A SIMPLE LABORATORY MODEL FOR INDUCING AND MEASURING PAIN IN SMALL EXPERIMENTAL ANIMALS

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ABSTRACT

Objective: Pain, an unpleasant sensation that we all experience in daily life, is an alert mechanism to prevent impending tissue injury. The animal models employed for screening of analgesic agents include pain-state models based on the use of thermal, mechanical electrical and chemical stimuli. This study was undertaken with an objective to design, develop and fabricate a new animal model for screening analgesics.

Methods: In the present study, a humble attempt is made to develop a new animal model for screening analgesics overcoming the limitations of earlier models. The utility of the newly developed laboratory model (M-model) of pain was compared with already established models.

Results: A simple laboratory model for screening of analgesics was developed in the present study. In this study, endurance time was defined as the time for which, the animals were able to endure the cold surface of ice-floor. The animals assumed a flinching posture and fled to M-Zone when they were unable to withstand the cold surface. Endurance time was significantly and consistently enhanced by different classes of analgesic agents such as pentazocine, butorphanol, tramadol, diclofenac, ketoprofen and meloxicam. The findings obtained using M-model was in line with those obtained using already established models.

Conclusion: An effective animal model for screening analgesics overcoming the limitations of earlier models was developed in this study. This model showed excellent face and predictive validity.

Keywords: Pain, Analgesics, Cold stimuli, M-model, Flinching posture, Endurance time

INTRODUCTION

Pain is a complex, unpleasant phenomenon composed of sensory and emotional experience that include time, intensity, emotion, cognition and motivation originating from damaged tissue [1]. It plays an important role as an alarm/signal that helps to protect the living organisms and provokes avoidance behaviour, which arrests the potentially damaging consequences and facilitates fundamental biological functions such as inflammation or healing [2]. One-third of the world's population suffers from persistent or recurrent pain. Nociceptors are the particular sensory receptors responsible for the detection of noxious stimuli, transforming the stimuli into electrical signals, which are then conducted to the CNS [3].

There are the free nerve endings of primary afferent Aδ and C fibres distributed throughout the body they can be stimulated by thermal, mechanical, electrical and chemical stimuli. Inflammatory mediators (eg. bradykinin, serotonin, prostaglandins, and cytokines) are released from damaged tissue and can stimulate nociceptors. They can also act by reducing the activation threshold of nociceptors so that the stimulation required to cause activation is less. In addition to the Aδ and C fibres that carry noxious sensory information, there are primary afferent Aβ fibres that bring non-noxious stimuli. These fibres possess different characteristics that allow the transmission of particular types of sensory information. Aβ fibres are highly myelinated and of large diameter, therefore allowing fast signal conduction. They have a low activation threshold and generally respond to light touch and transmit nonnoxious stimuli [4]. They respond to mechanical and thermal stimuli. C fibres are unmyelinated and are also the smallest type of primary afferent fibre. Hence, they allow the slowest conduction. C fibres respond to chemical, thermal and mechanical stimuli. Cold stimuli are that tactile sensibility and motor function deteriorate while pain perception persists. Nociception are the neural processes of encoding and processing noxious stimuli. Pain in response to a non-nociceptive stimulus is known as allodynia and increased pain sensitivity is hyperalgesia. Chronic pain is associated with

conditions such as back injury, migraine headaches, arthritis, diabetic neuropathy, and cancer. Many of the currently available pain therapies cause uncomfortable to deleterious side effects because of lack of perfect animal models for screening of different types of analgesics. Animal models serve as indispensable tools for discovering new medicines as well as for the analysis of the magnitude of causes, biomarkers, and pathophysiological changes, which bring about symptoms analogous to those of patients with a specific disorder. Since human life is precious, it becomes necessary to test new medicines in small animals before applying to human beings. Animal models provide an opportunity to decipher the relationships between the nervous system and animal behaviours as they serve as obligatory tools for screening of new drugs [5]. Pain cannot be monitored directly in animals, but can only be measured by examining their responses to nociceptive stimuli [6]. Noxious stimulus is often used in animal models to damage a specific tissue partially in order to produce pain or inflammation. The observed reactions are almost always motor responses ranging from spinal reflexes to complex behaviour.

Animal models serve as indispensable tools for discovering medicines useful in the treatment of human diseases. Tail-flick technique and hot-plate test are commonly used for screening analgesic agents, although both of these models use heat as the noxious stimulus. At present, there are several tests in literature, which employ cold stimulus for studying nociception in animal's viz. Paw or tail withdrawal after immersion in cold water [7, 8] or water-alcohol bath [9], ethyl chloride spray [10], direct application of cold acetone [11] or contact with a Peltier the mode [12].

