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Modulation of nociception by amitriptyline hydrochloride in the Speke's hinge-back tortoise (Kiniskys spekii)

Christopher M. Makau[1,2] | Philemon K. Towett[1] | Klas S.P. Abelson[2] | Titus I. Kanui[3]

[1]Department of Veterinary Anatomy and Physiology, University of Nairobi, Nairobi, Kenya

[2]Department of Experimental Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen N, Denmark

[3]School of Agriculture and Veterinary sciences, South Eastern Kenya University, Kitui, Kenya

Correspondence
Christopher M. Makau, Department of Veterinary Anatomy and Physiology, University of Nairobi, PO Box 30197-00100, Nairobi, Kenya.
Email: musembi06@yahoo.com

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Abstract

Background: There are limited studies on the utilization of analgesics in testudines. Management of pain in reptiles is by use of analgesics generally used in other vertebrate species. Evidently, some analgesics considered to be generally effective in reptiles are not effective in certain reptile species.

Objective: The purpose of this study was to examine the effect of amitriptyline hydrochloride on nociceptive behaviour in Speke's hinge-back tortoise.

Methods: Twenty-four adult Speke-hinged tortoises weighing 500–700 g were used. The effects of amitriptyline hydrochloride on nociception were evaluated using the formalin, capsaicin and hot plate nociceptive tests. Amitriptyline was administered intracoelomically at doses of 0.5, 1.0 and 3.0 mg/kg.

Results: The higher doses of amitriptyline hydrochloride caused an increase in nociceptive behaviour (time spent in hindlimb withdrawal) on the formalin and capsaicin nociceptive tests, suggesting a potentiating effect. However, the doses used had no significant change in nociceptive behaviour on withdrawal response in the hot plate test.

Conclusions: The study showed that amitriptyline hydrochloride which is widely used in management of neuropathic pain potentiates nociceptive effects in the formalin and capsaicin nociceptive tests in the Speke's hinge-back tortoise. The hot plate test, which previously has not been reported in these animals, gave results not in line with the other tests and therefore more testing and validation of the test is required. Amitriptyline modulates chemical and thermal pain differently.

KEYWORDS
amitryptyline hydrochloride, antinociception, capsaicin test, formalin test, hot plate test, hyperalgesia

1 | INTRODUCTION

The biggest challenge in utilization of analgesics in reptiles has always been species specificity in respect to their effectiveness. Some analgesics considered to be generally effective in reptiles are not effective in certain reptile species. Mostly, pain in reptiles is commonly treated by use of non-steroidal anti-inflammatory drugs (NSAIDs), opioids and alpha-2 agonists (Sladky & Mans, 2012). There is also a plethora of potential analgesics for reptiles that are currently available and used in other vertebrate species. Tricyclic antidepressants for example are

often the first-line treatment for neuropathic pain in humans (Dworkin et al., 2007; Saarto & Wiffen, 2010; Sindrup et al., 2005). Amitriptyline hydrochloride (tricyclic antidepressant) is commonly used to treat depression and neuropathic pain in humans and animal models (Kremer et al., 2018; Park et al., 2018). In veterinary medicine it has been used to manage neuropathic pain in a prairie falcon (Shaver et al., 2009), idiopathic cystitis in cats (Kraijer et al., 2003), canine separation anxiety (Sargisson, 2014) as well as psychogenic feather picking in companion birds. There is inclined utilization of amitriptyline hydrochloride in veterinary medicine for various indications. The drug is being repositioned for other indications which could be of benefit to animals. For example, amitriptyline has been found to have significant antimicrobial activity against many bacterial species (Serafin & Hörner, 2018).

Amitriptyline hydrochloride exhibits diverse pharmacological properties and thereby varied mechanisms of action. Its primary mechanism of action is through inhibition of serotonin and noradrenaline reuptake on the descending inhibitory pathways (Anser et al., 2018; Kremer et al., 2018; Meshalkina et al., 2018). This drug has considerable affinity for serotonin and norepinephrine reuptake transporters, 5-HT_{1A} and 5-HT_{2A} receptors, alpha-adrenergic receptors, muscarinic acetylcholine receptors and histamine receptors (Park et al., 2018). Amitriptyline hydrochloride is also a known voltage-gated Na^+ and K^+ channel blocker (Dick et al., 2007; Li et al., 2018; Song et al., 2000; Wang et al., 2004; Wolff et al., 2016). Some of its antinociceptive effects are as a result of inhibition of these voltage gated ion channels which impairs excitability of sensory neurons (Wolff et al., 2016). Therefore, the antinociceptive effects of amitriptyline hydrochloride appear to be through inhibitory effects on both the descending inhibitory and ascending pain pathways. Such mechanisms of action amongst other pharmacological properties enhance its potential as an analgesic drug for many animal species including testudines.

