Anti-inflammatory and analgesic effects of ethanolic leaf extract of *Newbouldialaevis* (P. Beauv.) Seemann Ex Bureau (Bignoniaceae)

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**ABSTRACT**

Various parts of *Newbouldialaevis* (Bignoniaceae) including the leaves have been used to relieve stomach pains, pelvic pain, haemorrhoids, and rheumatic swellings in Ghanaian and West African folk medicine with little or no scientific evidence. This study was therefore aimed at evaluating the scientific basis for the traditional use of *Newbouldialaevis* leaves as an anti-inflammatory agent and analgesic using animal models. The chick inflammation model, tail immersion test, Randall-Selitto test and formin test were used. One hour pre-treatment with the *Newbouldialaevis* extract (NLE) (30-300 mg kg\(^{-1}\); p.o.) significantly and dose dependently, inhibited foot edema in the chicks with maximal inhibition of 64.41±11.47%. In the tail immersion test, NLE (300 mg kg\(^{-1}\), p.o.) was able to significantly increase the withdrawal latency by 88.45±19.81%. Also, NLE (300 mg kg\(^{-1}\)) reversed the inflammatory-induced mechanical hyperalgesia with a maximum percentage effect of 37.60±7.26%. Treatment of mice with different doses of NLE (30–300 mg kg\(^{-1}\), p.o., 60 min before) produced a marked and dose-related inhibition of both phases of formalin-induced nociception with the highest dose given a maximal inhibition of 54.47±8.60% and 83.62±6.03% of the licking time in the first and second phases, respectively.

**Keywords:** chicks, edema, withdrawal, hyperalgesia, formalin

**INTRODUCTION**

*Newbouldialaevis* (Bignoniaceae) is a small tree that grows up to 15m high and a common plant across the forest regions of West Africa. Various parts of this plant including the leaves have been used traditional for medicinal purposes with little or no scientific evidence. The leaf decoction is used in Ghana as eye lotion for the treatment of sore eyes, conjunctivitis and trachoma. Hot application of pounded leaves and roots is used in the management of rheumatism [1]. The chewed leaves are also applied to snake bite [2].

A study on the effect of a methanolic leaf extract of *Newbouldialaevis* on spontaneous motor activity, exploratory behaviour, apomorphine-induced climbing behaviour in mice and pentobarbital induced hypnosis in rats led to the conclusion that the methanolic leaf extract may contain principles that have sedative effects [3]. The bactericidal activity against microbes implicated in toothache using an extract prepared from the fully matured leaves has been
reported[4]. The ethanol leaf extract of *Newbouldialaevis* demonstrated antidiabetic activity in rats and acute toxicity studies also show it to be relatively safe [5]. Woode and colleagues [6] have reported on the anti-artheritic and antioxidant properties of the ethanolic stem bark extract of this plant. In 2009, antinociceptive effects of the stem bark extract were reported [7].

The majority of clinically important medicines for the treatment of inflammation and pain belong to the steroidal or non-steroidal anti-inflammatory drugs [8]. Though these are very effective, they have various and severe adverse effects [9,10]. Agents of natural origin with very little or no side effects when identified as been effective can substitute some of these chemical therapeutics. The present study therefore reports for the first time the anti-inflammatory and analgesic effects of the ethanolic leaf extract of *Newbouldialaevis* using animal models.

**MATERIALS AND METHODS**

**Plant material**

Leaves of *Newbouldialaevis* were collected from the Botanic Gardens of Kwame Nkrumah University of Science and Technology (KNUST), Kumasi, Ghana, in October, 2011 and authenticated in the Department of Herbal medicine, KNUST where a voucher specimen is been kept.

**Preparation of extract**

The leaves were air-dried indoors for two weeks and pulverized with a hammer-mill. The powder was extracted by cold maceration using 70% (v/v) ethanol over a period of 72 hours. The resulting extract was concentrated under low temperature (60°C) and pressure to a syrupy mass in a rotary evaporator. The syrupy mass was then dried to a dark brown semi-solid mass using water bath and kept in a desiccators till it was ready to be used. The final yield was 18.3%. This is subsequently referred to as *Newbouldialaevis* extract (NLE) or extract.

