CHAPTER 2

INVESTIGATION OF ANALGESIC AND ANTI-INFLAMMATORY PROPERTIES OF BIRM IN ANIMAL MODELS OF ACUTE AND CHRONIC INFLAMMATION PAIN

INTRODUCTION

Inflammatory pain is the result of increased excitability of peripheral nociceptive sensory fibres produced by the action of inflammatory mediators (Linley *et al.*, 2010). Unlike nociceptive pain, wherein the pain sensation is not felt after removal of stimulus, in inflammatory pain, pain sensation resulting from tissue injury occurring due to exposure of high intensity stimulus continues long after the stimulus has been removed (Linley *et al.*, 2010). Moreover, increased sensitivity is observed to non-noxious stimuli when applied to the injured state and the inflammatory pain generally resolves with healing.

Mechanism of Inflammatory Pain

Injury or inflammation leads to an innate immune cascade at the site of injury yielding release of active factors from blood, local as well as migrating inflammatory cells and injured cells. These active factors play a role in sensitization of C-fibers and sustained afferent traffic leads to initiation of robust facilitation of dorsal horn output (Xu and Yaksh, 2011).

Peripheral sensitization: Tissue injury results in the release of inflammatory mediators from damaged cells such as ions (K^+, H^+), bradykinin, histamine, 5-hydroxytryptamine, ATP and nitric oxide (Dray, 1995). Furthermore, arachidonic acid biotransformation gets activated resulting in the release of prostanoids and leukotrienes (Ballou *et al.*, 2000). Immune cells which have been recruited due to tissue injury/inflammation further releases mediators such as cytokines and growth factors (Woolf and Thompson, 1991). This results in direct activation of peripheral nociceptors finally leading to spontaneous pain. Inflammatory mediators modify the response properties of primary afferent neurons to the stimuli, thus leading to peripheral

sensitization. The reason behind this process could be the result of changes to the sensitivity of receptor molecules or via modulation of voltage gated ion channels (Kidd and Urban, 2001).

Central sensitization: Repetitive activation of primary afferent fibers gives rise to change in function and activity of central neurogenic pathways. Inflammatory conditions lead to release of neurotransmitters (glutamate), neuropeptides (Substance P) and neurotrophic factors (BDNF) from central terminals of primary afferents. They act as co-transmitters and induce long lasting changes in spinal excitability know as central sensitization (Woolf, 1983). Their increased release leads to activation of second messenger systems resulting in phosphorylation of proteins and increased influx of Ca^{2+} ions. Transcriptional changes take place due to activation of kinases under prolonged inflammation condition (Woolf and Salter, 2000). These changes occurring in dorsal horn finally result in exaggerated responses to normal stimuli (hyperalgesia), expansion of receptive field size and reduction in the threshold level.

Apart from this, NMDA receptors are thought to play important role in central sensitization. A number of endogenous mediators such as prostaglandins, nitric oxide, opioids and adrenergic agonists also play mediating role in creating excitability of spinal neurons. Prostaglandins and nitric oxide are thought to facilitate spinal excitability. It has been established that the α2 adrenergic and opioid receptor agonists produce analgesia by presynaptic inhibition of C-fiber neurotransmitter release and post-synaptic hyperpolarization of second order neurons (Besson, 1999).

Animal models of Inflammatory Pain

Animal models of inflammatory pain are used to assess the production of inflammatory mediators at the sites of inflammation. They help us to understand the mechanisms of persistent pain and to identify anti-inflammatory properties of agents and assessing the efficacy of analgesic compounds in reversing the cutaneous hypersensitivity. Inflammatory hyperalgesia developed by the injection of inflammatory agents into the rat or mouse hind paw mimics human clinical pain conditions (Hargreaves *et al.*, 1988; Qui *et al.*, 1998).

Animal models of tissue injury and inflammation can be subdivided into those that produce inflammation of cutaneous and subcutaneous tissues, joint inflammation, inflammation of muscles and others (Ren and Dubner, 1999).

<u>Inflammation of cutaneous and subcutaneous tissues</u>: It includes models induced using phlogistic agents (irritants) such as brewer's yeast, formaldehyde, dextran, sulfated polysaccharides like carrageenan, zymosan or Complete Fruend's Adjuvant (CFA), etc. in the foot pad of hind paw causing edema and produces more persistent pain and hyperalgesia (Hargreaves *et al.*, 1988; Iadarola *et al.*, 1988; Meller and Gebhart, 1997).

<u>Joint inflammation</u>: It includes various models of arthritis (acute and chronic; inflammatory and non-inflammatory). Acute arthritis can be induced by injection of carrageenan and kaolin into the knee joint of cat or monkey just below the patella (Schaible *et al.*, 1987; Dougherty *et al.*, 1992). Inflammatory arthritis induced by injection of CFA into the rat tail (De castro Costa *et al.*, 1981) or by injection of urate crystals into the ankle joint of rat (Coderre and Wall, 1987) mimics chronic pain. Osteoarthritis is the form of non-inflammatory arthritis induced by intra-articular injection of monosodium iodoacetate (Williams and Brandt, 1985; Guingamp *et al.*, 1997) and by various other mechanisms. Progression of the non-inflammatory arthritis indirectly leads to inflammation due to wear and tear of the tissue.

<u>Muscle inflammation</u>: There are relatively fewer animal models to study the muscle pain. Myositis is induced by injection of carrageenan into the gastrocnemius-soleus muscle (Hoheisel *et al.*, 1994). Generally some algesic compounds such as bradykinin, prostaglandin E_2, hypertonic saline are used to elicit muscle pain (Mense, 1991; Stohler *et al.*, 1991).

OBJECTIVE OF THE STUDY

The overall objective of the current study was to assess anti-inflammatory properties of BIRM in acute and chronic pain conditions. Hence we tested the efficacy of BIRM in mitigating pain in animal models of carrageenan induced paw edema and monosodium-iodoacetate (MIA) induced osteoarthritis, which mimics acute and chronic pain conditions respectively. Although MIA induced osteoarthritis is a form of non-inflammatory arthritis but during the progression of diseases, inflammation develops due to wear and tear of tissues. Hence, to identify the efficacy of BIRM in mitigating the later stages of non-inflammatory disease and to study the anti-nociceptive properties of BIRM, MIA induced osteoarthritis was selected for further studies.

In order to achieve the above objective, the current study is planned and executed in two animal models one representing the acute inflammatory pain and the other chronic inflammatory pain. The details of the studies are given hereunder in two separate heads.

1. CARRAGEENAN INDUCED PAW EDEMA: MODEL FOR TESTING THE ROLE OF BIRM IN MITIGATING ACUTE INFLAMMATORY PAIN

Carrageenan-induced rat paw edema originally described by Winter *et al.* (1962) is a widely used test to determine anti-inflammatory activity and is a routine animal model for evaluation of pain at the site of inflammation without causing any injury or damage to the inflamed paw (Sugishita *et al.*, 1981; Henriques *et al.*, 1987). This method employs intraplantar injection of 1-3% lambda (λ) carrageenan solution prepared in saline. Normally 50-150µl as a single dose is commonly used (Salvemini *et al.*, 1996) to induce paw edema but higher concentrations have been used for the modeling of specific pathophysiological conditions such as muscle pain (Radhakrishnan *et al.*, 2004; Silva *et al.*, 2010). The inflammation response induced by carrageenan is usually quantified by increase in paw edema which is maximal around five hours post carrageenan injection and is modulated by inhibitors of specific molecules within the inflammatory cascade. Subcutaneous injection of carrageenan results into cardinal signs of inflammation such as edema, hyperalgesia and erythema, resulting from action of pro-inflammatory agents such as bradykinin, histamine and tachykinin as well as reactive anion species (Morris, 2003). During the current study we used a single dose of 100µl of 1% carrageenan diluted in Saline (λ-carrageenan, type IV, Sigma Aldrich USA) and mice as a test system.

