

The Suspended Formalin Test: A Method Designed for Studying Formalin-Induced Behaviour in the Speke's Hingeback Tortoise (*Kinixys spekii*)

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Summary

The present study aimed to develop a method for testing pain-related behaviour induced by formalin in the Speke's hingeback tortoise (*Kinixys spekii*). These animals retract their head and limbs into their shell when approached, making behavioural testing almost impossible. It was found that suspending the animals in the air, facing away from the observer, made the animals keep their limbs out of the shell. Subcutaneous injection of formalin induced easily identifiable and quantifiable behaviours that lasted for 20 minutes. Contrary to the biphasic effect of formalin observed in rats and mice, the response in tortoises was monophasic. The suspended formalin test may be useful for studying nociceptive mechanisms in tortoises, which in turn will be important for a further understanding of the nociceptive system in reptiles as well as in mammals.

Introduction

During recent years, an increased interest in studying pain-related behaviour in reptiles has emerged (Machin, 2001; Mitchell, 2002; Sneddon, 2004). A further understanding of the nociceptive system in these animals may contribute to an increased understanding of the development of the nociceptive system in mammals (Sneddon, 2004). In addition, reptiles are widely housed as companion animals. To be able to determine and improve the well-being of reptiles, as well as for conservation purposes, more information about the pain perception in these animals has to be obtained. To our knowledge, little research into the nociceptive system has been performed in animals of the order Chelonia, i.e. turtles,

terrapins and tortoises. The Speke's hingeback tortoise (*Kinixys spekii*), a small brownish-hinged tortoise, is recognised by its domed carapace with a flat dorsal surface and sloping sides. It is widely distributed in tropical Africa and lives in open woodland and savannah. Although the ecology and biology of the tortoise has been extensively studied (Hailey *et al.*, 1998; Hailey, 1998; Luiselli, 2003; Luiselli, 2005), little is known about nociception and pain-related behaviour in this species.

A common method for studying pain-related behaviour and nociceptive mechanisms in animal models is the formalin test. The formalin test was originally developed for cats and rats (Dubuisson & Dennis, 1977), and has since then been extensively used in several species to assess the pain-related response caused by injection of dilute formalin into the paw of an animal (Tjølsen *et al.*, 1992). In many animals, such as rats, the pain-related response caused by formalin is divided into a first immediate acute phase and a second inflammatory response. The first transpires during the first five minutes, followed by

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an almost silent response up to the 20-30th minute (Hunnskaar & Hole, 1987). Then, a second chronic phase occurs, caused by a peripheral inflammation and central sensitization. The formalin test has also been used in reptiles (Kanui *et al.*, 1990), but has not previously been performed in the Speke's hinge-back tortoise.

Studying behaviour in tortoises can be a difficult task, since the animals tend to retract their head and limbs into the shell when approached. In initial studies where the formalin test was applied in freely moving animals, it was not possible to score any pain-related behaviours, since the animals were quite often passive, completely retracted into the shell. However, we noticed that the tortoises were less willing to retract their limbs when lifted above the ground. Therefore, we decided to test whether it would be possible to study formalin-induced behaviour in the Speke's hingeback tortoise, after suspending the animals in the air and facing them away from the observer.

Materials and Methods

All experimentation was performed at the Department of Veterinary Anatomy and Physiology at the Chiromo Campus, University of Nairobi. All experiments were approved by the Kenyan Wildlife Society, KWS, before they were conducted.

Twelve tortoises (*Kinixys spekii*), were used in the study. They were collected from Machakos district, South-East of Nairobi, Kenya. The age ranged from an estimated 7 to 30 years, with an average weight of 729 ± 48 g (mean \pm SEM). The tortoises were housed in metallic cages measuring 1.25 m \times 0.9 m \times 0.58 m, with 22 cm of locally collected soil and stones. Each cage contained a bigger stone as enrichment. The tortoises were kept under 12 hours of natural light and 12 hours of darkness (6.30 am-6.30 pm), high relative humidity and temperature 22-28°C. The tortoises were labelled with a permanent marker on the scutes of the carapace and the plastron for identification purposes. The animals were attended to by a veterinarian on a regular basis. All tortoises were bathed, washed and weighed

weekly. Food was provided three to four times per week for six hours, and consisted of fresh, thinly sliced carrots, cabbages, tomatoes and Kikuyu grass (*Pennisetum clandestinum*). Tap water, which was changed daily, was provided *ad libitum*. All tortoises were acclimatized to the new environment for one month before experimentation. During this period and during the experimental period the tortoises were handled daily.