**Drugs**

Diclofenac sodium was purchased from Troge, Hamburg, Germany, morphine hydrochloride from Phyto-Riker, Accra, Ghana, carrageenan sodium salt and formalin were also obtained from Sigma-Aldrich Inc., St. Louis, MO, USA.

**Animals**

Cockerels (*Gallus gallus*; strain Shaver 579, Akropong Farms, Kumasi, Ghana) were obtained 1-day post-hatch and were housed in stainless steel cages (34×57×40 cm$^3$) at a population density of 12–13 chicks per cage. Food (Chick Mash, GAPCO, Tema, Ghana) and water were available *ad libitum* through 1-quart gravity-fed feeders and waterers. Room temperature was maintained at 29 °C, and overhead incandescent illumination was maintained on a 12-h light–dark cycle. Daily maintenance of the cages was conducted during the first quarter of the light cycle. Chicks were tested at 7 days of age. Group sample sizes of five were used throughout the study.

Sprague-Dawley rats of both sexes (155–190 g) and ICR mice (25-32 g) were purchased from Noguchi Memorial Institute for Medical Research, University of Ghana, Legon, Ghana and housed in the animal facility of the Department of Pharmacology, KNUST. The animals were housed in groups of six in stainless steel cages (34×47×18 cm$^3$) with soft wood shavings as bedding, fed with normal commercial pellet diet (GAPCO, Tema), given water *ad libitum* and maintained under laboratory conditions (temperature 24-28 °C, relative humidity 60-70%, and 12 hour light-dark cycle). All procedures and techniques used in these studies were in accordance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals (NIH, Department of Health and Human Services publication no. 85 - 23, revised 1985). All protocols used were approved by the Departmental Ethics Committee.

**Carrageenan-induced edema**

The carrageenan foot edema model of inflammation in the chick previously described by [11], and modified by [12], was used to evaluate the acute anti-inflammatory properties of the extract using the non-steroidal anti-inflammatory drug, diclofenac as a reference drug. Carrageenan (10 µl of a 2% suspension in saline) was injected subplantar into the right footpads of the chicks. Foot volume was measured before injection and at hourly intervals for 5 hours after injection by water displacement plethysmography as described by [13]. The edema component of inflammation was quantified by measuring the difference in foot volume before carrageenan injection and at the various time points.
The experiment was performed to study the effect of the drugs one hour after carrageenan challenge. Chicks were randomly selected for the following study groups: control; diclofenac (10, 30 and 100 mg kg\(^{-1}\), i.p.); and extract (30, 100 and 300 mg kg\(^{-1}\), p.o.). Extract was prepared in 2 % tragacanth mucilage. All drugs were freshly prepared.

**Tail immersion test**

The tail immersion test was carried out as described earlier by [14,15]. Tail withdrawal latency, defined by the time (in seconds) to withdraw the tail from hot water maintained at 50.0± 1.0 °C, was measured using a stopwatch. A cut-off time of 10 s was set to avoid tissue damage. Increase in tail withdrawal latency was defined as antinociception and calculated as % maximum possible effect (MPE). The maximum possible antinociceptive effect was reached when the animals did not show a tail withdrawal reaction within the cut-off time of 10 s. % MPE was calculated according to the formula: 

\[
\frac{(T_1-T_0)}{(T_2-T_0)} \times 100, \quad \text{where} \quad T_0 \text{ and } T_1 \text{ are the latencies obtained before and after drug treatment, and } T_2 \text{ is the cut-off time.}
\]

Animals were tested before and at 30, 60, 90, 120, 150 and 180 min after administration of NLE (30-300 mg kg\(^{-1}\), p.o), or diclofenac (10-100 mg kg\(^{-1}\), i.p). A single habituation test was used before baseline test to minimize learning effects.