However, most of these methods impose limitations on the testing conditions [13]. New laboratory models are difficult to develop particularly in the area of neuropharmacology, because of the complexity of the human neuronal network. Since the brain of animals is not so well developed as compared to human brain, it becomes a tough task to produce neurological disorders in laboratory animals. An ideal laboratory model for screening

analgesics agents should be able to evoke behavioral changes in animals, which can be quantified. These behavioral changes observed in animals should be reversible by the same analgesic agents, which are effective in human beings. In the light of the fact that pain is very frequently experienced by human beings in several disease states; there arises an urgent need to develop a suitable animal model for detecting different types of new analgesic agents. The present study was undertaken with an objective of designing, developing and fabrication of a simple laboratory model for screening of analgesic agents.

MATERIALS AND METHODS

Experimental animals

A total of 138 Swiss albino mice of either sex divided into 23 groups were employed in the present study. Each group consisted of a minimum of 6 animals. Adult (3-4 mo old) mice weighing around 20-25g were procured from the Disease-Free Small Animal House, Lala Lajpat Rai University of Veterinary and Animal Sciences (LUVAS) Hisar, Haryana (INDIA). The animals were acclimatized for at least 7 d to the laboratory conditions before behavioral experiments. Animals were housed under standard conditions of temperature (24±2 °C) and relative humidity (30-70%) with a 12:12 light: dark cycle. Experiments were carried out between 09:00 h-17:00 h. The experimental protocol was approved by Institutional Animal Ethics Committee (IAEC) as per guidelines of CPCSEA, Ministry of Forests and Environment, Government of India (Registration number 0436).

Induction and measurement of pain

A New Animal Model was designed and developed in the present study, for induction and measurement of pain in mice. This laboratory model (M-model) comprised of basically four components i) perspex-box ii) M-Zone iii) ice-tray and iv) ice-floor. Initially, the mouse was exposed to various parts of the M-model particularly M-Zone for around 60 sec, so that the mouse was aware of the presence of M-Zone before the start of the experiment. The animal was inserted from the top/ceiling of the perspex box. The ice-tray consisting of ice-block was slid onto the floor of the perspex box. The animal fled to M-Zone when it was not able to withstand or endure the cold surface of ice-floor. Endurance time was defined as the time taken by the animal to flee into the M-Zone (Flight-Zone) when placed on the ice-floor. Endurance time was recorded with the help of a stopwatch. Normally, mice take around 4-6 sec. to flee into the M-Zone to avoid ice-floor. The ice trays were changed for each group of mice so as to expose each mouse to a fresh ice-block. Separate groups of animals were pretreated with narcotics such as pentazocine (10 mg/kg, s. c.), butorphanol (partial opioid agonist, 2 mg/kg, s. c.), tramadol (opioid agonist, 5 mg/kg, s. c.) and non-narcotic analgesics such as diclofenac (non-selective COX inhibitor, 15 mg. kg, i. p), ketoprofen (non-selective COX inhibitor, 5 mg/kg, p. o) & meloxicam (preferential COX-2 inhibitor, 5 mg/kg, s. c) to determine their effect on Endurance-time. The endurance time was recorded at 0, 15, 30, 45, 60, 120, and 180 min after administration of the standard drugs.

Experimental design

The drugs and chemicals used in the study were procured from drug houses as given in the bracket. Diclofenac sodium (Voveran, Novartis, Mumbai), meloxicam (Meloxicam, Intas Pharmaceutical Ltd., Ahmadabad), Pentazocine lactate (Fortwin, Ranbaxy Laboratory Ltd., Ahmadabad), tramadol (Tramazac, Cadila, Ahmedabad), butorphanol tartrate (Butrum, Arito Pharmaceutical Pvt. Ltd., M. P.) and ketoprofen (Neon Laboratory Ltd Mumbai, India). Standard drugs such as diclofenac, meloxicam, pentazocine, tramadol, and butorphanol were diluted in distilled water, and ketoprofen was suspended in 2% of tween-80 solution.

Groups using hot-plate test

Group I: Control group: Distilled water was administered subcutaneously. The analgesic activity was recorded at 30 min after the administration of the Vehicle.

Groups II, III, IV and V: Test groups: Diclofenac (15 mg/kg, i. p), meloxicam (5 mg/kg, s. c), pentazocine (10 mg/kg, s. c) and tramadol (5 mg/kg, s. c) were administered to different groups. The analgesic activity was recorded at 30 min after the administration of these drugs.