Amitriptyline hydrochloride appears to be effective in management of various conditions in both humans and animals. Currently, there are limited studies on the utilization of amitriptyline in reptiles. There are no reported studies on its use as an analgesic in the testudine species. The purpose of this study, therefore, was to investigate the antinociceptive effects of amitriptyline hydrochloride in acute and chronic pain models in the Speke's hinge-back tortoise. It was hypothesized that amitriptyline hydrochloride would significantly decrease pain behaviour in the Speke's hinge-back tortoise.

2 | MATERIALS AND METHODS

2.1 | Experimental animals

Twenty-four adult Speke-hinged tortoises were used in the behavioural nociceptive tests. The animals used were males and females sourced from Kitui county of Kenya. The county lies at 1,141 m above sea level with an average annual temperature of 21.4°C. Kitui county receives about 1,068 mm of precipitation annually. Body weight, gender and plastron length of each animal were recorded on arrival. The age was determined by counting the number of growth rings in the carapace (Cheylan & Poitevin, 1998; Germano, 1988). For identification, the animals were marked on the carapace with specific numerals using a marker pen. The animals were housed in a well-ventilated room, with translucent windows.

The animals were kept in open metallic cages measuring 1.25 m × 1.0 m × 0.6 m. The cages were half-filled with sand and stones. Animals were fed thrice in a week on vegetables i.e., cabbages, kales, tomatoes, cucumber, carrots and cow pea leaves. The animals were also fed with fruits i.e. apples and melons once weekly. Vegetables were occasionally dusted with calcium and vitamin D3 powder. Drinking water was provided ad libitum. The animals were habituated to the laboratory conditions for one month before the start of experiments. During this period, they were adapted to restraining procedures. Animals were housed under standard laboratory conditions with a 12/12 hr light/dark cycle and at a temperature of 23–30°C. They were bathed twice a month to make them clean and to avoid diseases attributed to poor hygiene.

2.2 | Sample size

Four groups of six animals per group, i.e., a total of 24 animals were used per behavioural nociceptive test. Animals were stratified by weight, following which equal sample sizes from each stratum were obtained randomly to form the groups. The sample size i.e., 24 animals, was determined using the Resource Equation Method (Arifin & Zahiruddin, 2017). One of the four groups was used as the control. In the antinociceptive experiments, animals had a wash out period of at least 2 weeks between the experiments.

2.3 | Behavioural nociceptive testing

Behavioural nociceptive tests were performed to investigate the role of amitriptyline hydrochloride in nociception in the Speke's hinge-back tortoise. Before experimentation, tortoises were brushed with a soft brush to remove soil and sand. The animals were randomly picked from the groupings and restrained in very gentle way to avoid stressing them. Randomization was done using the standard randomization table and the numeric numbers marked on the carapace of the animals. Blinding was not possible due to logistical reasons. The study was approved by the departmental animal care and use committee and all ethical guidelines on animal research as provided for by the National research council guide for the care and use of laboratory animals, 8th edition; National Academies Press (revised 2010) were complied with.

2.3.1 | Formalin test

Animals picked for experiment were restrained on a stand with a string tied round its shell and then positioned facing away from the

investigator i.e. facing the wall as previously described by Dahlin et al. (2012). The procedure was performed in a sound attenuated room. The animals were then injected 100 μl of 10% formalin into the inter-claw space of the hindlimb using a micro litre syringe and a 29-gauge needle. The controls for formalin injection were given 100 μl of saline (0.9% NaCl) in a similar manner to that of formalin. The total time spent withdrawing the injected limb was scored over a one hour observation period. Scoring was performed in 12 blocks of 5 min each and the data were recorded as total time spent in pain behaviour after the injection of formalin or vehicle. Animal reuse was allowed solely after at least 2 weeks wash-out period following the previous formalin injection, and in that case injection was performed on an alternative paw.