**Carrageenan-induced mechanical hyperalgesia**

The test was carried out as described by [16,17]. Carrageenan (100 µl of a 20 mg ml\(^{-1}\) solution) was injected into the plantar surface of the right hind paw after which the mechanical pain threshold of the inflamed hind paw was determined with an analgesimeter (Model No.15776, UgoBasile, Comerio, Varese, Italy). The inflamed hind paw was placed on a small plinth and a blunt cone-shaped teflon piston was positioned on the convex surface of the paw. The pressure was progressively increased until the animal withdrew its leg. The paw withdrawal thresholds (PWT) pressure eliciting vigorous withdrawal movements was expressed in grams. A cut-off point of 250 g was used to prevent any tissue damage to the paw. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect (% MPE). On the test day, a baseline measurement was taken before animals were administered carrageenan. PWTs were determined again 2.0 h after carrageenan to establish that mechanical hyperalgesia had developed. NLE (30-300 mg kg\(^{-1}\), p.o) and diclofenac (10-100 mg kg\(^{-1}\), i.p) were then administered 3-h post-carrageenan, and PWTs were taken again at 3.5, 4, 4.5, 5, 5.5 and 6 h post-carrageenan.

**Formalin-induced nociception**

The formalin test, first described by [18], was carried out as described by [15]. Each animal was assigned and acclimatized to one of 20 formalin test chambers (a perspex chamber 15 × 15 × 15 cm) for thirty minutes before formalin injection [19]. The mice were then pre-treated with the test drugs (30 min for i. p. route and 1 h for oral route) before intraplantar injection of 10 µl of 5 % formalin. The animals were immediately returned individually into the testing chamber. A mirror angled at 45˚ below the floor of the chamber allowed a complete view of the paws. The behaviour of the animal was then captured (60 min) for analysis by a camcorder (Everio™ model GZ-MG1300, JVC, Tokyo, Japan) placed in front of the mirror. Pain response was scored for 60 min, in 5-min time block by measuring the amount of time spent biting/licking the injected paw immediately after formalin injection. Nociceptive behaviour was quantified by counting the incidents of spontaneous biting/licking of the injected paw using the public domain software JWatcher™ Version 1.0 (University of California, Los Angeles, USA and Macquarie University, Sydney, Australia available at http://www.jwatcher.ucla.edu/). The product of the frequency and duration of biting/licking was used as nociceptive score. Mice were randomly selected for one of the following study groups:

- **Group I** *Newbouldia* extract (30, 100 and 300 mg kg\(^{-1}\))
- **Group II** Morphine (1, 3 and 10 mg kg\(^{-1}\))
- **Group III** Diclofenac (10, 30 and 100 mg kg\(^{-1}\))
- **Group IV** Vehicle treated control

Extract was prepared in 2 % tragacanth mucilage. Drug solutions and suspensions were prepared such that not more than 1-ml of extract was given orally and not more than 0.5 ml of the standard drugs were injected intraperitoneally. All drugs were freshly prepared.

**Data analysis**

For the anti-inflammatory experiment, raw scores for right foot volumes were individually normalized as percentage of change from their values at time 0, then averaged for each treatment group. Raw data for the analgesic experiments was calculated as the percentage change in maximum possible effect (%MPE). The time-course curves
were subjected to two-way (treatment × time) repeated measures analysis of variance with Bonferroni’s post hoc test. Total foot volume and total nociceptive score for each treatment was calculated in arbitrary unit as the area under the curve (AUC) and to determine the percentage inhibition for each treatment, the following equation was used.

\[
\text{% inhibition} = \left(\frac{AUC_{\text{control}} - AUC_{\text{treatment}}}{AUC_{\text{control}}}\right) \times 100
\]

Differences in AUCs were analysed by ANOVA followed by Student-Newman-Keuls’ post hoc test.

Doses and concentrations responsible for 50% of the maximal effect (ED$_{50}$) for each drug were determined using an iterative computer least squares method, with the following nonlinear regression (three-parameter logistic) equation.

\[
Y = \frac{a + (b - a)}{1 + 10^{(\log ED_{50} - X)}}
\]

Where, $X$ is the logarithm of dose and $Y$ is the response. $Y$ starts at $a$ (the bottom) and goes to $b$ (the top) with a sigmoid shape.

