inflammatory effect. A dose- response curve is run for active drugs and *ED*50 values can be determined.[8][7][2]

6. Pleurisy tests: In experimental animals pleurisy can be induced by several irritants, such as histamine, bradykinin, prostaglandins, mast cell degranulators, dextran, enzymes, antigens, microbes, and nonspecific irritants, like turpentine and carrageenan. Carrageenan-induced pleurisy in rats is considered to be an excellent acute inflammatory model in which fluid extravasation, leukocyte migration and the various biochemical parameters involved in the inflammatory response can be measured easily in the exudate.

Procedure: The mouse pleurisy was induced by a single intrapleural injection of 0.1 ml of

carrageenan (1%). After 4 h the animals were killed with an overdose of ether, the thorax was opened and the pleural cavity was washed with 1.0 ml of sterile PBS, containing heparin (20 IU per ml). Samples of the pleural lavage were collected for determination ofexudation, myeloperoxidase , adenosine-deaminase activities, and nitric oxide levels, as well as for determination of total and differential leukocyte counts . Total leukocyte counts were performed in a Neubauer chamber. The cytospin preparations of pleural wash were stained with May–Grunwald Giemsa for the differential count which was performed under an oil immersion objective. The serum level of the C-reactive protein was also analysed. In another set of experiment animals were treated 30 min before carrageenan with a solution of Evans blue dye (25 mg/kg, i.v.) in order to evaluate the degree of exudation in the pleural space. A sample (500 µl) of the fluid leakage collected from the pleural cavity was stored in a freezer (−20 °C) to further determine the concentration of Evans blue dye. To this end, on the day of the experiments, a batch of samples was thawed at room temperature. and the amount of dye was estimated by colorimetry using an Elisa plate reader at 600 nm, by interpolation from a standard curve of Evans blue dye in the range of 0.01 to 50 µg/ml.

Evaluation: One ml (the added Hank's solution) is subtracted from the measured volume. The values of each experimental group are averaged and compared with the control group. *ED*50 values can be calculated using various doses.[9][2]

7. Granuloma pouch technique: With the introduction of an irritant substance into an s.c. air pocket, granulation tissue begins to proliferate and soon covers the whole inside of the pouch. This tissue consists of fibroblasts, endothelial cells and an infiltrate of macrophages and polymorphonuclear leukocytes. In the GPA this rapidly growing tissue can be exposed to carcinogenic and mutagenic sub-stances. One of the major advantages of the system is the possibility of bringing the test compounds into direct contact with the target cells, by injecting them into the air pocket. It is also possible to administer the material by the oral and parenteral routes. It does not provide quantitative information on cytotoxicity of the test compounds in vivo.

Procedure: Male or female Sprague-Dawley rats with a body weight between 150 and 200 g are used. Ten animals are taken for controls and for test groups. The back of the animals is shaved and disinfected. With a very thin needle a pneumoderma is made in the middle of the dorsal skin by injection of 20 ml of air under ether anesthesia. Into the resulting oval airpouch 0.5 ml of a 1% solution of Croton oil in sesame oil is injected avoiding any leakage of air. Forty-eight hours later the air is withdrawn from the pouch and 72 h later any resulting adhesions are broken. Instead of croton oil 1 ml of a 20% suspension of carrageenan in sesame oil can be used as irritant. Starting with the formation of the pouch, the animals are treated every day either orally or subcutaneously with the test compound or the standard. For testing local activity, the test compound is injected directly into the air sac at the same time as the irritant. On the 4th or the 5th day the animals are sacrificed under anesthesia. The pouch is opened and the exudate is collected in glass cylinders. The pouches are washed with 1 ml of saline, exudates are immediately cooled on ice and the volume is recorded. Total no. of leukocytes migrated into the pouch are evaluated after staining with Erythrosine B and remaining exudates is centrifuged at 3000 rpm for 10 min at 4 degrees and supernatant stored at -20 degrees until use.

Evaluation: The average value of the exudate of the controls and the test groups is calculated. Comparison is made by statistical means.[10][11][2]

Methods to evaluate proliferative phase of inflammation:

1. Cotton wool granuloma: The foreign body granulomas were provoked in rats by subcutaneous implantation of pellets of compressed cotton. After several days, histologically giant cells and undifferentiated connective tissue can be observed besides the fluid infiltration. The amount of newly formed connective tissue can be measured by weighing the dried pellets after removal. More intensive granuloma formation has been observed if the cotton pellets have been impregnated with carrageenin.

Procedure: Male rats weighing 180–200 g were used. Test drugs were administered orally on a once daily dosage regimen for 7 days, and the control group received vehicle. Two sterilized pellets of cotton wool were implanted subcutaneously, one on each side of abdomen of the animal, under the light ether anesthesia and sterile technique. The rats were sacrificed on the eighth day. The implanted pellets were dissected out and recorded for wet weight. Thymuses were also dissected out. Both pellet and thymus were dried at60 ◦C for 18 h and the dry weight was recorded.

Evaluation: The weight of the transudate and the granuloma as well as the percent granuloma inhibition of the test drugs were calculated. The body weight gain was also recorded.[12][13][2][7]

2. Glass rod granuloma: These reflects the chronic proliferative inflammation. Of the newly formed connective tissue not only wet and dry weight, but also chemical composition andvmechanical properties can be measured.

Procedure: Glass rods with a diameter of 6 mm are cut to a length of 40 mm and the ends rounded off by flame melting. Male Sprague-Dawley rats with an initial weight of 130 g are anaesthetized with

ether, the back skin shaved and disinfected. From an incision in the caudal region a subcutaneous tunnel is formed in cranial direction with a closed blunted forceps. One glass rod isintroduced into this tunnel finally lying on the back of the animal. The incision wound is closed by sutures. The animals are kept in separate cages. The rods remain *in situ* for 20 or 40 days. Treatment with drugs is either during the whole period or only during the last 10 or 2 days. At the end the animals are sacrificed under CO_2 anesthesia. The glass rods are prepared together with the surrounding connective tissue which forms a tube around the glass rod. By incision at one end the glass rod is extracted and the granuloma sac inverted forming a plain piece of pure connective tissue. Wet weight of the granuloma tissue is recorded. Finally, the granuloma tissue is dried and the dry weight is recorded. In addition, biochemical analyses, such as determination of collagen and glycosaminoglycans can be performed.

Evaluation:Several parameters can be determined by this method. Granuloma weight was reduced by test compound is compared with that of standard.[2]

"Cite this article"

Mitul Patel, Murugananthan, Shivalinge Gowda K,P "*In Vivo* Animal Models in Preclinical Evaluation of Anti-Inflammatory Activity-A Review" Int. J. of Pharm. Res. & All. Sci 2012; Volume 1, Issue 2, 01-05

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