2.3.2 | Capsaicin test

The experimental animals for this test were restrained as described above in the formalin test. The animal was then injected 12.5 mg capsaicin in a volume of 100 μl into the inter-claw space of the hindlimb using a micro litre syringe and a 29-gauge needle. The controls for capsaicin injection were given 100 μl of a solution of saline (0.9% NaCl) and ethanol in a ratio of 50:50 in a similar manner to that of capsaicin injection. The total time spent withdrawing the injected limb was scored over a 30 min observation period. Scoring was performed in 12 blocks of 5 min each and the data were recorded as total time spent in pain behaviour after the injection of formalin or vehicle. Animal reuse was allowed solely after at least 2 weeks wash-out period following the previous capsaicin injection, and in that case injection was performed on an alternative paw.

2.3.3 | Hot plate test

A transparent rectangular jar was placed on the apparatus covering the hot plate along its perimeter. The metal surface and the jar were cleaned with 70% ethanol before experiments. The hot plate apparatus (MOD 35D Hot plate) was switched on and left to warm to 63°C ± 1.0 before the experiments commenced. The sampled animal was put on the plate facing away from the investigator and simultaneously the stopwatch was started to measure the time latency to display of feet rubbing, alternating hind feet, escape or raising plastron. The stopwatch was stopped after the animal displayed the first nociceptive behaviour. The animal was then withdrawn from the hot plate apparatus. The cut-off time was set at 3 minutes. In the absence of any reaction, 3 minutes were considered as latency by default. The hot plate was cleaned with water and 70% ethanol before testing another animal. All the animals were given a baseline test to establish that they had similar sensitivities to thermal nociception prior to the drug injection. All experiments were performed at a room temperature of 24–25°C, and between 10.00 a.m. and 5.00 p.m.

2.4 | Drugs

Amitriptyline hydrochloride was used at doses of 0.5, 1.0 and 3.0 mg/kg dissolved in 0.9% saline. The doses used were identified during preliminary studies. Selection of the doses was based on extrapolation from doses used in other animal studies on nociception. Only the doses that did not cause sedation in these animals were used. The drugs or vehicle were administered intracoelomically 30 min before the start of the nociceptive tests. They were administered using a 22 gauge 3.75 cm needle. To inject the drugs, the animal was held in lateral recumbency and the area to inject was cleaned with alcohol. Injection was made specifically in the left prefemoral fossa; cranial to hindlimb to access coelomic cavity. The needle was advanced parallel to the body wall. The drug was then injected after aspiration free of blood, air or lymph.

2.5 | Data analysis

Data were analysed using SPSS version 24.0. Changes in time spend in nocifensive behaviour were analyzed using one-way ANOVA for repeated measures followed by Tukey test. All data were represented as mean ± *SEM*. $p \leq 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Formalin test

Subcutaneous injection of 100 μl of formalin (10%) induced an increase in the mean of time spend in pain-related behaviour (hindlimb withdrawal) compared to that for animals injected with saline ($p \leq 0.05$, *F*-test). The mean time spent in pain behaviour following 10% formalin administration was 14.92 ± 1.3 min ($n = 7$) while that for saline group was 0.37 ± 1.3 min ($n = 7$). The injection of formalin caused monophasic pain behaviour. Other behaviours observed after formalin injection were defecation, vocalization and urination. There was no significant change in the hindlimb withdrawal following saline injection (Figure 1).

The mean time spent in hindlimb withdrawal after intracoelomic administration of saline (100 μl) or amitriptyline hydrochloride at doses of 0.5, 1.0 or 3.0 mg/kg during formalin test were 14.92 ± 1.3, 18.47 ± 2.45, 48.01 ± 4.92 and 45.84 ± 4.84 min, respectively (Figure 2). Intracoelomic administration of amitriptyline hydrochloride at doses of 1.0 and 3.0 mg/kg induced an increase in the mean hindlimb withdrawal time compared to the saline control [F_{ANOVA} (3, 19) = 14.55; $p < 0.05$]. Amitriptyline hydrochloride 0.5 mg/kg did not induce a significant change in hindlimb withdrawal time.

3.2 | Capsaicin test

Subcutaneous injection of 12.5 mg of capsaicin in a volume of 100 μl induced an increase in the mean of time spend in pain-related

behaviour (hindlimb withdrawal) time compared to that for animals injected with saline only ($p \leq 0.05$, *F*-test). The mean time spent in pain behaviour following 12.5 mg capsaicin administration was 6.0 ± 1.4 min ($n = 6$) while that for saline group was 0.5 ± 1.3 min ($n = 6$). Other behaviours observed after formalin injection were defecation and urination. There was no significant change in the hindlimb withdrawal following saline injection (Figure 3).