The fitted midpoints (ED$_{50}$s) of the curves were compared statistically using F test [20,21]. GraphPad Prism for Windows version 5.0 (GraphPad Software, San Diego, CA, USA) was used for all statistical analyses and ED$_{50}$ determinations. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Carrageenan-induced edema**

The effect of the extract in acute inflammation was assessed in the chick carrageenan-induced foot edema using diclofenac as a reference drug. Administration of 10 μl 2% carrageenan intraplantar, induced moderate inflammation resulting in foot edema in the 7 day old chicks peaking at 2-3 h. Fig. 1 shows the time courses and the total edema response for the effects of NLE and diclofenac in carrageenan-induced edema. Two-way ANOVA (treatment x time) revealed a significant effect of drug treatment for NLE (F$_{3,64}$= 5.70, P=0.0075) and diclofenac (F$_{3,64}$= 4.50, P=0.0180). Total edema produced by each treatment is expressed in arbitrary units as AUC of the time-course curves (Fig. 1a and 1c). NLE (30–300 mg kg$^{-1}$) dose-dependently and significantly reduced the total foot edema with maximal effect of 64.41±11.47% for NLE administered curatively (Fig. 1b). Similarly the NSAID diclofenac (10-100 mg kg$^{-1}$) dose dependently reduced the edema with a maximal effect of 49.67±6.78% (Fig. 1d). Comparing the ED$_{50}$ values obtained from the dose response curves in Fig. 2, there was no significant difference (F$_{1,28}$= 0.278, P=0.6017) between the NLE (ED$_{50}$: 86.34±32.89 mg kg$^{-1}$) and that of diclofenac (ED$_{50}$: 67.35±21.18 mg kg$^{-1}$).

**Tail-immersion-induced nociception**

All the test drugs caused an increase in the tail withdrawal latency which was calculated as a percentage of the maximum possible effect (% MPE). NLE (30–300 mg kg$^{-1}$, p.o.) (Fig. 3a) caused a significant and dose dependent increase in the withdrawal latency of the tail as depicted in the time-course curve (F$_{3,48}$= 5.41, P=0.009). As shown in Fig. 3b, NLE (300 mg kg$^{-1}$, p.o.) was able to significantly (F$_{3,16}$= 6.02, P=0.006) increase the withdrawal latency by 88.45±19.81%. Similarly, diclofenac (10–100 mg kg$^{-1}$, i.p.; Fig. 3c) also produced a significant antinociceptive activity by dose dependently increasing the tail withdrawal latencies of animals pretreated with the drug (F$_{3,48}$= 3.82, P=0.0307) with the highest dose of 100 mg kg$^{-1}$ giving a significant (F$_{3,16}$= 5.03, P=0.012) percentage increase of 66.12±11.98% as shown in (Fig. 3d). Dose-response curves for the anti-nociceptive effects of NLE and diclofenac in the tail immersion test are shown in Fig 4. NLE displayed a biphasic, U-shaped dose response relationship with approximate ED$_{50}$ values of 59.70±22.75 and 150.66±57.41 mg kg$^{-1}$.

**Carrageenan-Induced Mechanical Hyperalgesia using Randall Sellito**

On the experiment day, animals showed baseline withdrawal thresholds of about 90 to 180 g. At 2.0 h after carrageenan injection, the ipsilateral paw exhibited marked mechanical hyperalgesia in all experiments which was maintained in vehicle- treated animals at all of the tested time points. A change in hyperalgesic state was calculated as a percentage of the maximum possible effect. NLE (30–300 mg kg$^{-1}$, p.o.) administered 3 h after carrageenan injection.
produced a significant and dose-dependent reversal of mechanical hyperalgesia ($F_{3,96} = 3.58, P=0.0376$; Fig. 5a). The highest dose of NLE 300 mg kg$^{-1}$ gave the highest nociceptive score of 37.60±7.26% ($F_{3,10} = 4.082, P=0.0249$) as shown in Fig. 5b. Intraperitoneal injection of diclofenac (10–100 mg kg$^{-1}$) significantly ($F_{3,10} = 5.46, P=0.0089$) and dose-dependently relieved the mechanical hyperalgesia($F_{3,16} = 13.13, P=0.0004$) with the highest dose of diclofenac also producing a total nociceptive score of 48.76±7.07% ($F_{3,10} = 6.183, P=0.0054$; Fig. 5d). Fig. 6 shows dose-response curves of the effects of the drugs under test. Even though the $ED_{50}$ for diclofenac ($ED_{50} = 47.86±9.60$ mg kg$^{-1}$) was smaller than that of NLE ($ED_{50} = 61.55±12.30$ mg kg$^{-1}$), they were not statistically different from each other ($F_{3,26} = 1.072, P=0.3100$).