MATERIAL AND METHOD

Animals and Housing Conditions

Male Swiss Albino mice (25-35g) were procured from CPCSEA and AAALAC approved vivarium facility at GVK Biosciences Pvt. Ltd., Hyderabad, India. They were allowed to acclimatize for a minimum duration of one week prior to initiation of testing. They were housed in groups of four in polypropylene cages under ambient conditions. Room temperature and humidity were maintained at 20-25°C and 65-70%, respectively. 12h light/dark cycle was maintained. Standard laboratory rodent diet and potable drinking water were provided *ad libitum*. Experimental protocols were approved by IAEC according to CPCSEA, India. All animal

procedures were performed in accordance with the ethical guidelines of CPCSEA. All efforts were made to minimize animal suffering and to utilize minimum number of animals in this study.

Test Compound and Treatment Regimen

BIRM was a gift from BIRM Inc., Quito, Ecuador. It is an aqueous extract of dried roots of a plant *S. dulcamara* and marketed as a greenish-brown suspension with a mild bittersweet smell. The inactive ingredients in BIRM comprise 16% solid particles, likely root fibers. The remainder is lipid-free liquid. BIRM is prepared by aqueous extraction of dried roots followed by oxidation/reduction of the extract. During this process, the amount of roots and the timing of oxidation/reduction are carefully controlled to minimize batch-to batch variation.

In the present study, BIRM samples from lot number 18.09.09 003PR were used and it was clarified by centrifugation at 10,000g prior to usage as described by Dandekar and co-workers (2003). Diclofenac and λ-Carrageenan (type IV) were obtained commercially from Sigma Aldrich, USA.

Study was conducted using twenty four male Swiss albino mice divided into three groups: Group I - Vehicle control (4 ml/kg, p.o., distilled water), Group II - BIRM (4 ml/kg, p.o., seven days pre-treatment) and Group III - Diclofenac (20 mg/kg, p.o.; single dose at 30 minutes pre-treatment).

Test Procedure

Paw edema was induced in male Swiss albino mice by injection of 100µl of 1% carrageenan diluted in saline in the plantar surface of left hind foot pad (Henriques *et al.*, 1987). In a similar manner, 100µl of 0.9% saline solution was administered in plantar surface of right hind foot pad to serve as control reference for the tested paw. The paw volume was measured through water displacement method using water plethysmometer (LE 7500, Panlab SI, Spain) immediately before intraplantar injection of carrageenan and at 2, 3, 4 and 5 hours thereafter. Each paw was marked at lateral malleolus in order to immerge it always at the same extent in the measurement chamber. The assessment of paw volume was performed in blind fashion. The change in paw volume was calculated by subtracting the initial paw volume of left hind paw (basal) from the paw volume of left hind foot measured at each time point. The percentage inhibition of paw

edema was calculated by using the following formula of Ravichandran and Panneerselvam (2014):

$$\text{Percentage of edema inhibition} = (Vc - \frac{Vt}{Vc}) \times 100$$

Vc = Volume of paw edema in control group

Vt = volume of paw edema in treated group

Statistical Analysis

Results were expressed as mean ± SEM (standard error of mean) of the change in paw volume measured. Data was analysed using Graphpad Prism (version 4.1). Two way repeated measures ANOVA followed by Bonferroni's multiple comparison test was used to analyse data generated from Carrageenan induced paw edema model. $p \leq 0.05$ was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the figure legends.

RESULTS

As expected, the intraplantar administration of carrageenan produced gradual increase in paw edema. However, repeated oral treatment of BIRM (4ml/kg) for seven days showed significant reduction in paw edema at 2h ($p \leq 0.05$), 3h, 4h and 5h ($p \leq 0.001$) post carrageenan treatment as compared to vehicle control. BIRM exhibited highest reduction of 71.89% in paw edema at 5h post carrageenan treatment. As reported, Diclofenac too showed significant reduction in paw edema at 3, 4 and 5 h ($p \leq 0.001$) post carrageenan treatment as compared to vehicle control (Niazi *et al.*, 2010; Sakat *et al.*, 2014). Diclofenac too exhibited highest reduction in paw edema at 5 h post carrageenan treatment (Table 1; Figure 1).

Table 1: Anti-inflammatory effect of repeated BIRM administration in paw edema model

Group	Change in paw volume of left hind paw post carrageenan administration at			
	2 h	3 h	4 h	5 h
Vehicle Control	0.17 ± 0.009	0.21 ± 0.015	0.26 ± 0.010	0.31 ± 0.010
BIRM (4 ml/kg; p.o.)	$0.12 \pm 0.015^{*}$	$0.13 \pm 0.011^{***}$	$0.11 \pm 0.011^{***}$	$0.09 \pm 0.006^{***}$
Diclofenac (20 mg/kg; p.o.)	0.13 ± 0.016	$0.09 \pm 0.010^{***}$	$0.09 \pm 0.007^{***}$	$0.05 \pm 0.005^{***}$

Data represented as mean $\pm$ S.E.M *p $\leq$ 0.05, ***p $\leq$ 0.001 as compared to vehicle control; (Two way repeated measures ANOVA followed by Bonferroni's posttest)
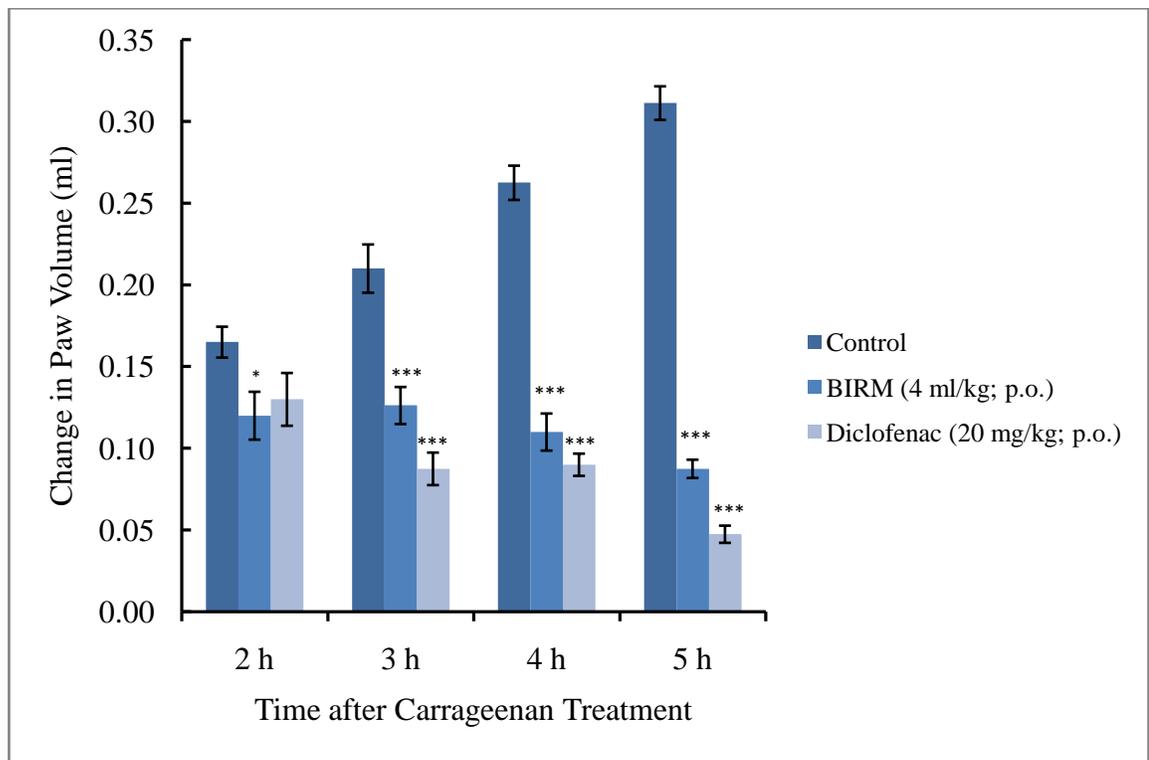