The mean time spent in hindlimb withdrawal after intracoelomic administration of saline-ethanol mixture (100 μl) or amitriptyline hydrochloride at doses of 0.7, 1.0 or 3.0 mg/kg in capsaicin test were 5.87 ± 0.99, 3.44 ± 0.47, 5.45 ± 0.83 and 26.38 ± 2.17 min, respectively (Figure 4). Intracoelomic administration of amitriptyline hydrochloride at a dose of 3.0 mg/kg induced an increase in the mean hindlimb withdrawal time compared to the saline control [F_{ANOVA} (3, 17) = 70.71; $p < 0.05$]. Amitriptyline hydrochloride at doses of 0.7 mg/kg and 1.0 mg/kg did not induce significant change in hindlimb withdrawal time.

3.3 | Hot plate test

The mean time spent in thermal-nociceptive behaviour after intracoelomic administration of saline (100 μl) or amitriptyline hydrochloride at doses of 0.5, 1.0 or 3.0 mg/kg in hotplate test was 1.12 ± 0.30 , 1.70 ± 0.34, 1.73 ± 0.33 and 1.00 ± 0.26 min, respectively (Figure 5). Amitriptyline hydrochloride of all the doses of amitriptyline hydrochloride used at the particular doses did not induce a change in plastron raising or resting time [F_{ANOVA} (3, 20) = 1.49; $p < 0.05$].

4 | DISCUSSION

In this study, the effects of systemic administration of amitriptyline hydrochloride were studied in tortoise models of experimental pain.

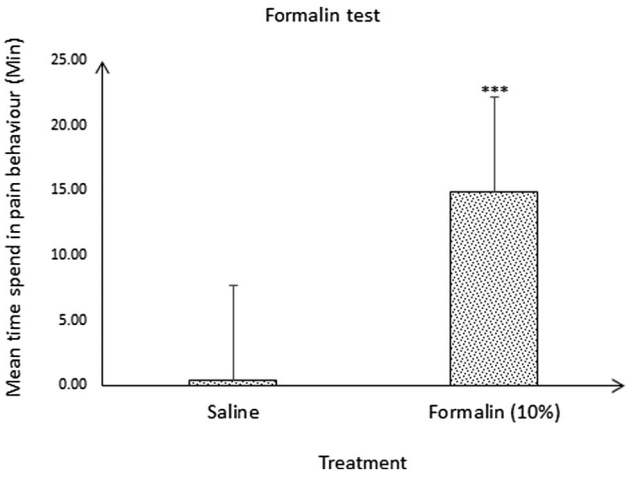

FIGURE 1 Effect of subcutaneous injection of saline (0.9%) or formalin (10%) on the mean hindlimb withdrawal time in the Speke hinged tortoise. Bars represent means ± *SEM* and $n = 7$ in each group. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. *** denotes $p < 0.001$ (saline group vs. formalin group)

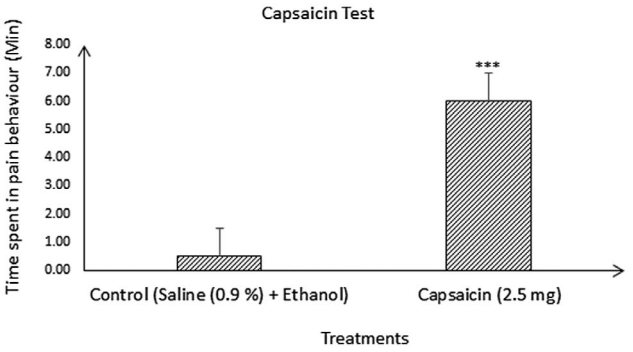

FIGURE 3 Effect of subcutaneous injection of saline (0.9%) or Capsaicin (2.5 mg) on the mean hindlimb withdrawal time in the Speke hinged tortoise. Bars represent means ± *SEM* and $n = 6$ in each group. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. *** denotes $p < 0.001$ (Saline group vs. Capsaicin group)

