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Research Article

A modified method for evaluating analgesic activity of drugs using *Rana tigrina* frog

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ABSTRACT

Background: Different species of frogs had been used by many researchers for evaluation of analgesic drugs e.g., *Rana pipiens* and African claw frog. In our study, we used *Rana tigrina,* which was never used for evaluation of analgesic activity of drugs. So by doing this project, we judged usefulness of *R. tigrina* to evaluate analgesic drugs.
Methods: Animals used were *R. tigrina* of either sex weighing 100-150 g. Glass flask with porous platform was used for observation of frog. All groups were treated with 4% NaCl solution S.C. on abdomen). Characteristic parameter i.e., number of eye blinkings (this parameter was observed during the pilot study after 4% NaCl S.C. injection on lower third of frogs abdominal wall) were observed before and after drug administration. Each observation was for 5 mins. Centrally and periphrally acting drugs effect was tested on the number of blinks and buccal oscillations.
Results: Centrally acting drug inhibit rise in number of eye blinkings and buccal oscillation significantly. Piroxicam diclofenac sodium and ketoprofen decreased rise in number of blinks; but, it was not significant as compared with control.
Conclusion: This animal may be used alternative to existing methods of evaluating analgesics in Indian setup.

Keywords: *Rana tigrina*, Writhing, Analgesic activity, Animal model

INTRODUCTION

Opioids and non-opioids are the main group of drugs used for the treatment of pain. Some antidepressants and anticonvulsants are also used for the treatment of pain.[1] Opioids acts on central nervous system (block synaptic transmission of impulse signaling pain) for producing analgesia. Non-opioids acts on peripheral nervous system (block impulse generation at pain receptor) for producing analgesia.[2] Many animals and methods are used for evaluating analgesic activity of a test substance e.g. rats,[3] mice,[4] dogs,[5] and monkeys.[6] Frog is also used as alternative to mammalian pain models. For this purpose, many species of frogs were used by many researchers e.g. *Rana pipiens* was used by Stevens in his studies on pain.[7,8] African claw frog was used by Coble et al.[9] In our study, we used *Rana tigrina* (Indian bull frog), which was never used for evaluation of analgesic activity of drugs. Hence, this study was done to evaluate reproducibility, advantages and disadvantages with using *R. tigrina* as animals model for evaluation of analgesic drugs.

METHODS

Frogs of either sex weighing 100-150 g were procured from local supplier. The care of frogs were taken according to guideline given by Tayler (2009).[10] Each frog was kept in a separate cage with 2 L of water. Water was replaced every day. To attract insects toward the cage, bulb of 10 w was kept glowing above each cage. Apart from this frogs were also fed with household cockroaches. Drugs were purchased from the hospital pharmacy.

Commonly used irritants for producing pain in animal are phenylquinone, acetic acid, and sodium chloride. In our

study, we used sodium chloride solution to induce pain in frogs. 4% NaCl solution was used by Fukawa et al.[11] to produce writhing rapidly in rats. After injection of 4% sodium chloride solution (0.5 ml) to we saw characteristic responses to this irritant stimulus. These responses were an increase in change in number of eye blinking and buccal oscillations (Figures 1 and 2). To determine exact concentration of sodium chloride, which produces maximum number of both these parameters frogs were divided into different groups as shown in Table 1.

Sodium chloride solutions of concentration 1, 2, 4, and 8% were formed. First to fourth group received increasing concentration of sodium chloride solution subcutaneously on lower third of frogs abdominal wall. 4% solution showed maximum number of blinking and buccal oscillations. Hence, further experiment was carried with the same concentration (Tables 2 and 3).

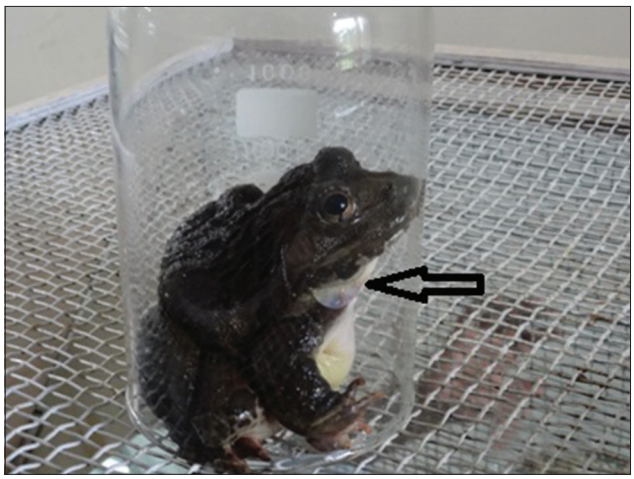

Figure 1: Frog in observation chamber before injection of NaCl. Arrow shows site where buccal oscillation was observed.

