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Original Research Article

A comparative study for antinociceptive potential of vitamin D_3 with diclofenac in animal models

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ABSTRACT

Background: Calcitriol is one of the active forms of vitamin D. It not only acts on calcium metabolism but might have a role in treating various disorders also through vitamin D receptors that are present in many tissues besides intestine and bone. This study was conducted to compare antinociceptive activity of Calcitriol with Diclofenac and Morphine in animal models.
Methods: In this study, healthy Swiss albino mice were taken after permission from IAEC. Mice were divided into six groups as one control- treated with normal saline, two standards - treated with diclofenac and treated with morphine while three tests - treated with Calcitriol in dose of 15µg /kg/mice, 30µg/kg/mice and 60µg/kg/mice respectively. Comparison of antinociception was done using Tail pinch and writhing method.
Results: Tail pinch and Writhing methods were used for comparison of antinociceptive activity. In tail pinch model, Calcitriol showed some analgesia at 30 and 60µg/Kg doses, which was more than control but not comparable with the standard Morphine. In writhing method, test doses of Calcitriol (15 and 30µg/Kg) failed to show analgesic efficacy in inflammatory pain but test dose of 60µg/Kg showed some analgesic activity which was not comparable with standard Diclofenac.
Conclusions: Antinociception was exhibited at higher doses of Calcitriol by tail pinch method while in writhing method analgesic activity was shown with only 60 µg/Kg dose of Calcitriol. The results obtained from this study needs to be further evaluated by planning extensive animal experimentation.

Keywords: Antinociception, Calcitriol, Diclofenac, Tail pinch, Writhing

INTRODUCTION

Vitamin D is a group of fat-soluble secosteroids synthesized in adequate amounts by all mammals from sunlight. The major endogenous source of Vitamin D for humans is the epidermis.[1] Calcitriol (Vitamin D_3), also known as the "sunshine vitamin" is one of the major physiologically active forms of Vitamin D. Vitamin D_3 is manufactured by the irradiation of 7-dehydrocholesterol from lanolin and the chemical conversion of cholesterol in the epidermis.[2] It promotes intestinal absorption of calcium and regulates bone mineralization.[3] The discovery of vitamin D receptors in many tissues besides intestine and bone- including brain, heart, pancreas, breast, prostate, lymphocytes, and other tissues- implies that vitamin D supplementation might have applications for treating a number of disorders.[4,5] These non-classic tissues are therefore potential targets for the active metabolite of Vitamin D, 1,25(OH)2D which may provide number of potential new clinical applications of Calcitriol and its analogs. One of such non-classic action of calcitriol is the regulatory effect on some of the key molecular pathways involved in inflammation, such as inhibition of Prostaglandin synthesis and actions. Calcitriol inhibits the synthesis and biological actions of pro-inflammatory PGs by mechanisms like suppression of the expression of cyclooxygenase-2, up-regulation of the expression of 15-hydroxyprostaglandin

dehydrogenase and down-regulation of the expression of PG receptors.[3]

The drugs in clinical practice for inflammatory pain such as NSAIDs act by the mechanism that coincides with vitamin D_3 action on PGs. Nonsteroidal anti-inflammatory drugs (NSAIDs) are commonly used for 'peripheral' analgesia. Agents such as aspirin, diclofenac sodium and other NSAIDs provide their anti-inflammatory action by blocking the cyclo-oxygenase pathway in periphery.[6] For central analgesic action, opioids group of drugs like Morphine are used which modulates nociceptive inputs at many sites (supraspinal and spinal) in the CNS through opioid receptors.[7] Experimental evaluation of any agent for the analgesic effects is most commonly carried out using various animal models for nociception. For mechanical nociception, pain is produced by applying pinch cock or artery clip to the root of animal tail and response of biting the clip in order to remove is noted. Acetic acid induced writhing reflex is recorded for inflammation induced pain. These responses are measured and recorded before and after the drug administration and can be compared with the standard. Keeping in mind the effect of Calcitriol on the mediators of pain it was thought to carry out an experimental comparison of analgesic action of Calcitriol using pain models in albino mice.

