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The Formalin Test in African Toad (*Bufo regularis*) - a Novel Pain Model in Amphibians

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Abstract: The formalin test was evaluated in African toad (*Bufo regularis*) with 37% formaldehyde (100% formalin) using a multi-dimensional motor activity scoring. The responses were biphasic like those obtained in rodents but with longer first phase, longer interval and longer second phase. Acetylsalicylic acid (70 mg kg^{-1}) reduced significantly responses in both phases while indomethacin only reduced the second phase responses. The first phase is likely due to direct activation of nociceptors by formalin while the second phase suggests the involvement of inflammation. These results are similar to what has been observed in rodents although the physiological mechanisms may not be exactly the same. It is suggested that formalin test in toads is a valid experimental pain model in amphibians for the evaluation of longer-acting analgesics.

Key words: Formalin · African toad · Bufo regualaris · Acetylsalicylic acid · indomethacin · analgesic

INTRODUCTION

A number of behavioural methods have been developed in order to study nociception in animals. Several of these tests: the tail flick test, the flinch-jump test, the pinch test and the hot plate test measure the response to a brief, noxious stimulus. Dubuisson and Dennis presented the formalin test in 1977 [1].

A small amount of diluted formalin (50 µL of 5% formalin) injected into one of the animal's forepaws elicited a sequence of pain-related responses. The responses were rated according to 4 categories and the total score assessed as a weighted average of time spent in the 4 categories during an observation period of 30-60 min after formalin injection. An important feature of the formalin test in rodents is that the animal shows two phases of nociceptive behaviour which seem to involve two distinctly different stimuli [1, 2]. At present, different modifications and implementations of the test are in use.

Most studies have used rodents, predominantly rats. In recent years an increasing number of studies have employed mice [2, 3], Cats [4], primates [5], rabbits [6], guinea pigs [7], crocodiles [8], domestic fowls [9] and Octodon degus [10]. As far as we know there is no report of the test in amphibians. Since pain is a multi-dimensional event, we believe a multi-specie modeling should also be adopted in its understanding. This fact is further underscored by the fact that except in rodents, where the time course and pharmacologic influences are similar, it is doubtful whether the time course and thus the physiological mechanisms in other species are similar to what obtains in rodents [11].

Only the acetic acid-induced wiping response has been documented as a valid behavioural pain model in frogs [12, 13]. Therefore the aim of the present study was to investigate the formalin test in African toad (*Bufo regularis*) and to explore the possibility of its use as a valid pain model.

MATERIALS AND METHODS

Animals: Adult male toads weighing 30-40 g were used in the experiments. They were housed in colony cages with free access to food and water. Prior to the experiments, they were maintained in light-controlled room with 12/12 h dark /light for at least 2 weeks prior to the experiments. The animals were brought to the test room the day before testing and were allowed to adapt to the testing environment for at least 18 h. Two hours before testing, the animals were placed individually in transparent plastic cages (30 x 20 x 30 cm), which also served as observation chambers after formalin injection.

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Drugs and administration route: The method of Rosland *et al*. [14] was followed. Acetylsalicylic acid (70 mg kg^{-1}) (Kunimed pharmachem, Nigeria) and indomethacin (5 mg kg^{-1}) (Guangdong medicines and health, China) were dissolved in distilled water and administered intraperitioneally 30 min before formalin injection. In all experiments, 0.3 mL distilled water served as vehicle in control animals.

Testing procedure: The original design of Dubuisson and Dennis [1] was followed although with some modifications. Previous pilot studies using lower formalin concentrations did not produce easily detectable responses, therefore 37% formaldehyde (100% formalin) was used similar to what was done in rabbits by Farabollini *et al*. [15]. In all experiments, 0.03 mL of the formalin was injected subcutaneously into the dorsal surface of the right forepaw of the toad, using a microsyringe with a 26-gauge needle. After injection the animal was immediately put back into the observation chamber where recording of its behavioural responses was done at 5 min intervals for 60 min.

Assessment of nociceptive behaviour (scoring): Pain is a composite of several nociceptive modalities which may elicit different behavioural patterns and scoring of several behavioural patterns in the animal allows a more valid identification of nociceptive behaviour [11]. We therefore chose to measure the overall behavioural responses of the animal as opposed to a one-paradigm measurement adopted by some authors. The time spent by the animal at rest during each 5 min observation period was calculated and deducted from the observation time interval (i.e. 5 min) because previous observations of untreated toads revealed a state of almost perfect inactivity in the absence of any disturbing stimulus. This is consistent with the specie attributes. Thus what was measured was the total time of motor performance in line with Tjolsen *et al*. [11]. A graph of the response against time was plotted to determine the pattern of the responses as reported by Dubuisson and Dennis [1]. Each animal served as its own control few hours before the experiment and received 0.03 mL of distilled water.