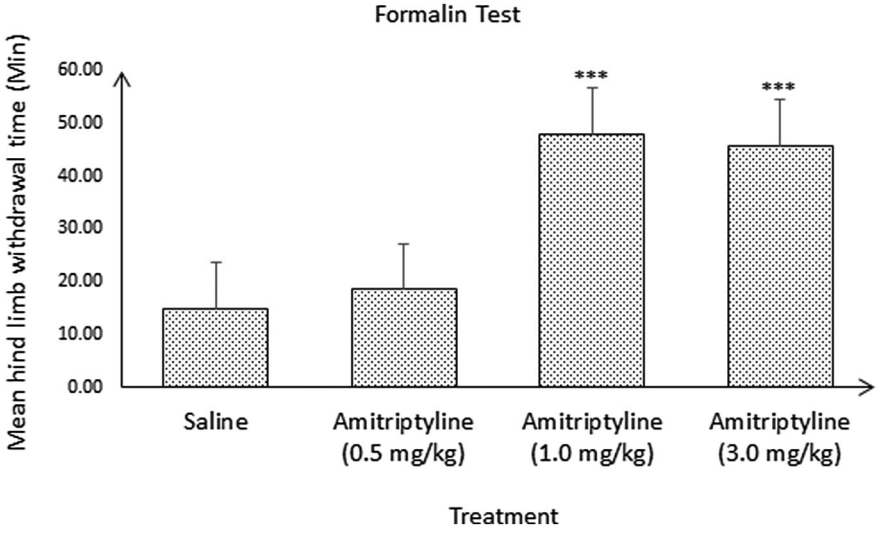

FIGURE 2 Effect of intracoelomic administration of saline or amitriptyline hydrochloride (0.5, 1.0 or 3.0 mg/kg) on the mean hindlimb withdrawal time in the Speke hinged tortoise. Bars represent means ± *SEM* and $n = 6$ in each group. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. *** denotes $p < 0.001$ (saline group vs. treatment group)

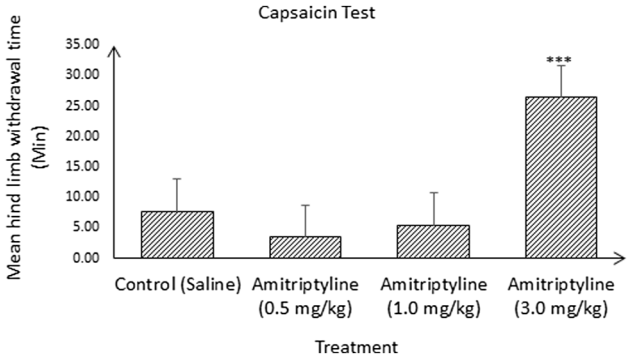

FIGURE 4 Effect of intracoelomic administration of a mixture of saline and ethanol or amitriptyline hydrochloride (0.5, 1.0 or 3.0 mg/kg) on the mean hindlimb withdrawal time in the Speke hinged tortoise. Bars represent means ± *SEM* and $n = 6$ in each group. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. *** denotes $p < 0.001$ (Control group vs. treatment group)

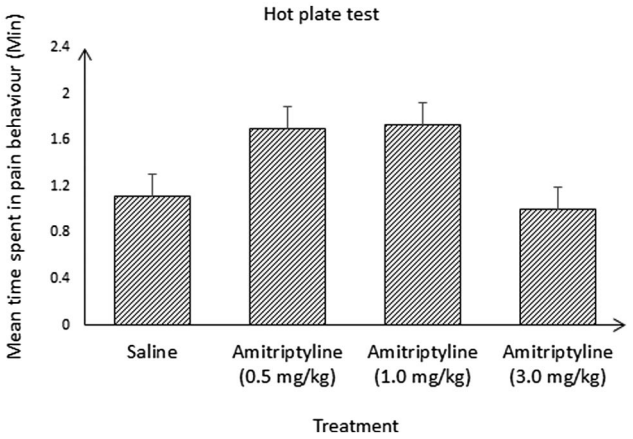

FIGURE 5 Effect of intracoelomic administration of saline or amitriptyline hydrochloride (0.5, 1.0 or 3.0 mg/kg) on the mean of time spend in pain related behaviour in the Speke hinged tortoise. Bars represent means ± *SEM* and $n = 6$ in each group. Treatment means were compared using Dunnett's (2-sided) test, subsequent to ANOVA. *** denotes $p < 0.001$ (Saline vs. treatment group)