Figure 1: Effect of repeated administration of BIRM (4 ml/kg, p.o. for seven days) on inflammation induced by intraplantar injection of carrageenan in hind foot pad of Swiss Albino mice. *p $\leq$ 0.05 and ***p $\leq$ 0.001 as compared to vehicle control. Data was analysed using two way repeated measure ANOVA followed by Bonferroni post hoc test

DISCUSSION

Carrageenan-induced paw edema test is commonly used as an experimental model for acute inflammation and is observed to be biphasic. Edema observed in the first phase (mainly 1-6 hours) of the carrageenan model is believed to be of little intensity as compared to the second phase (24-72 hours) with more pronounced edema (Henriques *et al.*, 1987; Posadas *et al.*, 2004). However, Posadas *et al.* (2004) have reported age and weight of mice as the critical issue while studying this model. They have clearly shown through their studies that biphasic edema and consistent inflammatory pattern to carrageenan was observed in first phase in animals of 7-8 weeks with weight range of 32-35g as compared to their younger counterpart (4-5 weeks age with 18-20g weight range). Hence, in the present study, our observation was limited up to first phase only.

Carrageenan induced inflammation model is used extensively in the development of NSAIDs and selective COX-2 inhibitors and assessing the contribution of mediators involved in vascular changes associated with acute inflammation. Acute inflammation also leads to leakage of plasma elements from blood vessels to the inflamed tissue and also the infiltration of neutrophils (Zhou *et al.*, 2006; Thakare *et al.*, 2010). Histamine, serotonin, bradykinin, prostaglandins, hydrogen sulfide and nitric oxide are some of the inflammatory mediators which are reported to play role in the genesis of inflammation in this model (Zhou *et al.*, 2006). There are several reports indicating COX-2 mediated increase in PGE_2 production in the central nervous system (CNS) as the major player in inducing inflammatory pattern and pain response in carrageenan - induced paw edema model (Salvemini *et al.*, 1996; Guay *et al.*, 2004). Administration of carrageenan in the paw leads to increased mRNA levels of COX-2 in the spinal cord and other regions of CNS thus indicating its major role in induction of inflammation (Ichitani *et al.*, 1997). Several other studies by Seibert *et al.* (1994), Ibuki *et al.* (2003) and Guay *et al.* (2004) also have shown elevated levels of COX-2 very early on (1-6 hours) in paw tissues and in the CNS following carrageenan-induced inflammation. Diclofenac and other NSAIDs drugs such as Indomethacin and celecoxib are found efficacious in mitigating inflammatory pain through their inhibitory action on COX-2. BIRM has shown significant reduction in paw edema at all time points throughout the study. This observation is in agreement with the reference drug, Diclofenac used in this study. Diclofenac too, as widely reported is able to prevent the inflammation exhibited through reduced paw edema through the study course. As observed by Jaggi *et al.* (2004),

mother tincture of *Solanum dulcamara*, which is the source of BIRM too, is found to inhibit prostaglandin production via COX-2. Hence the anti-inflammatory property exhibited by BIRM in carrageenan induced paw edema model could be attributed to its inhibitory action on COX-2 and thereby inhibiting the production of PGE_2 - the major mediator of inflammation or BIRM having the possible ability of hindering the endogenous synthesis or release of inflammatory mediators such as prostaglandins, histamine, serotonin, bradykinin and leukotrienes. The later however, needs to be validated in future through a carefully controlled mechanistic study and is beyond the scope of the current study.

2. MIA INDUCED OSTEOARTHRITIS: MODEL FOR TESTING THE POTENTIAL OF BIRM IN CHRONIC PAIN MANAGEMENT

Osteoarthritis (OA), the major form of joint diseases leading to chronic pain and disability, is widely prevalent in the elderly population world over (Witter and Dionne, 2004; Sarzi-Puttini *et al.*, 2005). Patients suffering from OA experience joint stiffness and excruciating pain with weight bearing or physical activity. The etiology of OA is multifactorial including several factors such as biomechanical forces affecting the articular cartilage and subchondral bone as well as biochemical changes in the articular cartilage and synovial membrane. In addition, genetics too is assumed to play a pivotal role in the prognosis of osteoarthritis (Mow *et al.*, 1995; Poole *et al.*, 1995; Holderbaum *et al.*, 1999).

From a structural point of view, joints are designed to provide flexibility, support, stability and protection. These functions, essential for normal and painless movement, are primarily supplied by specific parts of the joint namely the synovium and cartilage. Synovium is the membrane that surrounds the entire joint. It is filled with synovial fluid, a lubricating liquid that supplies nutrients and oxygen to cartilage. Cartilage is a slippery tissue that coats the ends of the bones. It is one of the few tissues in the body that does not have its own blood supply but has number of essential components such as chondrocytes, proteoglycans, collagen and water. Chondrocytes being the basic cartilage cells are critical for balance and function. Proteoglycans are large molecules that help to cushion the cartilage and improve the ability of cartilage to bond with water hence, maintain the high fluid content in cartilage. Collagen being the critical protein in cartilage forms a mesh to give support and flexibility to the joint. The combination of the

collagen meshwork and the high water content, tightly bound by proteoglycans, creates a resilient and slippery pad in the joint which helps in resisting the compression between bones during muscle movement (Marker and Pomonis, 2012).
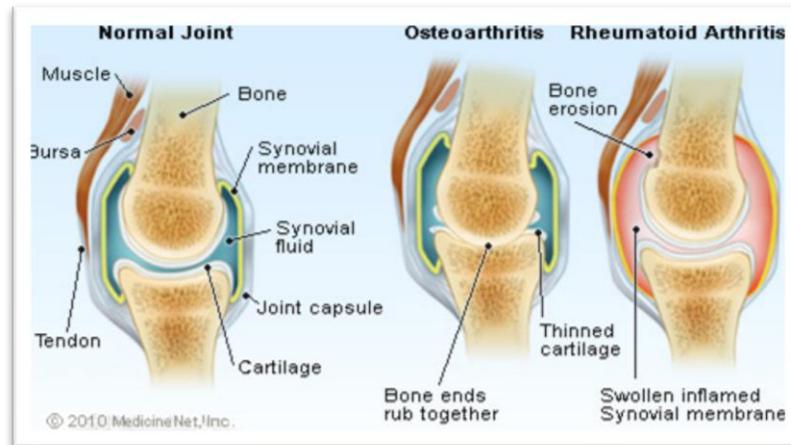

Figure 2: Image showing the characteristics of normal and arthritic joints. Image taken from: http://www.medicinenet.com/osteoarthritis/article.htm

However, the fluidity of the joints will be severely challenged during osteoarthritis - a disease of the joints. Unlike many other forms of arthritis that are systemic illness, such as rheumatoid arthritis and systemic lupus, OA does not affect other organs of the body. The most common symptom of OA is pain in the affected joint after repetitive use. Joint pain gets worse as the day progresses. There can be swelling, warmth and creaking of the affected joints. Pain and stiffness of the joints can also occur after long periods of inactivity. In severe osteoarthritis, complete loss of the cartilage causes friction between bones, causing pain at rest or pain with limited movement (Creamer and Hochverg, 1997).