Figure 2: Frog in observation chamber after injection of NaCl (Blinking can be clearly seen).

After this, the centrally and peripherally acting drugs were given to see their effects on sodium chloride induced changes in blinking and buccal oscillations.

1. Effect of centrally acting drugs:
   For this purpose, frogs were divided into four groups (each containing 6 animals)
   a. Control group: received 2 ml distilled water followed by 4% NaCl subcutaneously
   b. Test Group 1: received buprenorphine followed by 4% NaCl subcutaneously
   c. Test Group 2: received pentazocine followed by 4% NaCl subcutaneously
   d. Test Group 3: received fentanyl followed by 4% NaCl subcutaneously.
2. Effect of peripherally acting drug:
   For this purpose, frogs were divided into four groups (each containing 6 animals):
   a. Control group: received 2 ml distilled water followed by 4% NaCl subcutaneously
   b. Test Group 1: received piroxicam followed by 4% NaCl subcutaneously
   c. Test Group 2: received diclofenac sodium followed by 4% NaCl subcutaneously
   d. Test Group 3: received ketoprofen followed by 4% NaCl subcutaneously.

Test groups received drugs by subcutaneous route in dorsal lymph sac.[9] Each drug were given 30 mins before subcutaneous injection of 4% NaCl on lower third of frogs abdominal wall. For observation glass flask and porous platform was used. Each frog was observed for 5 mins after subcutaneous injection of sodium chloride solution. Same procedure was repeated after 2 weeks of washout period for 4 times using same frogs.

Statistical analysis

Statistical analysis was done using Prism Pad software. One-way ANOVA test was done followed by Dunnet's multiple comparison test. $p<0.05$ was considered to be significant.

Table 1: Different groups of animals used for determination of effective concentration of sodium chloride.

Group I	Control	6 animals	2 ml of distilled water by S.C. route
Group II	Test	6 animals	1% Nacl solution by S.C. route (0.5 ml)
Group II	Test	6 animals	2% Nacl solution by S.C. route (0.5 ml)
Group III	Test	6 animals	4% Nacl solution by S.C. route (0.5 ml)
Group IV	Test	6 animals	8% Nacl solution by S.C. route (0.5 ml)

Table 2: Determination of effective concentration of NaCl solution (number of blinks).

Groups (n=6)	2 ml subcutaneously	Number of blinks before receiving NaCl (in 5 mins)	Number of blinks after receiving NaCl (in 5 mins)	Percentage of increase in blinking
Control	Distilled water	1.83±0.30	3.83±0.60	116.7
Test	1% Nacl	2.16±0.47	7.66±0.95	304.2
Test	2% Nacl	2.5±0.42	12.5±1.28	454.2
Test	4% Nacl	2.83±0.47	27.5±1.20	1068***
Test	8% Nacl	2.5±0.42	11.33±0.98	422

n: number of animals, ***p<0.001

Table 3: Determination of effective concentration of NaCl solution (number of buccal oscillations).

Groups (n=6)	2 ml subcutaneously	Number of buccal oscillations before receiving NaCl (per minute)	Number of buccal oscillations after receiving NaCl (per minute)	Percentage of increase in buccal oscillations
Control	Distilled water	49±2.59	54±2.36	10.58
Test	1% NaCl	50.33±3.20	67.33±4.26	34.15
Test	2% NaCl	55.33±2.10	88.83±1.83	61.87
Test	4% NaCl	54.67±2.36	114.30±3.70	111****
Test	8% NaCl	54.33±1.83	73±1.46	34.76

****p<0.0001

RESULTS

Determination of effective concentration of NaCl solution

When sodium chloride solution was given in increasing concentration, the number of blinks and buccal oscillations were increased, and 4% concentration showed maximum number in both parameters. Next concentration i.e. 8% showed a decrease in number of both parameters. With both parameters percentage of rise was highly significant with 4% concentration (During statistical analysis percentage of the rise in both parameters after giving sodium chloride solution was calculated for each animal and then mean of percentage rise was compared). Hence, whole experiment was conducted with 4% concentration.

Effect of drugs with peripheral mechanisms on eye blinking and buccal oscillations

Piroxicam diclofenac sodium and ketoprofen decreased rise in number of blinks and buccal oscillations, but it was not significant in comparison to control (Tables 6 and 7).