Groups using tail-immersion test

Group VI: Control group: Distilled water was administered subcutaneously. The analgesic activity was recorded at 30 min after the administration of the vehicle.

Groups VII, VIII, IX and X: Test groups: Diclofenac (15 mg/kg, i. p), meloxicam (5 mg/kg, s. c), pentazocine (10 mg/kg, s. c) and tramadol (5 mg/kg, s. c) were administered to different groups. The analgesic activity was recorded at 30 min after the administration of these drugs.

Groups for tail-flick technique

Group XI: Control group: Distilled water was administered subcutaneously. The analgesic activity was recorded at 30 min after the administration of the vehicle.

Groups XII, XIII, XIV and XV: Test groups: Diclofenac (15 mg/kg, i. p), meloxicam (5 mg/kg, s. c), pentazocine (10 mg/kg, s. c) and tramadol (5 mg/kg, s. c) were administered to different groups. The analgesic activity was recorded at 30 min after the administration of these drugs.

Groups for designing and fabrication of a New, laboratory model

Group XVI: Control group: Distilled water was administered subcutaneously. The analgesic activity was recorded at 30 min after the administration of the vehicle.

Group XVII: Control group: tween-80 (2%) was administered per oral with the help of an oral needle. The analgesic activity was recorded at 30 min after the administration of the vehicle.

Groups XVIII, XIX, XX, XXI, XXII and XXIII: Test groups: Diclofenac (15 mg/kg, i. p), ketoprofen (5 mg/kg, p. o), meloxicam (5 mg/kg, s. c), pentazocine (10 mg/kg, s. c), tramadol (5 mg/kg, s. c) and butorphanol (2 mg/kg, s. c) were administered to different groups. The analgesic activity was recorded at 30 min after the administration of these drugs. The established models such as hot-plate test, tail-immersion test, and tail-flick technique were employed for comparing the utility of the newly developed model using narcotic (pentazocine, tramadol) and non-narcotic agents (diclofenac, meloxicam).

Statistical analyses

All the results were expressed as a Mean+Standard error of mean (S. E. M). Data were analyzed using one-way ANOVA followed by Dunnett's test. $P<0.01$ was considered as statistically significant.

RESULTS

Description of newly developed laboratory model

A New animal model was designed and developed in the present study, for inducing and measuring the pain of mice. We have named this newly developed model as M-model, since the shape of the M-Zone (Flight-Zone) is like letter "M" and the first letter of the most powerful analgesic agent morphine is also "M".

Functions of various parts of the device are described below

i) Perspex-box: Perspex-box (36 x 18 x 18 cm³) consists of three transparent walls made up of Perspex so that animal behavior can be easily observed. The fourth wall of the box is made up of aluminum sheet for accommodating "M" shaped Flight-Zone.

ii) M-Zone: The animal flees to M-Zone (Flight-Zone), which is situated at a height of 12 cm from the ice-floor, when, it is not able to withstand or endure the cold surface of ice-floor, which has been portrayed in fig. 1. The arms (18 x 3 x 3 x 18 cm⁴) of M-Zone provide safe and comfortable grip to the animal.

iii) Ice-tray: Ice-tray made up of aluminum sheet is fitted at the base of the perspex box. It comprises of ice-block to provide cold

surface. This cold surface forms the ice-floor for the animals. The purpose of ice-floor is to induce mild pain in mice.

iv) Ice-floor: Ice-floor comprised of ice spread over aluminum sheet to provide a cold surface.

Fig. 1: Showing various components of the model

Endpoint

Endurance time is defined as the time for which, the animals were able to endure the cold surface of ice-floor. Under the influence of analgesics, the animals were able to withstand cold surface of ice-floor for a longer period. The animals assume a flinching posture and flee to M-Zone when they are unable to withstand the cold surface.