Pain associated with chronic arthropathies is the result of a complex and orchestrated series of mechanisms integrated at all levels of the neuraxis from the periphery via the dorsal horn to higher cerebral structures. There is a growing body of evidence showing neurotransmitters released at the site of tissue damage exerting a modulatory effect on cutaneous mechanoreceptors and nociceptors, leading to their sensitization and consequently altering their function (Hendiani *et al.*, 2003).

Nociceptors on the other hand are peripheral sensory organs which are activated when a nociceptive stimuli cause tissue damage. These nociceptors are unspecialized, naked nerve endings found close to small blood vessels and mast cells. There are four different types of nerves that innervate the joint and there is a rich supply of myelinated and unmyelinated fibers innervating the joint capsule, subchondral bone, periosteum, ligaments and menisci. These four fibers include Type 1 (Aα), Type 2(Aβ), Type 3 (Aδ) and Type 4 (C). Studies indicate presence of Aδ and C fibers in the most joint structures with the exception of articular cartilage (Enohumah and Imarengiave, 2008).

As described earlier, synovium provides nutrients to cartilage, since it does not have its own blood vessels. Hence, irrespective of its role in the pathogenesis of joint damage, cartilage could not be the tissue from where the OA pain originates. The subchondral bone, periosteum, synovium, ligaments and the joint capsule contain nerve endings that could be the source of nociceptive stimuli in OA. Irritation of the periosteum as a result of remodeling, denuded bone, compression of soft tissue by osteophytes, microfractures of the subchondral bone, effusion and spasm of surrounding muscles has been shown to contribute to the pain that may be felt by patients suffering from OA (Enohumah and Imarengiave, 2008; Gwilym *et al.*, 2008). Pain and inflammation are the predominant clinical features of osteoarthritis, yet therapy is ineffective for many patients. One of the main mechanisms responsible for the generation of joint pain is the activation of nociceptors located on the terminal branches of joint - Aδ fibers and C fibers primary afferents.

As described earlier (Chapter 1), Aδ fibers initiate the sharp pain associated with acute injury while C fibers are responsible for the less well-defined aching pain. Primary afferent fibers at the site of tissue injury upon getting stimulated, causes release of Substance P (SP), activation of cells in the dorsal horn of the spinal cord and transmission of the nerve impulse to the midbrain and the cortex. Transmission of sensory information gets modulated in the nervous system by neurons causing peripheral nociceptor sensitization and release of chemical mediators such as PGs and leukotrienes at the site of injury or damage. These damaged fibers release inflammatory agents causing a spread of increased sensitivity around the area of tissue damage causing hypersensitivity of nerves. The free-ending receptors of sensitized nerves may have different sensitivities to chemical, mechanical and thermal stimuli (Gwilym *et al.*, 2008). These afferent

nerve fibers show increased activity when a noxious stimulus is applied to the innervated tissue. In animal models of inflammatory joint disease and OA, it has been shown that these primary afferent nerves in joint becomes sensitized, causing enhanced mechano-sensation in the affected joint leading to allodynia, hyperalgesia and spontaneous ongoing pain (Guzman *et al.*, 2003; Schuelert and McDougall, 2009).

Drugs that modify the disease progression represent the ultimate goal of treatment but are not clinically available. Treatment is currently based on symptomatic relief of pain and inflammation associated with osteoarthritis to increase joint function. Nonsteroidal anti-inflammatory drugs (NSAIDs) and acetaminophen are the most widely used drugs but cyclooxygenase-2 inhibitors, steroids and opiates are prescribed as well (Beyreuther *et al.*, 2007).

It is therefore important to try and elucidate the mechanisms responsible for the induction and maintenance of these pain states to help in the development of more effective analgesics for the treatment of OA.

As prelude to such a screening, several animal models have been established to study arthritis. Animal models are considered as useful tools for investigating pain and underlying mechanisms thus providing proof of concept in the development of pharmacologic and biologic agents that may modify structural damage. Animal models of OA have been used fairly extensively for testing of potential anti-arthritic agents and disease modifying effects have been reported for agents currently used to treat patients with OA (Bendele, 2001). There are several animal models of osteoarthritis which are widely used but mainly limited to study of pathophysiology and progression of joint damage. Pain being the predominant clinical feature need arises to study its link with OA. Different types of animal models used for study of OA are detailed as below:

<u>Naturally occurring models of OA</u>**:** Naturally occurring OA is mainly seen in the medial compartment of the knee joint of male and female Hartley albino guinea pigs as well as other strains of guinea pigs (Bendele and Hulman, 1988; Smith *et al.*, 1999). Other commonly used laboratory animals that develop spontaneous OA include Syrian hamsters, dogs, non human primates and many strains of mice (Silberberg *et al.*, 1952).

Genetically modified OA animal models: Genetic models are useful for studying the function of a specific gene and its interaction with components in the tissue (Little and Zaki, 2012). Knockout or transgenic mouse models are used to address the roles of certain molecules in OA pathogenesis. Polymorphism or mutations in genes encoding extracellular matrix genes and signaling molecules are linked with OA susceptibility (Nakamura *et al.*, 2007). Thus the loss or mutation of such single gene may lead to cartilage degeneration similar to that in OA patients.

Medial meniscal tear: Unilateral medial meniscal tear in 300-400g rats/guinea pig (Bendele *et al.*, 1999) results in rapidly progressive cartilage degenerative changes characterized by chondrocyte and proteoglycan loss, fibrillation, osteophyte formation and chondrocyte cloning.

Meniscectomy: Partial meniscectomy surgery on the medial aspect of the joint in New Zealand white rabbits weighing approximately 4kg or dogs generally results in relatively mild to moderate degenerative changes. Lesions occurring in this model resemble to those occurring in human OA and this model has been used extensively for testing of potential chondroprotective agents (Moskowitz *et al.*, 1973).

Anterior cruciate ligament transaction: Transection of the anterior cruciate ligament in dogs results in a true instability-induced OA lesion that mimics OA occurring naturally in dogs or humans following traumatic injury (Pond and Nuki, 1973; Marshall and Chan, 1996; Visco *et al.*, 1996).

Papain induced: Papain, a proteolytic enzyme, degrades proteoglycans in cartilage resulting in the release of chondroitin sulphate from the matrix in many species such as mice, rats and rabbits at different dosages and intervals thus enabling to study different stages of osteoarthritic progression (Miyauchi *et al.*, 1993). It is being used less frequently.

Collagenase induced: Intra-articular injection of collagenase leads to development of patellar dislocation on the medial side initiating joint instability and development of OA by damaging joint structures containing collagen I (Schunke *et al.*, 1988).

Quinolone induced: Quinolone antibiotics administration causes arthropathy and tendinopathy. It also results in gait disorders, irreversible loss of proteoglycans, chondrocytes and extracellular matrix (Sendzik *et al.*, 2009).

Monosodium iodoacetate induced: Intra-articular injection of MIA leads to a decrease in number of chondrocytes and subsequent histological and morphological articular alterations are similar to human osteoarthritic changes (Lampropoulou-Adamidou *et al.*, 2013).