Formalin-induced nociception

Formalin administration produced a typical pattern of flinching and licking behaviour. The first phase started immediately after administration of formalin and then diminished gradually in about 10 min. The second phase started at about 15 min and lasted until 1 h. Treatment of mice with different doses of NLE (30–300 mg kg$^{-1}$, p.o., 60 min before; Fig. 7a&b) produced a marked and dose-related inhibition of both phases of formalin-induced nociception (Phase 1: $F_{3,16} = 6.65, P<0.0033$; Phase 2: $F_{3,16} = 9.24, P<0.0009$) with the highest dose exhibiting a maximal inhibition of 54.47±8.60% and 83.62±6.03% of the licking time in the first and second phases, respectively(Fig. 7a&b). Similarly, morphine (1-10 mg kg$^{-1}$, i.p.) produced marked inhibition of both the neurogenic ($F_{3,16} = 3.90, P<0.0289$) and inflammatory($F_{3,16} = 3.55, P<0.0384$) pain phases (Fig. 7c&d). Morphine, was able to reduce the duration of formalin evoked nociceptive behavior by a maximum percentage of 42.48±10.10% in the early phase and 60.38±10.77% in the late phase of the formalin test (Fig. 7c&d). However, diclofenac, (10-100 mg kg$^{-1}$, i.p.) was only effective in inhibiting the formalin-induced pain only in the first phase ($F_{3,16} = 6.43, P=0.0046$) (Fig. 7e&f) with a maximal inhibition of 60.89±11.58% but not the second phase ($F_{3,16} = 3.19, P=0.0522$).

Figure 8 shows the dose-response curves of the drugs under test in both phases of formalin-induced pain. Comparing the $ED_{50}$ obtained by non-linear regression, the extract was seven times more potent in the second phase ($ED_{50} = 26.22±10.69$ mg kg$^{-1}$) than the first phase ($ED_{50} = 189.80±58.63$ mg kg$^{-1}$). Similarly, morphine was also five times more potent in the second phase ($ED_{50} = 2.64±1.05$ mg kg$^{-1}$) than the first phase ($ED_{50} = 12.60±1.03$ mg kg$^{-1}$). Diclofenac was also four times more potent in the second phase ($ED_{50} = 18.88±10.45$ mg kg$^{-1}$) than the first phase ($ED_{50} = 69.56±36.24$ mg kg$^{-1}$).

DISCUSSION

Results presented here indicate that the ethanolic leaf extract of *Newbouldialaevis* has anti-inflammatory and analgesic properties.

The *Newbouldialaevis* extract (NLE) was able to significantly reduce paw edema induced by carrageenan, and these effects were similar to those exhibited by the group of chicks treated with diclofenac. Carrageenan causes a reproducible inflammatory reaction and remains the standard irritant for examining acute inflammation and anti-inflammatory drugs [22]. Inflammation induced by carrageenan develops immediately following subplantar injections, resulting from the combined action of prostaglandins, bradykinin, histamine, serotonin and tachykinins[23,24]. This inflammatory response is usually quantified by the increase in paw size (edema) and is modulated by inhibitors of specific molecules within the inflammatory cascade, such as non-steroidal anti-inflammatory drugs as shown with diclofenac in this study [25,26]. From the above results, it is suggested that the anti-inflammatory effects of NLE on carrageenan-induced paw edema may be related to inhibition of inflammation mediator formation.

Several behavioral pain models were selected for this study such that both centrally and peripherally mediated effects could be investigated. The ethanolic leaf extract exhibited potent analgesic activity at all the doses tested which was evident in all the pain models used.

Even though the tail immersion test is considered to be a more selective model for centrally mediated [27], the extract as well as the standard drug used all showed significant analgesic activities. This may be indicative that, NLE may act via centrally mediated (spinal and/or supra spinal) analgesic mechanisms[28,29,30].