Flinching posture comprised of following components (fig. 2)

i) Straightening of the tail ii) Recoiling (Shrinking) of the body

iii) Licking of the paws iv) Ready to jump

Induction and measurement of pain in mice using M-model

Initially, the mouse was exposed to various parts of the M-model particularly M-Zone and base of the perspex box for around 60 sec, so that the mouse was aware of the presence of M-Zone before the

start of the experiment. The ice-tray consisting of ice-block was slid onto the floor of the perspex box. The animal fled to M-Zone when it was not able to withstand or endure the cold surface of ice-floor. Normally, mice take around 4-6 sec. to flee into the M-Zone to avoid ice-floor. This Endurance time was significantly increased (beyond 12 sec.) under the influence of an analgesic agent. A camera may also be fitted at the ceiling of the box for video recording of the behavior of the mouse. Separate groups of animals were pretreated with narcotics such as pentazocine (10 mg/kg, s. c), butorphanol (partial opioid agonist, 2 mg/kg, s. c), tramadol (opioid agonist, 5 mg/kg, s. c) and non-narcotic analgesics such as diclofenac (non-selective COX inhibitor, 15 mg. kg. i. p) ketoprofen (non-selective COX inhibitor, 5 mg/kg, p. o) & meloxicam (preferential COX-2 inhibitor, 5 mg/kg, s. c) to determine their effect on Endurance–time.
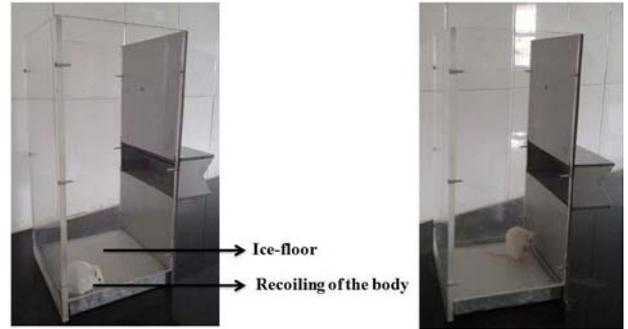

Fig. 2: Showing flinching posture of mouse on ice-floor

The endurance time was recorded at 0, 15, 30, 45, 60, 120, and 180 min after administration of the standard drugs. Endurance time was significantly ($p<0.01$) and consistently enhanced by different classes of analgesic agents such as pentazocine, butorphanol, tramadol, diclofenac, ketoprofen and meloxicam at 30, 45, and 60 minute time interval. This enhancement of endurance latency reflects the anti-nociceptive effect of various analgesics, which has been depicted in fig. 3 and 4.
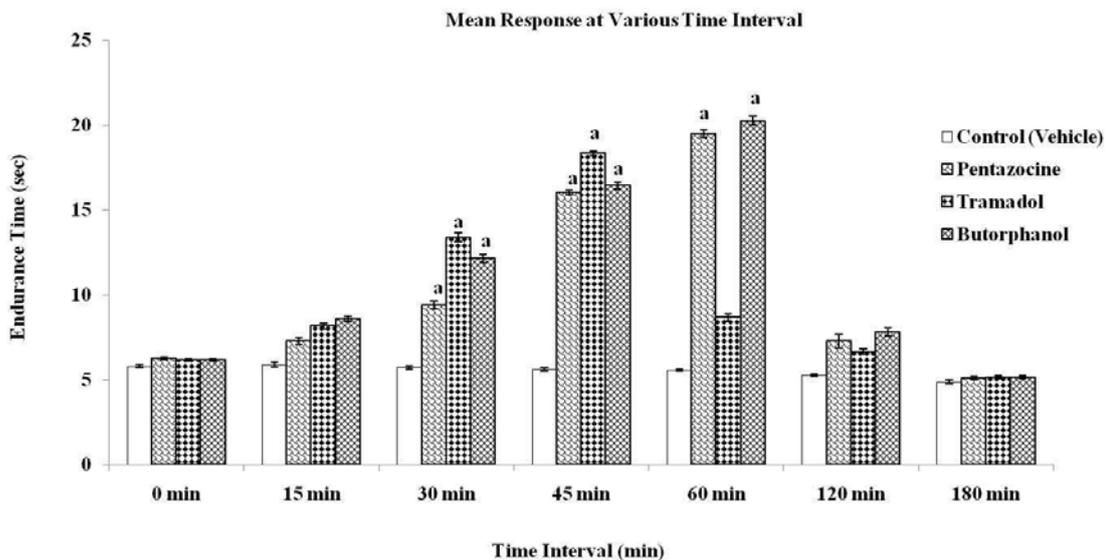