Preliminary tests revealed that intradermal injection of formalin was unreliable since the formalin had a tendency to leak out of the dermis. Therefore, as previously performed in the crocodile (Kanui *et al.*, 1990), formalin was injected subcutaneously. The concentration of formalin used was 10 %, based on previous studies where the formalin test has been adapted to reptiles (Kanui *et al.*, 1990). Formalin was injected with a 28½ gauge needle (U-100 Insulin Syringe) at a volume of 100 μ l into one of the hind paws between digit IV and V. Note that the first digit is absent in *Kinixys spekii*. (Crumly & Sanchez-Villagra, 2004).

The tortoises were suspended by a thin cotton string (ARTCCOT, CT-613) around the shell, and the string was attached to a metallic stand. The tortoises were suspended horizontally and the head and the limbs were allowed to move freely according to Fig 1, facing away from the observer. After a few minutes of acclimatisation, the tortoises kept their heads and limbs out of the shell, enabling injection of formalin and observation of altered behaviour.



Figure 1. A Speke's Hingeback tortoise suspended for the formalin test.

The data were analysed with Wilcoxon signed rank test to test whether the median of the responses differed from score 0 (i.e. no pain score), and Friedman's test with Dunn's multiple comparison post-hoc test to test whether the responses differed between each time interval. All statistics were calculated using SPSS version 14.0.

Results

Injection of formalin into the paw of the suspended tortoises resulted in a retraction of the paw into the shell. The retraction could be partial or complete, and could occur either while the animal was still or while moving its limbs. When the animal was still, it was possible to distinguish five different responses: injected limb completely retracted; almost completely retracted; half-way out; almost completely out; or completely out. These five behaviours are demonstrated in Fig 2. While limbs were moving, it was more difficult to distinguish between each of the five behaviours. Therefore, the response when

moving was defined as three different behaviours: Moving all limbs except for the injected limb; moving all limbs, but the injected limbs only carefully; or moving all limbs.

Each of the behaviours was given a specific score, according to Table 1, where behaviour considered to indicate a greater nociceptive response was given a higher score. In addition to the above mentioned parameters, the integrated movement in seconds throughout the experiment was measured, in order to assess whether the animals were sedated by any drug treatment (data not shown).

After injection of formalin, pain-related behaviour was studied in blocks of 10 minutes. Each behaviour during an interval was noted, and the average of behavioural scores during an interval was set as the pain-related score for that specific interval. Figure 3 shows the pain-related score for the suspended formalin test using 10 % formalin injected subcutaneously into the hind paw of the animal. Since the experimental data were limited by a minimal and a