METHODS

This was a prospective, experimental study conducted at Animal resource centre for medical research, Rural Medical College, Pravara Institute of Medical Sciences, Loni. This study was carried out as per the CPCSEA guidelines after obtaining approval from Institutional Animal Ethics Committee (IAEC).

Selection of animals

Swiss albino mice of both sex of average weight (25-30gms) and age 12 weeks were taken for study. Animal caring, housing and handling requirements were promptly observed as per the CPCSEA protocol. All animals were maintained under standard laboratory conditions of food and water before start of the experiment.

The animals were acclimatized for 1 week; they were maintained under standard condition at temperature of 25±10 $^{\circ}$ C, 60±5% relative humidity and 12 hours light dark cycle. All experiments were carried out between 9.00 am and 5.00 pm according to the guidelines for the care of laboratory animals. Animals were also kept under observation for 7 days, after the completion of experiment to observe any acute or sub acute toxicity.

Chemicals/ drugs used

1. Injection Diclofenac sodium (75mg/ml)-DYNAFORD (Stanford Laboratories Pvt. Ltd.)
2. Inj. Morphine sulphate (10mg/ml)-MORPHITROY*10 (Troikaa Pharmaceuticals Ltd.)
3. Inj. Calcitriol [6,00,000 I.U. (15mg)] - Bone-D_3 injection (KEEN Health care (P) Ltd.)
4. Normal saline

Allocation of animals

The animals were weighed and then divided into six groups (group A to F) each having 6 animals, according to block randomization method. Thus 36 animals were allocated for each experimental model. (6 albino mice X 6 groups (A-F) X 2 experimental models, n=72). The animals were divided as follows-

- Group A- Control group: Treated with Normal saline Dose: 10ml/kg/mice
- Group B- Positive control group I: Treated with Diclofenac Dose: 15mg/kg/mice
- Group C- Positive control group II: Treated with Morphine Dose: 1mg/kg/mice
- Group D- Test group I: Treated with injection Calcitriol Dose: 15µg/kg/mice
- Group E- Test group II: Treated with injection Calcitriol Dose: 30µg/kg/mice
- Group F- Test group III: Treated with Injection Calcitriol Dose: 60µg/kg/mice

Tail pinch method

The tail pinch method was used for central analgesic action. In this animals were selected by noting their response to nociceptive stimuli and those showing positive response were included. Pain was produced mechanically by an artery clip which was applied approximately 2 cm away from the base of the tail.

The animal quickly responds to this noxious stimulus by biting the clip or the tail near the location of the clip as a continuous attempt to remove it. The time between stimulation onset (application of clip) and response (biting, chewing) was calculated. The drugs were injected through i.p. route according to their respective drug group. The reaction time in response to noxious stimuli (biting, chewing) before and after 30, 60, 120 and 240 minutes after administration of the drug with the use of a stopwatch.[8,9]

Interpretation

Prolongation of the reaction time in the drug treated animals compared to the control indicates analgesic effect. The percentage increase or decrease in reaction time (as index of analgesia) at each time was calculated.[10]

Percentage increase in reaction time = [Rt/Rc- 1] x 100

Where, Rt = reaction time in treated group
Rc = reaction time in control group

Writhing method

Writhing method was used for peripheral analgesic action. The experiment was conducted in the dim lighted and noise free laboratory. It was done by injecting 0.1ml acetic acid 0.6% v/v intraperitoneally which produces severe inflammatory pain. The animals were observed for Writhing response which is characterized by typical stretching behaviour. Writhing is defined as stretch torsion to one side, drawing up of hind limbs, retraction of the abdomen opisthotonus, as mice tries to touch its belly to the floor.[8,9]

The drugs were injected through i.p. route according to their respective drug group. All animals received the test drug 30 minutes before the experiment than 0.1 ml acetic acid i.p was injected.