Fig. 3: Effect of various narcotic agents on M-model

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, pentazocine (10 mg/kg, s. c), tramadol (5 mg/kg, s. c) and butorphanol (2 mg/kg, s. c) were dissolved in distilled water (vehicle) and administered to mice. "a" denotes P<0.01 as compared to control group
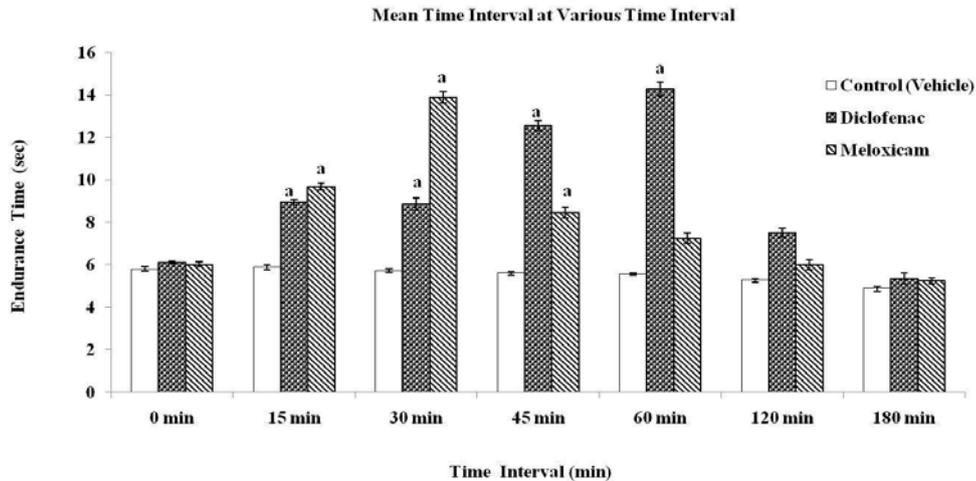
Fig. 4: Effect of various non-narcotic agents on M-model

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, diclofenac (15 mg/kg, i. p), meloxicam (5 mg/kg, s. c), were dissolved in distilled water (vehicle) and administered acutely to mice. "a" denotes $P<0.01$ as compared to control group

Comparison of Newly developed model (M-model) with established models such as hot-plate model, tail-immersion test and tail-flick technique

The utility of Newly developed laboratory model (M-model) of pain was compared with already established standard models viz. hot-plate model, tail-immersion test and tail-flick technique

using different classes of analgesic agents such as pentazocine (10 mg kg^{-1}, s. c), tramadol (5 mg kg^{-1}, s. c), diclofenac (15 mg kg^{-1}, i. p) & meloxicam (5 mg kg^{-1}, s. c). The findings obtained using M-model were in line with those obtained using already established models such as hot-plate model (fig. 5 and fig. 8), tail-immersion test (fig. 6 and fig. 9) and tail-flick technique (fig. 7 and fig. 10).
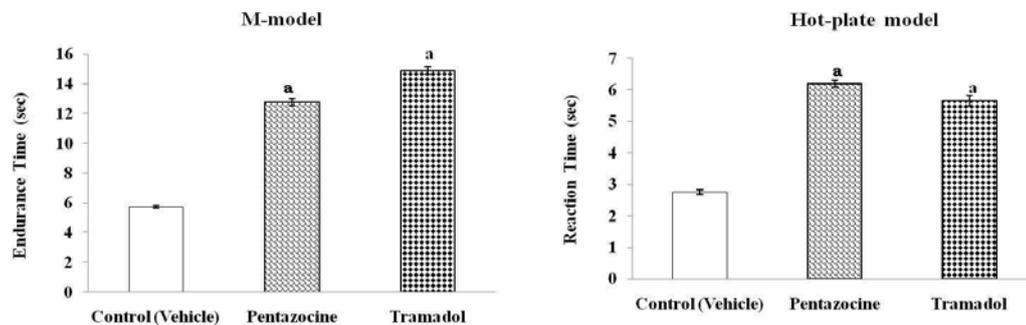

Fig. 5: Comparison of M-model with hot-plate model using narcotic agents

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, pentazocine (10 mg/kg, s. c) and tramadol (5 mg/kg, s. c) were dissolved in distilled water (vehicle) and administered to mice. "a" denotes $P<0.01$ as compared to control group
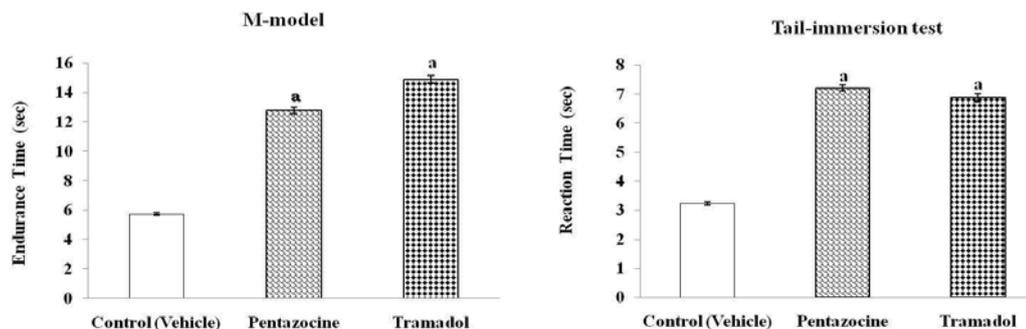