Amitriptyline in the higher doses used caused a significant increase in pain related behaviour in these animals in the formalin test. These findings are contrary with the known analgesic effects of amitriptyline in both humans and animal models of pain. Amitriptyline has been used effectively in treatment of neuropathic pain in humans (Kakoei et al., 2018; Rossignol et al., 2019; Siddall & Hall, 2018; Soomro et al., 2018). In veterinary medicine, amitriptyline is used effectively in management of pain in cats and dogs (Sutayatram et al., 2018). The findings of the current study are however similar with findings of previous studies in rat formalin tests (Bomholt et al., 2005; Heughan et al., 2002; Sawynok et al., 1999). Amitriptyline increases second phase flinching behaviour in the formalin test in rats indicative of hyper-algesic effects (Bomholt et al., 2005). There is also evidence showing that amitriptyline in higher doses, dose-dependently induces hyper-algesic effects in morphine-dependent rats in formalin test (Akbari et al., 2018). The key question therefore is why amitriptyline hydrochloride demonstrates differential effects on nociception in the different animals and different models.

The pharmacological effects of amitriptyline depend on several pathophysiological factors, including the level of drug exposure, drug metabolism capacity, concurrent disease and cellular responsiveness (Sutayatram et al., 2018). Anti-nociceptive effect of amitriptyline involves the suppression of ERK1/2 and cAMP-response element binding protein (CREB) signalling proteins (Kim et al., 2019). CREB regulates transcription of genes like the c-fos gene which is implicated in nociception following formalin injection. Subcutaneous injection of formalin (2.5%) into the rat hind paw, increases FOS phosphoprotein immunoreactivity in laminae I–VI of the dorsal L5 spinal cord in rats (Heughan et al., 2002). Therefore, it appears that amitriptyline suppresses CREB which decreases transcription of c-fos gene; minimizing FOS proteins and thereby decrease pain. In this study such a mechanism seems not to be the case with amitriptyline having hyperalgesic effects in these animals. It is worth noting that the hyperalgesic effects of amitriptyline reported occur in the second phase of the formalin test in the rat pain model. The second phase is attributed to the development of the increased input from primary afferent fibre and local inflammatory response at the injection site (Dickenson & Sullivan, 1987). Anti-inflammatory properties of amitriptyline has been demonstrated in rat models (Fattahian et al., 2016; Rafiee et al., 2017). Co-administration of amitriptyline with high concentration of formalin at 2.5% produces a dose-related reduction in flinching behaviours in rats (Heughan et al., 2002; Sawynok et al., 1999). The mechanism of amitriptyline-induced increase in nociceptive behaviour in the formalin test in these animals is not clear at the moment. However, a number of speculations can be made. The most plausible argument is that amitriptyline stimulates an increase in inflammatory mediators in the injection site. There is a possibility that secretion of dynorphin protein is enhanced. FOS protein is known to enhance expression of dynorphin gene and subsequently dynorphin protein which is implicated in nociception (Ahmad & Ismail, 2002). There is a likelihood that the released FOS protein following formalin injection causes enhanced release of dynorphin protein and thereby potentiating pain.

Amitriptyline is a 5-hydroxytryptamine and noradrenaline reuptake inhibitor, an antagonist of alpha1-adrenergic, histamine, muscarinic receptors (Dean, 2017). There is evidence that alpha-adrenergic, histamine, excitatory amino acid or opioid receptors are not involved in the antinociceptive action of amitriptyline (Sawynok et al., 1999). Therefore, the potentiation of pain due to amitriptyline is most likely not due to antagonistic effects to these receptors. Tortoises have a robust descending bulbo spinal pathway as indicated in other studies (Makau et al., 2017). Amitriptyline being a noradrenergic and serotonergic re-uptake inhibitor ought to have demonstrated analgesic effects in these animals. Perhaps, amitriptyline did not stimulate sufficient antinociceptive effect through inhibition of 5-hydroxytryptamine and noradrenaline reuptake.

Amitriptyline in the higher doses used caused a significant increase in pain related behaviour in the capsaicin test. A capsaicin test involves peripheral nociception and its use in reptiles as a nociceptive test has not been reported widely. Subcutaneous injection of capsaicin produces behaviour similar to that elicited by formalin. Capsaicin selectively binds to TRPV1, the vanilloid subtype 1 of the superfamily of transient receptor potential ion channels, which is highly expressed in pain-transmitting C fibres (Colvin et al., 2011). The findings in this experiment reflect similar findings in the formalin test. Therefore, amitriptyline seems to trigger a similar mechanism of action resulting in hyperalgesia in both the capsaicin and formalin test. In humans, systemic administration of amitriptyline does not have antinociceptive effect following intradermal injection of capsaicin (Eisenach et al., 1997). Interestingly, in other animal species capsaicin has been found to potentiate amitriptyline anti-nociceptive effects when applied intra dermally. Intradermal co-administration of capsaicin with amitriptyline in rats results in prolonged cutaneous analgesia compared with amitriptyline alone. It has been suggested that during co-administration of capsaicin with amitriptyline the activation of the TRPV1 channel by capsaicin facilitates the passage of amitriptyline into nociceptors (Colvin et al., 2011). The differences in the effects observed could be due to the method of administration of amitriptyline and the type of analgesiometric test used.