The extract together with diclofenac at all doses tested exhibited significant analgesic activity in the Randall–Selitto paw pressure test pain model which is often used to distinguish between central and peripheral analgesic actions. Inflammation is known to lower the thresholds of various mechanoreceptors and mechanotransduction pathways.
[31]. Carrageenan-induced inflammatory pain is known to involve sequential release of bradykinin, histamine, leukotrienes and prostaglandins [32]. Therefore the inhibitory effects of NLE on carrageenan-induced hyperalgesia could possibly be due to the inhibition of pain mediators and/or attenuation in sequential release of various inflammatory mediators that trigger pain response at the periphery.

Formalin test uses a sufficient painful stimulus indicating its sensitivity towards the commonly used analgesics [33]). This test helps in identifying different types of pain since it employs a chemical nociceptive stimulus which involves two distinctive phases[34]. In the formalin test, the early phase, which is also known as non-inflammatory pain or neurogenic pain, occurs within seconds of formalin injection and is considered to be a direct result of stimulation of paw nociceptors where the prostaglandins do not play an important role, while the late phase, which is also termed as inflammatory pain, occurs as a result of on-going activity in primary afferents, is due to release of serotonin, histamine, bradykinin and prostaglandins associated with peripheral inflammation [35,36, 37].
Opioids and other centrally acting drugs inhibit both phases equally [38] as shown in this study. However, NSAIDs, such as diclofenac which block prostaglandin synthesis, reduce nociception mostly in the late phase but can also affect the early stage [39].

NLE inhibited both phases of the formalin test but more effectively the second than the first. This gives an indication that NLE is effective against both neurogenic and inflammatory pain.

Generally, the present results demonstrate that the ethanolic leaf extract of *Neboudaialaevis* has anti-inflammatory effects as well as analgesic effect that might partially or wholly be due to centrally or peripherally mediated.
FIGURE 3: Effect of NLE (30-300 mg kg\(^{-1}\) p.o) and diclofenac (10-100 mg kg\(^{-1}\) i.p) on time course curve of tail immersion method of pain (a and c) and the AUC (b and d) Data was presented as mean ± S.E.M. (n=5); ***P<0.001; ** P<0.01; *P<0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni post hoc test). †††P<0.0001 ††P<0.05 compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keuls post hoc test).
FIGURE 4: Dose response curves for diclofenac (10-100 mg kg$^{-1}$i.p.) and NLE (30-300 mg kg$^{-1}$p.o.) on tail immersion method of pain
FIGURE 5: Effect of NLE (30-300 mg kg\(^{-1}\) p.o) and diclofenac (10-100 mg kg\(^{-1}\) i.p) on time course curve in carrageenan-induced mechanical hyperalgesia in the rat using the Randall sellito model (a and c) and the AUC (b and d).

Data was presented as mean ± S.E.M. (n = 5); ***P < 0.001; **P < 0.01; *P < 0.05 compared to vehicle-treated group (Two-way ANOVA followed by Bonferroni’s post hoc test). †††P<0.0001 †P<0.05 compared to vehicle-treated group (One-way ANOVA followed by Neuman-Keul’s post hoc test)
FIGURE 6: Dose response curves for diclofenac (10-100 mg kg\(^{-1}\) i.p) and NLE (30-300 mg kg\(^{-1}\) p.o.) on carrageenan-induced mechanical hyperalgesia
FIGURE 7 (a) Effect of NLE (30-300 mg kg\(^{-1}\) p.o.), (c) Morphine (1-10 mg kg\(^{-1}\) i.p.) and (e) diclofenac (10-100 mg kg\(^{-1}\) i.p.) on the time course of formalin induced pain in mice. Nociceptive/pain scores are shown in 5min blocks up to 60min post formalin injection. Each point represents Mean ± S.E.M (n = 5). *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 compared to respective controls (two-way repeated measures ANOVA followed by Bonferroni post hoc); (b, d and f) represent the AUC (total response) for phase 1 and phase 2. Each column in b, d and f represent the mean ± S.E.M. †P ≤ 0.05, ††P ≤ 0.01, †††P ≤ 0.001 (one-way ANOVA followed by Newman-Keuls post hoc).
FIGURE 8 Dose response curves for NLE (30-300 mg kg$^{-1}$ p.o.), diclofenac (10-100 mg kg$^{-1}$i.p) and Morphine (1-10 mg kg$^{-1}$i.p) on Phase 1 and 2 of formalin induced pain in mice

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