Fig. 6: Comparison of M-model with tail-immersion test using narcotic agents

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, pentazocine (10 mg/kg, s. c) and tramadol (5 mg/kg, s. c) were dissolved in distilled water (vehicle) and administered acutely to mice. "a" denotes $P<0.01$ as compared to control group.
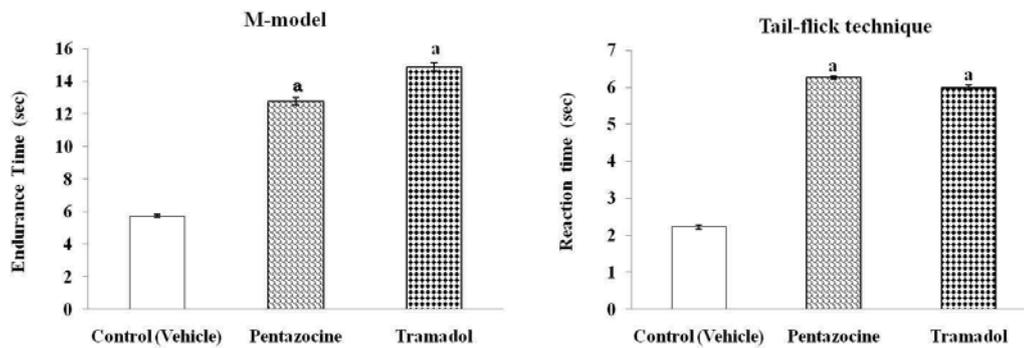
Fig. 7: Comparison of M-model with tail-flick technique using narcotic agents

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, pentazocine (10 mg/kg, s. c) and tramadol (5 mg/kg, s. c) were dissolved in distilled water (vehicle) and administered to mice. "a" denotes $P<0.01$ as compared to control group
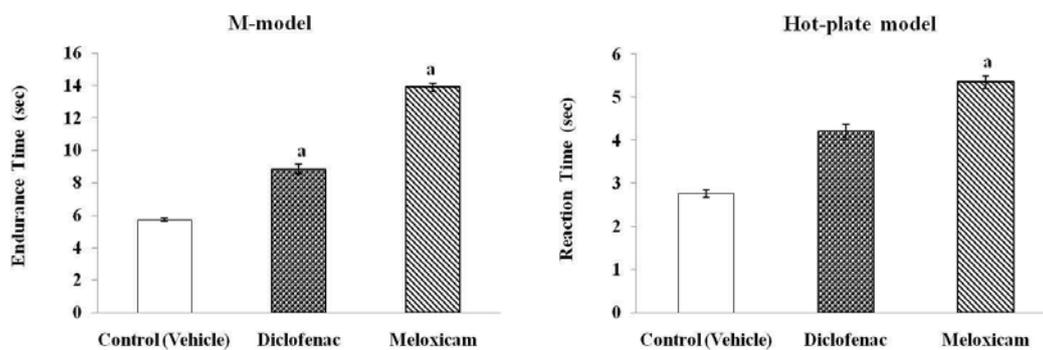

Fig. 8: Comparison of M-model with hot-plate model using non-narcotic agents

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, diclofenac (15 mg/kg, i. p) and meloxicam (5 mg/kg, s. c), were dissolved in distilled water (vehicle) and administered to mice. "a" denotes $P<0.01$ as compared to control group