Mechanistically, MIA inhibits the activity of glyceraldehydes-3-phosphate dehydrogenase in chondrocytes, resulting in disruption of glycolysis and eventually in cell death. Chondrocytes in the mature articular cartilage is the only type of cell that is capable for production and maintenance of extra cellular matrix. Thus progressive loss of chondrocytes results in histological and morphological changes of the articular cartilage, closely resembling those seen in OA patients. As this degenerative model progresses, chondrocyte death, articular cartilage-fibrillation, exposure of subchondral bone, osteolysis, reduction in bone mineral content and density occur, thus generating joint impairment with associated pain and mechanical hypersensitivity (Beyreuther *et al.*, 2007; Harvey and Disckenson, 2009). Intra-articluar injection of MIA also results in sensitization of primary afferent nerves resulting in spontaneous pain, hyperalgesia and allodynia. Schuelert and McDougall (2009) has observed concentration dependent increase of mechanosenstivity in the nerves.

The MIA model of OA mimics pathological changes and pain of OA in humans. Both punctuate allodynia and weight bearing deficits are present in this model and these measureable endpoints may be of use to differentiate between analgesic compounds. Additionally, analgesic drug studies indicate that this model may be useful for the investigation of chronic nociceptive pain (Schuelert and McDougall, 2009; Marker and Pomonis, 2012). Close association between evaluation of disease progression in the animal model and information of the molecular mechanisms involved in OA pain is essential to elucidate the pathophysiology of this disease (Combe *et al.*, 2004).

MIA induced OA is one of the best characterized rat models for analyzing properties of drugs on the pathology of osteoarthritis through the intra-articular injection of the metabolic inhibitor monosodium iodoacetate into the femorotibial joint. It is also a model to study cartilage degeneration and to study pain in OA. Therefore this model is selected for the current experiment wherein MIA-induced OA model of rats was used to assess the analgesic efficacy of BIRM and compared it with the reference drug Celecoxib, routinely used to treat the chronic nociceptive pain.

MATERIAL AND METHODS

Animals: Adult male Sprague Dawley rats weighing 250-350g were used and given free access to food and water. They were housed individually in solid bottom cages in a room with controlled temperature ($20 \pm 3°C$) and 12h light and dark cycle. Adequate measures were taken to minimize pain or discomfort in animals and were allowed to retain full mobility and grow normally. All experimental procedures in the present study were performed in accordance with the ethical guidelines for the study of experimental pain in conscious animals (Zimmermann, 1983).

The experimental protocol was duly approved by IAEC and care of the animals was carried out as per the guidelines CPCSEA, Ministry of Environment and Forest, Government of India.

Treatment Regimen: Twenty animals were divided randomly into four groups (n=5) as given in a tabular form below:

Groups	Treatment
Group I (Control group)	Intra-articular injection of 50 µl Normal saline (p.o.)
Group II (Arthritic control group)	Intra-articular injection of MIA (50 µl of 60 mg/ml of MIA) Normal saline (p.o.)
Group III (Test group)	Animals injected with MIA and treated with BIRM (4 ml/kg; p.o.)
Group IV (Reference Drug)	Animals injected with MIA and treated with Celecoxib (30 mg/kg, p.o.)

Induction of Osteoarthritis: The animals were deeply anesthetized under 2% Isoflurane (Baxter)-O_2 mixture and administered with a single intra-articular injection of MIA through the patellar ligament into the joint space of the right knee. MIA was dissolved in 0.9% sterile saline and administered in a volume of 50µl of 60mg/ml using a 27½ gauge needle. Animals from control group were given a single intra-articular injection of 50µl 0.9% sterile saline into the right knee (Combe *et al.*, 2004). All the animal except for control group (G-I) were administered with MIA. The dose of MIA (3 mg/joint) was chosen on the basis of previous reports (Beyreuther *et al.*, 2007; Ferreira-Gomes *et al.*, 2008; Schuelert and McDougall, 2009; Al-Saffar *et al.*, 2010).

The day of OA induction was considered as day 0. On day 15 post MIA treatment, the test drug and reference drug was administered orally with a feeding catheter until two weeks up to day 28 to evaluate any effects of the treatment.

Pharmacological Evaluation

Behavioral Testing: Testing was performed in a blinded manner. Animals were habituated to the experimenter and to the behavioral testing conditions for at least 1 week prior to the start of the experiment and 10 to 15 minutes before each testing, until exploration activities ceased. For each rat and each test, testing was done before the intra-articular injection (day 0), to assess the baseline response of each animal, and on day 3, 7, 14, 21 and 28 after the MIA induction. All tests were done bilaterally.

Secondary Mechanical Allodynia (Up and Down method): Mechanical allodynia was assessed by modified Dixon's Up and Down method using a set of von-Frey filaments (0.4, 0.6, 1.0, 2.0, 4.0, 6.0, 8.0 and 15.0 g). Briefly, the rats were placed in clear plexiglass chambers on a wire mesh floor and allowed to acclimatize for at least 20 minutes. The testing was initiated with the 2.0g filament. The filament was applied once to the mid-plantar surface of right and left hind paw in a perpendicular fashion and until slight buckling occurred for 6-8 seconds. Positive responses include an abrupt withdrawal of the paw from the stimulus or flinching behavior immediately following removal of the stimulus. Stimuli were always presented in a consecutive fashion, whether ascending or descending. In the absence of a paw withdrawal response to the initially selected filament, a stronger stimulus was applied; in the event of paw withdrawal, the next weaker stimulus was chosen. The test was completed when four measurements were made after the initial change in behavior or after five consecutive negative or four positive responses has occurred (Ferreira-Gomes *et al.*, 2008; Harvey and Disckenson, 2009). The resulting pattern of positive and negative responses was tabulated using the convention, X = withdrawal; O = no withdrawal and the 50% response threshold was interpolated using the formula:

$$50\% \text{ g Threshold} = \frac{(10\,[Xf + k\delta])}{10{,}000}$$

Where Xf = value (in log units) of the final von Frey filament used; k = tabular value (Appendix 1 in Chaplan *et al.*, 1994) for the pattern of positive/negative responses; and δ = mean difference (in log units) between stimuli. Data were reported and plotted as 50% g threshold values.

The percentage effect was calculated using 50 % g threshold values using the following formula.

$$\% \text{ Protection} = \frac{(50\% \text{ PWT posttreatment} - 50\% \text{ PWT pretreatment})}{(\text{Maximum possible } 50\% \text{ PWT i. e. } 15 - 50\% \text{ PWT pretreatment})}$$

Knee-Bend Test: To assess the sensitivity to the normal movement of each knee, knee-bend test of nociception for monoarthritic rats was performed. For this purpose, animals were gently restrained, allowing access to both hind limbs while at the same time restricting movement. The test consisted on the recording of the number of squeaks and/or struggle reactions in response to 5 flexions and 5 extensions of the knee joint and the total numbers of vocalizations were recorded. The score of the test was determined according to the type of reaction, squeaks and/or struggle and the type of manipulation that originated the reaction, according to the following evaluation scale: 0 = no response to any kind of extension or flexion of the joint, 0.5 = struggle occurs to maximal flexion/extension, 1= struggle occurs to moderate flexion/extension and also in vocalizations to maximal flexion/extension, 2= squeak reactions in response to moderate manipulations (flexions and extensions) of the joint. The sum of the recorded reactions, giving maximal values of 20, represented the knee bend score, an indication of the animal's nociception (Ferreira-Gomes *et al.*, 2008).

Thermal Hyperalgesia (Hargreaves Method): Thermal response was determined by measuring hind paw withdrawal latency of paw employing Hargreaves' plantar test. Rats were allowed to acclimatize within plexiglass enclosures on a clear glass plate maintained at 30°C for 15-30 minutes before testing. A radiant heat source controlled with a timer was focused onto the plantar surface of hind paw encompassing the glabrous skin. On response of paw withdrawal, both heat source and timer were stopped and paw withdrawal latency was recorded. Five trials, 1-2 minutes apart were conducted and average of three trials excluding maximum and minimum response was taken as paw withdrawal latency. The cut-off limit for exposure was 20 seconds (Hargreaves *et al.*, 1988).