Statistical analysis: Data were examined by paired t-test when the same animals served as both the experimental and control and by unpaired t-test when different animals served as the control. Statistical significance was accepted at the 5% level ($p<0.05$).

RESULTS

Effect of formalin on the nociceptive response: From Fig. 1 formalin induced a statistically significant increase in nociceptive responses of the animal. Two distinct phases are identifiable, a period of high nociceptive responses lasting about 10 min separated by about 20 min from a second period lasting about 20 min.

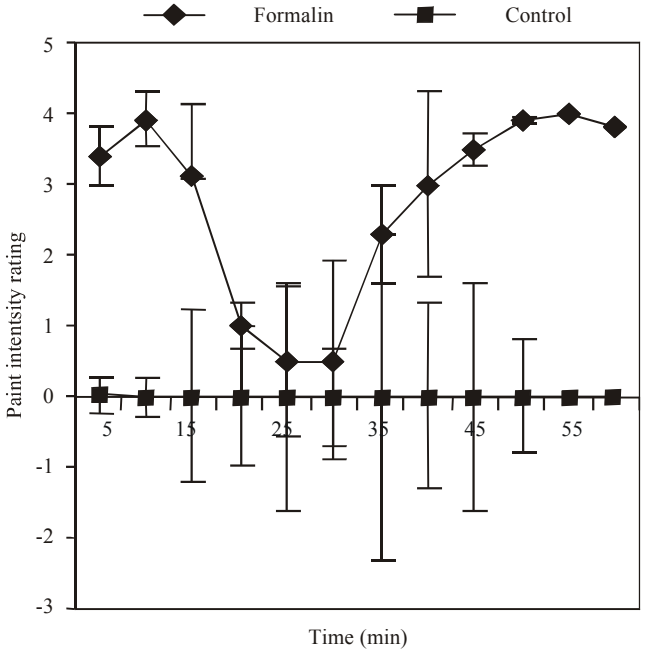

Fig. 1: Pain intensity ratings for formalin and control groups. Each point represents the mean ± SEM of ten toads

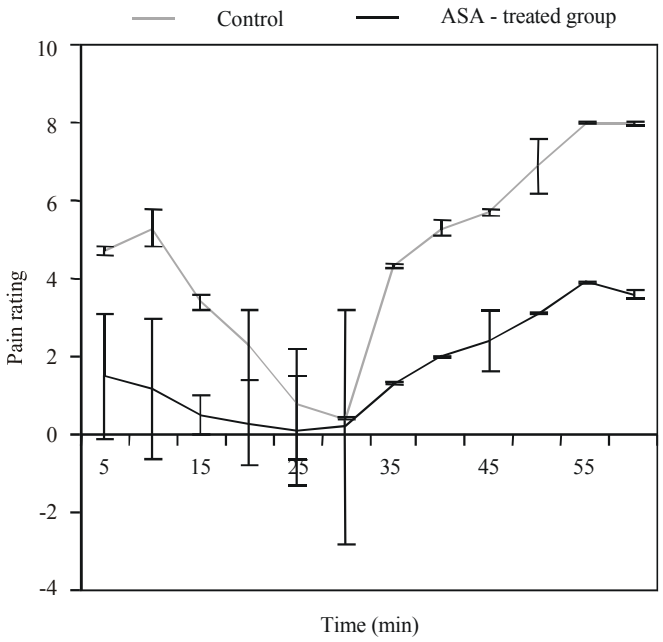

Fig. 2: Pain intensity ratings for acetylsalicylic acid (ASA) - treated and control groups of animals. Each point represents the mean ± SEM of ten toads