Fig. 9: Comparison of M-model with tail-immersion test using non-narcotic agents

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, diclofenac (15 mg/kg, i. p) and meloxicam (5 mg/kg, s. c), were dissolved in distilled water (vehicle) and administered to mice. "a" denotes $P<0.01$ as compared to control group
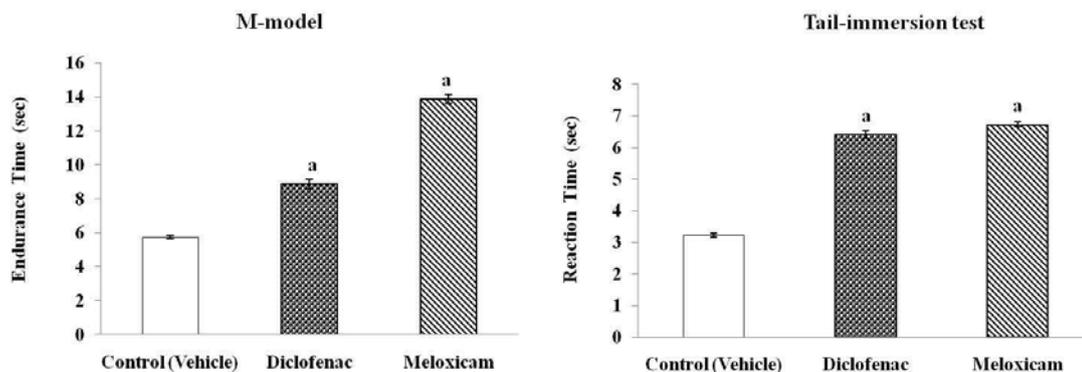
DISCUSSION

Tail-flick technique and hot-plate test are commonly used for screening analgesic agents, although both of these models use heat as the noxious stimulus. Furthermore, these models are not sensitive enough to detect non-narcotic analgesics. At present, there are several tests in literature, which employ cold stimulus for studying nociception in animals viz. paw or tail withdrawal after immersion in cold water and water-alcohol bath, ethyl chloride spray, direct application of cold acetone and contact with a Peltier the mode [14-17].

However, most of these tests have limitations on the testing conditions. For instance, immersion tests or Peltier devices require the animals to be restrained/ anesthetized. Acetone application or ethyl chloride spray produced an olfactory or auditory stimulus; both of these can lead to conditioned responses. Moreover, the stimulated area in many of these tests is possible to vary from one trial to the other, an important consideration given that specific area of the paw are differently sensitive to nociceptive stimuli. Bennett and Xie (1988) first used a 4 °C cold plate on a limited scale with the CCI model one month post-surgery. The authors quantified

nociceptive behavior by monitoring the duration of paw lifts, a scoring method adopted by; however, this scoring method was found to be unreliable and produce variability in results [18, 19]. Moreover, there exists a possibility wherein; animals climb on the ridge of cold plate to avoid cool temperature, thereby interfering in the recording of withdrawal time. Hama and Sagen (1993) used same parameters of cold plate testing as Bennett and Xie (1988), with the exception that scoring took into account the number of

contralateral responses. Thus, there was gross inconsistency in recording the end point. In the present study, a new laboratory model for screening of analgesic agents was designed, developed and fabricated to overcome this inconsistency using Ice-floor to provide cold stimulus. Although there is no agreement in the literature as to when cold begins to be nociceptive, temperatures from-12 °C to 20 °C have been investigated for their nociceptive ability in both humans [20] and rats.
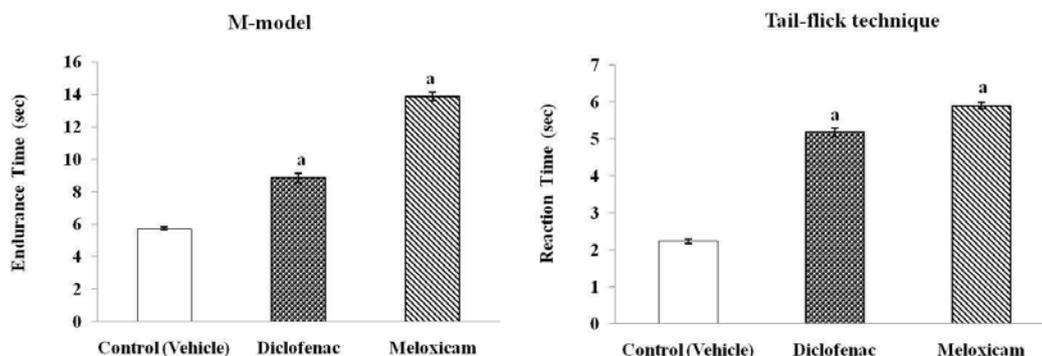

Fig. 10: Comparison of M-model with tail-flick technique using non-narcotic agents

Values are expressed as mean±SEM (n=6). Data was analyzed by one-way ANOVA followed by Dunnett's t-test, diclofenac (15 mg/kg, i. p) and meloxicam (5 mg/kg, s. c), were dissolved in distilled water (vehicle) and administered to mice. "a" denotes $P<0.01$ as compared to control group