RESULTS

Mechanical allodynia

Mechanical allodynia was detected in all MIA-treated animals from day 14 and it gradually developed until day 28 post MIA injection. Treatment of BIRM and Celecoxib was initiated on

day 15 post MIA injection. There was significant increase in paw withdrawal threshold in animal treated with BIRM on day 28 (62% inhibition) as compared to MIA-Vehicle control group. This increase in paw withdrawal threshold was comparable to Celecoxib treated group which is a standard first line of treatment used in the current time. Celecoxib however, significantly attenuated tactile allodynia throughout the study duration ($p \leq 0.01$ on days 15 and day21, $p \leq 0.001$ on day 28). Rats treated with saline showed normal paw withdrawal threshold for the entire tenure of study (Table 2; Figure 3).

Thermal hyperalgesia

Paw withdrawal latencies were measured prior to MIA injection and also at defined times after MIA injection. Intra-articular injection of MIA into the right knee joint produced marked and significant reduction of paw withdrawal latencies to noxious radiant heat stimuli as compared to normal control animals. Even though, animals treated with BIRM showed improvement in the paw withdrawal latency on first day of its administration i.e., on day 15 post MIA injection, decrease in paw withdrawal latency was observed thereafter on day 21 and 28 moreover it was significantly higher ($p \leq 0.05$) as compared to MIA-vehicle control group. Celecoxib did show significant improvement in the paw withdrawal latency on day 28 ($p \leq 0.05$) as compared to MIA-vehicle control group (Table 3; Figure 4).

Knee Bend test

Ankle bend test is one way to measure the nociception occurring due to movement in monoarthritic rats and is found to be an appropriate method to assess the anti-nociceptive effects on analgesics. This method is adapted in OA animals by replacing the flexion and extension of ankle by flexion and extension of the knee joint. In the current study, as expected. it was observed that knee bend score was significantly higher in MIA-induced OA animals as compared to saline administered animals ($p \leq 0.001$) on day three onwards till day 28. Moreover, the elevated score remained consistent throughout the study duration. However, repeated treatment of BIRM was able to reduce nociception as evident from the significantly ($p \leq 0.001$) reduced knee bend score on day 21 and day 28 as compared to MIA-vehicle control group. Furthermore, the reduction in knee bend score observed in BIRM treated rats was found to be quite comparable to that shown by the standard drug of choice the Celecoxib treated animals (Table 4; Figure 5).

Statistical Analysis

Results were expressed as mean ± SEM (standard error of mean) of the change in paw volume measured. Data was analysed using Graphpad Prism (version 4.1). One way ANOVA followed by Tukey's multiple comparison test was used to analyse data generated from MIA induced osteoarthritis model. $p \leq 0.05$ was considered statistically significant. For ease of reading, the basic statistical values are shown in the text while the more extensive statistical information can be found in the figure legends.

Table 2: Attenuation of mechanical allodynia on repeated administration of BIRM in MIA induced osteoarthritic rats

Group	50% Paw Withdrawal (g) Threshold (PWT) on Days				
	0	14	15	21	28
Normal Control (Group I)	13.99 ± 0.64	13.24 ± 0.82	13.74 ± 0.82	13.24 ± 0.82	13.49 ± 0.68
MIA -Vehicle Control (Group II)	13.47 ± 0.96	3.49 ± 0.36	4.08 ± 0.25	3.89 ± 0.21	4.63 ± 0.41
BIRM (Group III)	13.49 ± 0.96	4.57 ± 0.20	9.47 ± 2.33	8.75 ± 1.80	11.09 ± 1.29[**]
Celecoxib (Group IV)	13.74 ± 0.82	3.72 ± 0.31	12.22± 1.42[**]	10.85 ± 1.45[**]	11.99 ± 1.38[***]

Values represented as mean ± SEM. **p ≤ 0.01, ***p ≤ 0.001 as compared to MIA-Vehicle control. Data analysed by One way ANOVA followed by Tukey's Multiple Comparison post test.

Figure 3: Effect of BIRM on mechanical allodynia in MIA induced osteoarthritic rats
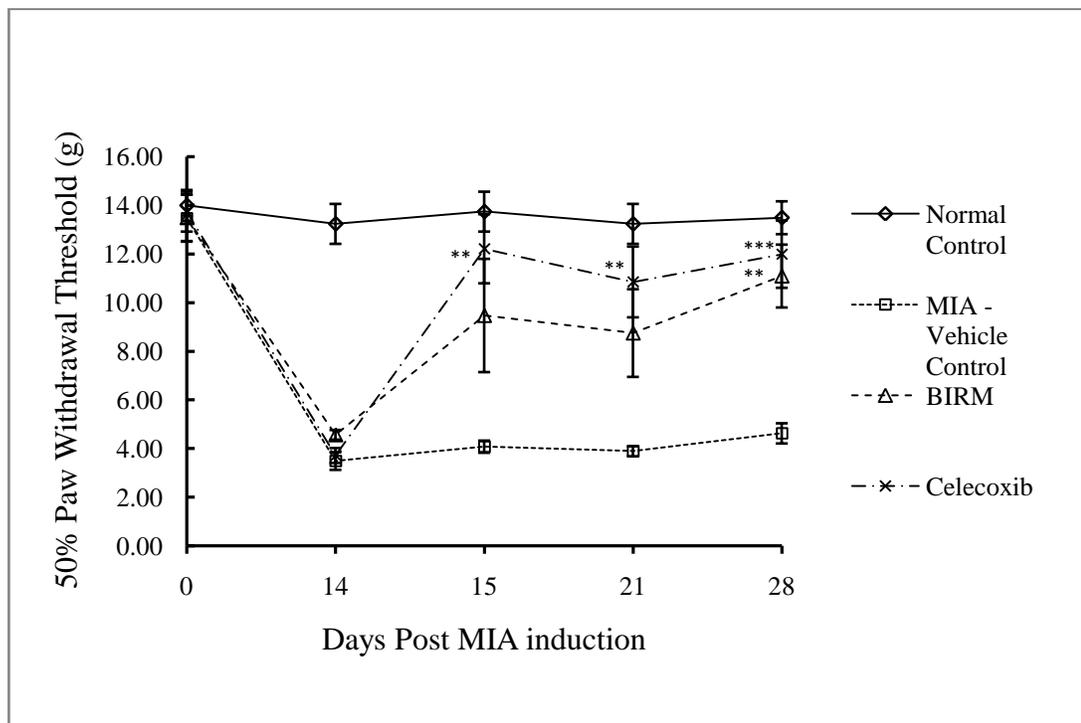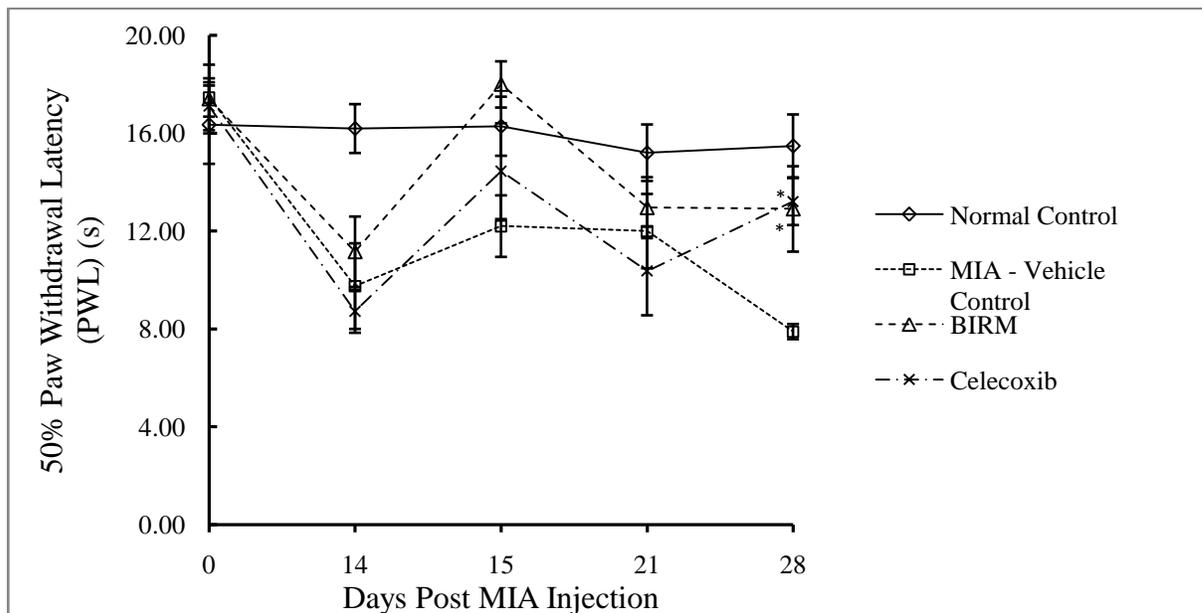