In this study, systemic administration of amitriptyline in the tortoise did not result in a significant change in the mean time spent in pain behaviour during the hot plate test. This concurs with data reported by other researchers in rats in the tail-flick and hotplate tests (Bomholt et al., 2005). In other studies, however, thermal hyperalgesia of the injured hind paw in rats was fully reversed by amitriptyline in the chronic constriction injury model of neuropathic pain (Bomholt et al., 2005). Similarly, findings by Esser and Sawynok (1999) suggest that systemic (intraperitoneal and subcutaneous) and spinal (intrathecal cannula), administration of amitriptyline at varied dosages produces anti-hyperalgesic effect in the injured paw. Amitriptyline seems to be effective in management of thermal hyperalgesic indications but is less effective in thermal nociception.

Hotplate test is an effective and relevant nociceptive test because the behavioural responses in this test are supraspinally organized. However, hotplate nociceptive test in tortoise has not been reported previously, and further validation of the test may be needed to ensure proper validity in tortoises. It has been reported that reptiles do not demonstrate withdrawal reflex when placed in hot objects in a similar way to mammals (Mader, 2008). Thermal burns in reptiles are one of the most common injuries seen by herpetology veterinarians (Mader, 2008). This is based on their failure to respond accordingly when exposed to heat or hot objects which results to the animals being burned more often. This characteristic can affect the response of reptiles to thermo nociceptive tests. However, thermo nociceptive tests have been conducted successfully to assess the efficacy of certain analgesics (Baker et al., 2011; Sladky et al., 2009).

Amitriptyline has been found to have several adverse effects in varied animals. In this study, drowsiness was observed following administration of amitriptyline in doses of above 3 mg/kg. Similar effects have been observed in experimental amitriptyline intoxication studies in conscious dogs. Foaming around the mouth was observed at higher doses that were not used in this study. In dogs, vomiting, restlessness and seizures have also been observed following amitriptyline administration (Boeck & Jørgensen, 1980). Furthermore, amitriptyline in dogs is known to cause cardiovascular toxicity (Sutayatram et al., 2018). Amitriptyline has been found to cause skin irritation when applied transdermally (Colvin et al., 2011). It seems that amitriptyline has considerable side effects notably observed in higher doses in the Speke's hinge-back tortoise and in other animals as well. The side effects in the respective animals is due to the varied pharmacological effects of amitriptyline hydrochloride.

4.1 | Conclusion

Amitriptyline hydrochloride potentiates the nociceptive effects in inflammatory pain models of the Speke's hinge-back tortoise. Similar effects have been observed in other animal species. This study also affirms that tricyclic antidepressants and other drugs for similar indications in other vertebrates cannot be generalized for use in testudine and reptiles at large. Further studies on the effect of amitriptyline on inflammatory mediators in the Speke's hinge-back tortoise are recommended. The mechanism of action of amitriptyline hydrochloride is through interaction with varied receptors, ion channels and neurotransmitters. The degree and prioritization of involvement of the varied mechanisms of action by amitriptyline is unknown. Lastly, there is need for more tests and validation of the hot plate nociceptive test in these animals because in this experiment, the results from the test were not consistent with the other tests. The shell of these animals makes it difficult to assess pain behaviour by tail flick or other type of thermo nociceptive tests.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Christopher Musembi Makau: Conceptualization; Formal analysis; Investigation; Methodology; Validation; Writing-original draft. **Philemon K. Towett:** Conceptualization; Formal analysis; Methodology; Supervision; Writing-review & editing. **Klas S. P. Abelson:** Conceptualization; Methodology; Resources; Supervision; Visualization; Writing-review & editing. **Titus I. Kanui:** Conceptualization; Funding acquisition; Methodology; Supervision; Writing-review & editing.

Peer Review

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ORCID

Christopher M. Makau https://orcid.org/0000-0001-9548-2345

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