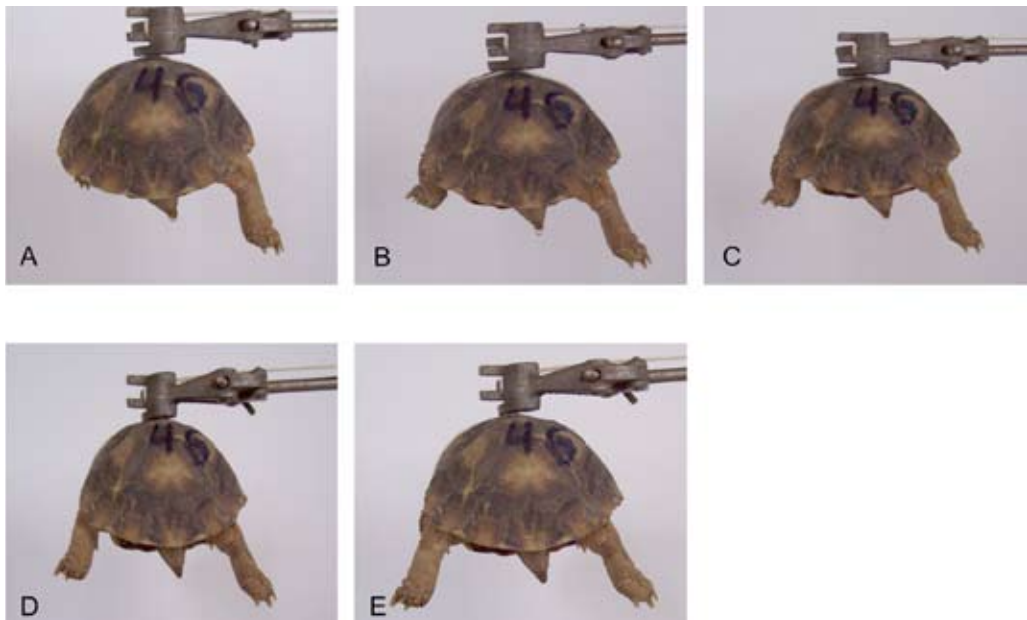


Figure 2. The formalin-induced behavioural response, while the tortoise is still. (A) The injected limb completely retracted, (B) the injected limb almost completely retracted, (C) the injected limb half-out, (D) the injected limb almost completely out and (E) the injected limb completely out.

Table 1. Pain-related scores based on formalin-induced behaviour.

Behaviour	Score
Animal being still	
Injected limb completely retracted	5
Injected limb almost completely retracted	3.75
Injected limb half-way out	2.5
Injected limb almost completely out	1.25
Injected limb completely out	0
Animal in motion	
Moving all limbs except for the injected limb	5
Moving all limbs, but the injected limbs only carefully	2.5
Moving all limbs	0

maximal score, and since D’Agostino and Pearson’s omnibus test for normality showed that the data did not follow a Gaussian distribution, the data was ana-

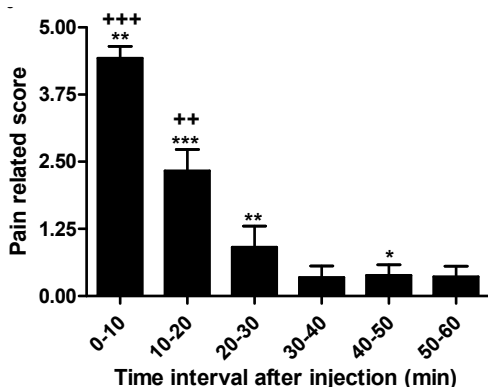


Figure 3. The pain-related behavioural score in *Kinixys spekii* after 10 % formalin injection studied in 10-min blocks during 60 min, presented as mean score ± standard error of the mean (SEM). Asterisks represent median score significantly different from score 0 according to Wilcoxon signed rank test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Plus-signs represent mean score significantly different from mean scores of any of the fourth, fifth or sixth time intervals, according to Friedman’s test with Dunn’s multiple comparison post-hoc test (+ $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$).

lyzed with non-parametric tests. During the first 10 min interval, an increased pain-related behaviour was observed, that was significantly different from score 0 according to Wilcoxon signed rank test. The second interval also showed a significant difference from score 0, and the pain-related score during this interval was not significantly different from the first interval, according to Friedman’s test with Dunn’s multiple comparison test. The pain-related score of the third interval was significantly different from score 0 and from the first, but not from any of the other intervals. The fifth interval was significantly different from score 0, but not different from the fourth and the sixth intervals, which were not significantly different from score 0. Hence, the fourth, fifth and sixth interval were considered not to reflect any response to noxious stimuli. As a consequence, the first and second intervals were considered to reflect a response to noxious stimuli, but not the third interval. Subcutaneous injection of saline did not induce any pain-related behaviour (data not shown).

Discussion

The present study demonstrates that the formalin test with suspended animals seems to be a good method for studying pain-related behaviour in tortoises, since the animals are less eager to retract their head and limbs during suspension. Although

suspension of the tortoises does not represent a natural situation, it makes the behavioural response to formalin injection easily identified and quantified. The formalin-induced response is clearly visible during the first 20 minutes after injection.

Contrary to what is observed in rats and mice, there was no indication of a biphasic response, since only a first acute response was observed, although this acute response lasted longer than what has been observed in rodents (*Hunnskaar & Hole, 1987*). Plausible explanations for the lack of a second response may be that the inflammatory phase coincides with the acute one; that the inflammation is not sufficiently severe to cause pain; or that the inflammatory phase actually does not exist. In addition, the fact that formalin was injected subcutaneously in the tortoises instead of intradermally could also have affected the response in comparison to rodents. However, this remains to be investigated.

In conclusion, the suspended formalin test in the Speke's hingeback tortoise opens the way for future studies, where the effects of opioids and other analgesics will be investigated. This will profoundly lead to an increased understanding of the function of the nociceptive system in Chelonians.

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