MIA-induced osteoarthritic rats gradually developed allodynia to mechanical stimulus post intra-articular injection of MIA. Repeated administration of BIRM improves the paw withdrawal threshold (PWT) of osteoarthritic rats on day 28. **p ≤ 0.01, ***p ≤ 0.001 as compared to MIA-Vehicle Control. One way ANOVA followed by Tukey's multiple comparison post test

Table 3: Attenuation of thermal hyperalgesia on repeated administration of BIRM in MIA induced osteoarthritic rats.

Group	50% Paw Withdrawal Latency (PWL) (s) on Days				
	0	14	15	21	28
Normal Control (Group I)	16.34 ± 1.60	16.19 ± 1.00	16.28 ± 1.21	15.20 ± 1.15	15.48 ± 1.29
MIA -Vehicle Control (Group II)	17.46 ± 0.78	9.75 ± 1.75	12.20 ± 1.26	11.99 ± 1.52	7.89 ± 0.31
BIRM (Group III)	17.39 ± 1.40	11.15 ± 1.44	17.99 ± 0.94	12.95 ± 1.25	12.90 ± 1.75[*]
Celecoxib (Group IV)	17.10 ± 0.98	8.72 ± 0.88	14.44 ± 1.96	10.36 ± 1.81	13.20 ± 0.96[*]

Values represented as mean ± SEM. *p ≤ 0.05 as compared to MIA-Vehicle control. Data analysed by One way ANOVA followed by Tukey's Multiple Comparison post test.

**Figure 4**: Effect of BIRM on thermal hyperalgesia in MIA-induced osteoarthritic rats

MIA-induced osteoarthritic rats gradually developed hyperalgesia to noxious thermal stimulus post intra-articular injection of MIA. Repeated administration of BIRM improves the paw withdrawal latency (PWL) of osteoarthritic rats on day 28. *p ≤ 0.05 as compared to MIA-Vehicle Control. One way ANOVA followed by Tukey's multiple comparison post test

Table 4: Repeated administration of BIRM ameliorates knee bend score in MIA induced osteoarthritis

Group	Knee Bend Score on Days					
	0	3	14	15	21	28
Normal Control (Group I)	0.75 ± 0.21	0.92 ± 0.15	1.50 ± 0.18	1.25 ± 0.21	0.67 ± 0.25	1.42 ± 0.20
MIA-Vehicle Control (Group II)	0.90 ± 0.19	11.50 ± 0.65	13.00 ± 0.42	13.70 ± 0.58	13.50 ± 0.61	13.40 ± 0.29
BIRM (Group III)	0.80 ± 0.25	12.90 ± 0.80	12.50 ± 0.61	12.40 ± 0.66	7.90 ± 0.83[***]	6.20 ± 0.68[***]
Celecoxib (Group IV)	0.67 ± 0.25	11.92 ± 0.47	13.08 ± 0.98	11.33 ± 0.54[*]	7.17 ± 0.51[***]	5.67 ± 0.25[***]

Values represented as Mean ± SEM. *$p \leq 0.05$, ***$p \leq 0.001$ as compared to MIA-Vehicle control. Data analysed by One way ANOVA followed by Tukey's Multiple Comparison post test.

Figure 5: Effect of MIA-induced osteoarthritis on normal function of knee joint (flexion/ extension)
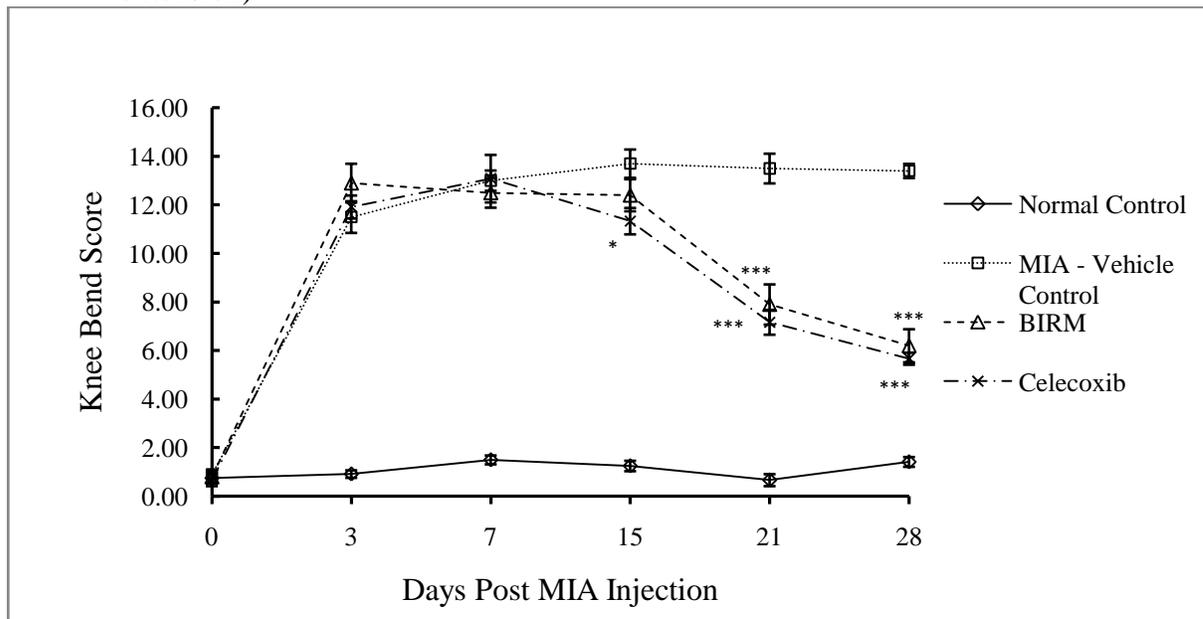
MIA-induced osteoarthritic rats gradually exhibited more sensitivity towards the normal movement like flexion or extension of the knee joint post intra-articular injection of MIA right from day 3 onwards. Repeated adminsitration of BIRM helped in reduction of the knee bend score thus improving the sensitization to normal movement.
*$p \leq 0.05$, ***$p \leq 0.001$ as compared to MIA-Vehicle Control. One way ANOVA followed by Tukey's multiple comparison post test