Essentially, the perception of a cold stimulus for inducing nociceptive behaviour depends on considerably upon the testing conditions, such as duration of the stimulus, continuous exposure, skin temperatures, location of the stimulated area and medium of stimulation (solid, liquid and gaseous). There is also sufficient evidence to indicate that threshold temperatures for inducing discomfort are slightly higher in humans, usually around 10 °C, than in animals at around 5 °C. This discrepancy in thresholds is likely due to the fact that humans can verbally express discomfort before the urge to withdraw is felt. In rats, the actual withdrawal response is likely to be triggered by recruitment of a greater number of cold nociceptive afferents than those needed strictly for the awareness of cold nociceptive stimulus. The latency to withdrawal becomes shorter as the testing temperature is reduced. Primary afferent Aδ and C fibres distributed throughout the body they can be stimulated by thermal stimuli such as heat or cold. In the present study, ice-block was used to provide cold stimuli for producing mild pain/discomfort in mice (peculiar nociceptive behaviour), which was easily quantified. This observation is in agreement with the study of Jasmine el at., (1998) and Bennett and Xie (1988), who employed a cold plate for studying nociceptive behaviours in neuropathic injury and inflammatory pain models.

The functioning of M-model is based on the fact that the mice flee to M-Zone, when they are not able to withstand or endure the cold surface of ice-floor. Endurance time was defined as the time taken by the animal to flee into the M-Zone (Flight-Zone) when placed on the ice-floor. Under the influence of analgesics, the animals are able to endure cold surface of ice-floor for a longer period. The animals assumed a flinching posture, just before fleeing to M-Zone, when they were unable to endure the cold surface. We had named this newly developed model as M-model, since the shape of the Flight-Zone is like letter "M" and the first letter of the most powerful analgesic agent Morphine is also "M". An ideal animal model should meet the fundamental criteria of good face validity, predictive validity and construct validity [21]. In the newly developed model (M-model), the pain was produced in mice by using ice-floor. The Ice-floor provides cold stimulus evoking mild pain sensation in mice equivalent to human pain experience. Animals as well as humans, when confronted with a physical threat exhibit behaviours that enable them to escape from the source of danger. This fact imparts excellent face validity to M-model. The animals assumed a flinching

posture in the present study, just before fleeing to M-Zone, when they were unable to endure the cold surface. This typical behavioral response was delayed by different classes of therapeutic regimens effective in pain. Different categories of analgesic agents used such as, pentazocine, butorphanol, tramadol, diclofenac, ketoprofen & meloxicam successfully enhanced the endurance time of mice in the present study. Whereas, the vehicle did not have any significant effect on the endurance time. This observation confers good predictive validity to the M-model. The theoretical rationale underlying the animal model of pain and human pain experience contributes to the construct validity of the model. In the present study, the physical causes of pain, as well as the biological factors underlying the pain, appear to be similar in animals and human beings. Therefore, the construct validity of the M-model appears to be fairly good. Furthermore, the new model represents a very simple and cost-effective method for screening of analgesic agents. This model has immense practical and commercial applications [22].

The efficacy of the newly developed model was compared in the present study with the established models such as hot-plate test, tail-immersion test and tail-flick technique. It is noteworthy that M-model was at least as effective as the previous models. The newly developed model permits a fixed temperature setting and provides freedom to the animal allowing observation of spontaneous behaviors. This is a very simple & cost effective model. No special skill is required in handling of this device. Fleeing movement, flinching posture and endurance time observed in this model can be easily recorded and quantified. There was consistency while observing these nociceptive behaviours. There are no noxious stimuli such as electric shock/intense heat to the animals in this model. M-model developed in the present study was effective in detecting the analgesic activity of currently, marketed Narcotic analgesics such as pentazocine, butorphanol (partial-opioid agonist) tramadol (opioid agonist) and non-narcotic analgesics such as diclofenac (nonselective COX inhibitor), ketoprofen (nonselective COX inhibitor) & meloxicam (preferential COX-2 inhibitor). Ice-floor of the M-model produces mild unpleasant sensory experience (nociception), which is similar to pain experience of human beings. There is no need to anesthetize the animals or surgical procedure during the experiment. Thus, new substances possessing analgesic potential can be screened effectively using the newly developed model designed in this study.

CONCLUSION

An effective animal model for screening analgesics overcoming the limitations of earlier models was developed in this study. The findings obtained using M-model was in line with those obtained using already established models. This model showed excellent face and predictive validity.

CONFLICT OF INTERESTS

This paper is jointly prepared by both the authors and there are no conflicts of interest.

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