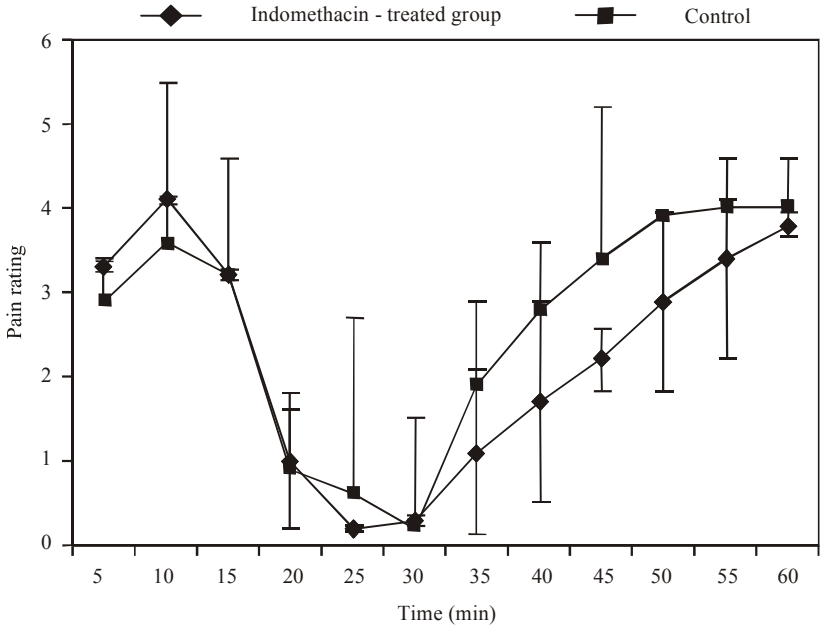

Fig. 3: Pain intensity rating for indomethacin-treated and control groups of animals. Each point represents the mean ± SEM of ten toads

Effect of analgesics on the nociceptive response: Acetylsalicyclic acid (70 mg kg^{-1}) caused a statistically significant inhibition of both phases of the nociceptive responses when compared with the control (Fig. 2). Indomethacin (5 mg kg^{-1}) did not produce any statistically significant effects on the first phase of the nociceptive responses while it reduced the second phase when compared with the control (Fig. 3).

DISCUSSION

Formalin induced a biphasic response in toads. This is similar to what has been obtained in rodents and other animals. It is imperative to note that in this study, a very high concentration was used because lower concentrations did not produce any measurable responses for reasons which could not be immediately verified scientifically. One of the reasons however could be a behavioural resistance of the specie. Even then the 100% concentration used in this study has earlier been used in rabbits with valid results [15].

In other animals, the first phase lasted about 5 min and was separated by a period of about 15 min from a second phase lasting about 20 min [11]. Our results showed a longer first phase, (10 min), a longer second phase, (25 min) and a longer interval, (20 min), between the two phases. The first phase in rodents was due to direct chemical stimulation of nociceptive fibres [1] and experimental data indicated that formalin predominantly evoked activity in C fibres and not in A δ afferents [16]. Hamamoto and Simone [13] recently established the existence of a difference in the population of primary afferents in the frog hind limb with A beta > A delta > C fibres. Although it has been shown by Rosland *et al*. [14] that formalin concentrations higher than 0.2% did not produce higher responses in mice, it is likely that the observed higher first phase in toads was due to the high concentration of formalin used. It might also be due to higher stimulation of C fibres, the population of which might be more in toads than in rodents. Experimental results have also indicated that substance P and bradykinin participate in the early phase in rats and the concentration of these substances display species variation [17].

The second phase in rat is mainly due to peripheral inflammatory processes mediated by histamine, serotonin and prostaglandins [17]. It has been recently demonstrated that central changes induced by the early phase may contribute to the development of the late phase, suggesting that mechanisms other than inflammation may also be involved [18]. The second phase obtained in the toad was longer probably reflecting the high concentration of formalin used. The possibility exists that firing in primary afferents continues a little longer, due to the noxious effect of high formalin concentration on peripheral nerve fibres. This is consistent with our observation that the resultant inflammation was visible in less than 20 min and peaked in about 8 h. These facts might make the model suitable for testing long acting analgesic and anti-inflammatory substances.

Acetylsalicylic acid significantly reduced the nociceptive responses in both phases, a fact in similarity with the reports of several investigators using rats and mice [11, 16]. The non steroidal anti-inflammatory drug (NSAID) indomethacin reduced the nociceptive responses of the second phase while it had no effect on the first phase, this is similar to the finding in mice [19]. While these effects possibly established the formalin test in toad as a valid pain model in amphibians, we are very reluctant to accept that the physiological mechanisms of the observed responses are the same in both rodents and toads. This is of relevance because the different time course observed in toads may be suggestive of different physiological mechanisms.

In conclusion, the formalin test in toad has been shown to be biphasic like in rodents although the time course is slightly different, suggesting different physiological mechanisms. However, the similarities in phases and time course with those obtained in rodents and humans suggest that the responses are closely related to the animal's experience of pain. In addition, the assessment of multi-dimensional motor activities as nociceptive responses that we adopted, coupled with similarity in results of pharmacological interventions between this model and other established ones, make this a possible pain model in amphibians, especially for the evaluation of long acting analgesic substances.

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