DISCUSSION

Osteoarthritis being a chronic condition is widely prevalent in elderly population. Pain associated with OA usually gets worsen with weight bearing and activity. It is believed that subchondral bone, periosteum, synovium, ligaments and the joint capsule are richly innervated and contain nerve endings which may be the sources for nociceptive stimuli (Heppelmann, 1997; Mach *et al.*, 2002). In addition to this, Schaible *et al.* (2002) have reported occurrence of peripheral and central pain sensitization in OA. As of now, due to lack of disease modifying drugs, pharmacological treatment is being aimed at alleviation of pain, maintenance or improvement of joint mobility and reduction of functional impairment. Free nerve endings in the synovium may be the cause of joint pain sensation observed in many states of osteoarthritis (Saito and Koshino, 2000). MIA-induced OA model reflects the different pain states observed in clinical conditions. In the initial duration of the MIA-induced OA model implies a transient synovial inflammation involving role of macrophages (Haywood *et al.*, 2003). Inflammation normally gets resolved in the joints after one week post MIA injection and biomechanical forces affecting the articular cartilage and subchondral bone are the reason behind pain sensation. There are many proinflammatory cytokines, oxidants and other factors exerting action in initiation and development of OA. It is hard to obtain complete therapeutic effects by blocking activity of one or two cytokines. In such scenario, developing therapeutics from herbal sources may reduce the risk of toxicity or adverse effects and may exert strong multifunctional anti-inflammatory effect (Kim *et al.*, 2012).

Of the pharmacological interventions available, analgesics and NSAIDs have been proven to be highly effective in the controlling the symptoms and signs of OA. But, these drugs have potential adverse effects which makes them unfavorable for long time consumption. In comparison to these, herbal formulations though are not yet among the recommended treatment options, are used widely in the treatment of OA in oral or topical forms. The better efficacy of these botanicals could be due to their broader mechanism of action as compared to their currently used counterpart like NSAIDs or other analgesics used in the treatment of OA. Exact mechanism through which these herbal medicines exert their potency however, remains elusive and needs to be studied in detailed but most of them act through several pathways which include inhibition of COX and/or lipoxygenase, cytokine release, elastase or hyaluronidase and may also induce anti-oxidative activity (Cameron *et al.*, 2009b). The crude aqueous extract of *Solanum dulcamara* the

source of drug of choice for the current study the BIRM, is reported to limit the local PGE_2 production by inhibiting COX-2 at the site of inflammation (Jaggi *et al.*, 2004). Based on this information we expect BIRM to show anti-nociceptive potentials in OA.

In this study we have carefully characterized the widely used MIA model of OA in relation to development of pain related behavior and investigated effects of BIRM on the clinical and behavioral changes associates with MIA-induced OA. It was noticed that single dose of MIA induced osteoarthritis in animals accompanied with pain behavior. Swelling in the knee joint and limping gait was observed in the first week post MIA injection. In addition, abnormally increased response to non-noxious mechanical stimulus (tactile allodynia) and heightened sensitivity to noxious radiant heat source was observed in animals injected with MIA. We also observed that MIA treated animals reacted more profoundly when they were subjected to normal movement such as flexion and extension of knee joint. Nevertheless, repeated oral administration of BIRM not only reduced the swelling in affected joint but also significantly reversed the nociception-related behavior as assessed by the knee bend test at day 21 and day 28. Although it could not lower the score comparable to saline treated animals, the effect produced by BIRM was found much similar to that of the standard drug, celecoxib treated animals. It was also able to inhibit tactile allodynia by increasing the paw withdrawal threshold significantly on day 28. Though increased response to the noxious thermal stimulus was not shown at a consistent scale by MIA-induced animals during the tenure of the study, BIRM treatment showed improvement in paw withdrawal latency thus inhibiting thermal hyperalgesia to some extent. Our observation with respect to inconsistent hypersensitivity to noxious thermal stimulus throughout the study duration in the MIA-induced OA animals is in line with the findings of the other studies conducted in rats (Combe *et al.*, 2004; Vonsy *et al.*, 2009) as well as with clinical observations. These results suggest that BIRM may have the potential to be used as therapeutic for OA.

While performing tactile allodynia test using von Frey filaments, due to experimental hindrances, we applied filaments to plantar surface and not on the skin above the knee joint. Studies performed by Salo and Theriault (1997) and Bajrovic and Sketelj (1998) have shown that L3-L5 pre-dominantly receive primary afferent input from the knee and L3, L4 and L5 DRGs receive hind paw afferents thus hypothesizing that cell bodies of afferents from the knee are thought to co-localize in DRGs. This concurrence permits the crosstalk responsible for pain transmitted by

the afferents supplying the knee causing pain in the hind paw thus justifying the location of filament exposure.

As mentioned previously, cartilage do not have any nerve supply but dense innervations of A- and C-fibers is found in maximum load bearing region of bone such as proximal and distal head of the femur (Mach *et al.*, 2002). In OA, pro-inflammatory agents such as bradykinin, SP and PGs get released into the joint leading to sensitization of afferent fiber and reduction in fiber threshold causing peripheral sensitization (Scott *et al.*, 1994). These could be the reason behind silent nociceptors which do not usually respond to stimuli gets activated in response to tissue damage/inflammation which in turn increases nociceptive drive onto dorsal horn neurons thus facilitating central mechanisms of hypersensitivity such as wind-up. Hence, the increased in-put, wind up, sensitization of A- and C-fiber resulting in hypersensitized behavioral response to mechanical stimuli implicates the role of central sensitization in the MIA-induced OA pain. Ziegler *et al.* (1999) and Magrel *et al.* (2001) have reported involvement of Aβ-fiber mechanoreceptors in mechanical allodynia whereas punctuate stimuli is mediated by capsaicin-insensitive Aδ-fiber nociceptors.

Most of the time, methods such as the paw pressure test and von Frey filament are used to measure secondary hyperalgesia and allodynia for nociceptive behavioral test in OA model. However, they measure the secondary hyperalgesia occurring away from the affected knee joint and do not evaluate the primary hyperalgesia taking place in the OA joints. Hence, ankle knee bend test is employed for evaluating nociception in OA animals. In knee bend test, OA animals exhibit profound response through vocalization when their affected joint is moved normally through flexion or extension. Ferreira-Gomes *et al.* (2008) have observed slightly higher knee bend score on first day after saline injection in control animals, indicating reaction of the intra-articular injection, thus establishing this method to be useful, sensitive and reliable test to evaluate nociception exacerbated by the movement of a diseased joint - a common trait observed in OA.

CONCLUSION

Firstly, when using animal models of OA, nociceptive tests including mechanical allodynia using von Frey filaments, thermal hyperalgesia and knee bend test have proved to be useful methods for the evaluation of nociceptive symptoms and movement related nociception and allows direct assessment of the activation of joint nociceptors in OA. These tests allow us the measurement of nociception in a clinically relevant way.

Secondly, BIRM seems to manifests its anti-nociceptive effect in MIA-induced OA model through inhibiting mechanical allodynia (primary and secondary) and thermal hyperalgesia. Prochazkova *et al.* (2009) have reported increased COX-1 and COX-2 mRNA levels in spinal cord of OA animals. COX-2 being the inflammatory mediator may serve as the precursor to the subsequent chain of reaction giving rise to peripheral and central sensitization leading to chronic pain. Although the exact mechanism of anti-nociceptive/anti-inflammatory action of BIRM in OA remains unknown, BIRM's reported inhibitory effect on COX-2 activity may play a crucial role in exerting its therapeutic potential in MIA-induced OA model.