Table 4: Effect of centrally acting drugs on eye blinking.

Group (n=6)	Number of blinks	% of inhibition
Distilled water	24±0.85	0
Buprenorphine (5mcg S.C.)	11.83±0.70	50.70****
Pentazocine (0.54 mg S.C.)	16.50±1.56	31.25***
Fentanyl (1.26 mcg S.C.)	17.50±1.20	27.08**

**p<0.01, ***p<0.001, ****p<0.0001

Table 5: Effect of centrally acting drugs on buccal oscillations.

Group (n=6)	Number of buccal oscillations	% of inhibition
Distilled water	118±2.76	0
Buprenorphine (5mcg S.C.)	58±2.62	50.84****
Pentazocine (0.54 mg S.C.)	73.83±2.12	37.43*
Fentanyl (1.26 mcg S.C.)	69.17±3.34	41.38**

**p<0.01, ***p<0.001, ****p<0.0001

DISCUSSION

Many researchers did experiments on amphibians for detecting analgesic activity of drugs. Though human and frog brain has many differences, these researchers showed that their findings can be used to predict action of drugs in human beings. Pezalla et al. in his experiment on *Rana pipines* showed how to assess the nociceptive threshold (NT) in frogs using the acetic acid test. Nociceptive testing is done by placing, with a Pasteur pipette, a single drop of acid on the dorsal surface of the frog's thigh. Testing begins with the lowest concentration and proceeds with increasing concentrations until the NT is reached. The NT is defined as the lowest concentration of acid that causes the frog to vigorously wipe the treated leg. In this test, the frog is exposed to various concentrations of acetic acid until it shows response. This process may damage tissue of the thigh.[12] Suckow et al. show hypothermia induced analgesia where they used tourniquetion of test leg and ice water (6°C) for producing hypothermia.[13] Such procedure

Table 6: Effect of peripherally acting drugs on eye blinking.

Group (n=6)	Number of blinks	% of inhibition
Distilled water	25.5±1.08	0
Piroxicam (0.36 mg S.C.)	21.83±1.22	14.39
Diclofenac sodium (1.35 mg S.C.)	22.17±1.53	13.05
Ketorolac (0.54 mg S.C.)	22.5±0.92	11.76

Table 7: Effect of peripherally acting drugs on buccal oscillations.

Group (n=6)	Number of buccal oscillations	% of inhibition
Distilled water	118±2.76	0
Piroxicam	114.3±4.17	3.13
Diclofenac sodium	113.5±3.41	3.81
Ketorolac	117.3±3.20	0.59

can damage the tissue and animals will suffer a lot. In our study, we gave 0.5 ml of 4% NaCl subcutaneously to the frog. It is the same concentration that was used by Fukawa et al. in their experiment.[11] The quantity and concentration of sodium chloride solution is very low, and it will not harm the animal anyway.

Stevens (1996) demonstrated action of different opioid drugs on *R. pipiens*.[14] He also showed that effect of NSAIDs like indomethacin and ketorolac on *R. pipiens*.[8] But in our study, we got significant result only with opioid analgesics. This may be due to variations in animal species. These variations should be compared in further studies. In comparison to other animals, which are commonly used for detecting analgesic activity frog is less costly and easily available. The maintenance also is very easy. Writhing response, which is produced by various chemicals in mammals is difficult to understand for beginners. On the contrary, in our experiment the parameters taken were a number of blinking of eye and buccal oscillatations, which can be easily counted by a beginner. Furthermore, the animal is not sacrificed during this experiment. Hence, this study may be helpful to give an alternative animal model by using frog commonly available in India without endangering the animal.

There were some problems to which we came across during this study:

1. We could not number the frog and because of it we arranged separate cage for each frog and numbered that cage for identification of the particular frog
2. Frog skin was very slippery, and it was very difficult to inject drugs.

One characteristic phenomenon, we saw during this experiment was increased secretions from the skin of frog after injection of NaCl. We could not explain this phenomenon. The secretions can be collected, and concentration of NaCl can be checked in further studies to detect whether this response is because of irritation due NaCl injection. The frog might have this natural ability to excrete toxin from its body whenever it is exposed to them. We could test only six analgesic drugs. In further studies, other drugs can be tested to know the reliability of this method.

CONCLUSION

We want to state that *R. tigrina* (Indian bull frog) can be used as an alternative pain model to evaluate centrally acting analgesics by this modified method.

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Ethical approval: The study was approved by the Institutional Animal Ethics Committee

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