Mice were then placed in plexi glass funnel for 45 min. Animal behaviour was observed for: Onset, duration of writhing and total number of writhing in 45 minutes.[8,9]

Interpretation

Prolongation in onset, decrease in number and duration of writhings in test group compared with standard treated and control treated groups indicate analgesic activity.[10]

The percentage inhibition was calculated by using the formula:

Percentage inhibition = [1- Nt/Nc] x 100

Where, Nt = average number of writhing in treated group
Nc = average number of writhing in control group

All values were expressed as mean±SD and compared with the corresponding control values. P- Values are calculated by using one- way ANOVA followed by Tukey - Kramer multiple comparison tests. P <0.05 was regarded as statistically significant.

RESULTS

For Tail Pinch method, by applying Student's Paired 't' test significant prolongation of response time was observed at 30 - 240 mins as compared to the response time before giving the drug in group B, C, E and F. On comparison of mean and SD values of Response (In Seconds) by applying Student's Unpaired 't' test in Tail Pinch method there was highly significant difference in favour of group C showing highest analgesic activity followed by group B, group F, group E, group D and group A when compared with each other (i.e. p<0.01). Thus group B, F, E, D showed analgesia not comparable with the standard group C at 30, 60, 120 and 240 minutes. The results of Tail Pinch method revealed significant prolongation of response time at 30- 240 mins in group B, C, E and F and there was highly significant difference in favour of group C showing highest analgesic activity followed by group B, group F, group E, group D and group A when compared with each other (Table 1). Thus group B, F, E, D showed analgesia not comparable with the standard group C at 30, 60, 120 and 240 mins. Thus with Tail pinch method Calcitriol showed no analgesic activity at 15µg/Kg but showed some analgesia at 30-60µg/Kg which was more than control Normal saline but not at all comparable with the standard Morphine for thermal type of pains. Diclofenac sodium was consistent as weak analgesic also for mechanical pain induced by tail pinch.

Table 1: Comparison of mean and SD values of response (in seconds) by tail pinch method in all groups under study from before and after drug effect.

Group	DRUGS	Response Mean ± SD (In Seconds)				
		Before	After			
			30 Min	60 min	120 min	240 min
A	Normal saline (10 ml/kg)	5.05 ± 0.33	5.15 ± 0.60	5.15 ± 0.47	5.00 ± 0.61	4.90 ± 0.65
B	Diclofenac (15 mg/kg)	5.26 ± 0.13	7.61 ± 0.11* (47.77)	7.33 ± 0.14* (42.33)	7.10 ± 0.11* (42.00)	6.93 ± 0.14* (41.43)
C	Morphine (1 mg /kg)	5.84 ± 0.09	24.92 ± 1.97* (383.88)	18.47 ± 3.08* (258.64)	15.47 ± 0.74* (209.40)	12.49 ± 2.73* (154.90)
D	Calcitriol (15 µg/kg)	5.45 ± 0.29	5.58 ± 0.20 (12.62)	5.52 ± 0.27 (7.18)	5.44 ± 0.24 (8.80)	5.40 ± 0.26 (10.20)
E	Calcitriol (30 µg/kg)	5.57 ± 0.22	5.89 ± 0.36* (15.37)	5.84 ± 0.52* (13.40)	5.74 ± 0.43* (14.80)	5.65 ± 0.41* (14.31)
F	Calcitriol (60 µg/kg)	5.62 ± 0.23	6.80 ± 1.06* (32.04)	6.53 ± 0.79* (26.80)	6.40 ± 0.80* (28.00)	6.21 ± 0.76* (26.73)

(Numbers in parenthesis indicate percentage increase in reaction time when compared with control. *p<0.01 when compared with control

In the writhing method, the mean values of onset of writhe were significantly prolonged in group B, followed by group C, group F, group E, group A and group D when all groups compared to each other. The mean

values of duration is significantly decreased in group B, followed by group C, group F, group A, group D and group E when all groups compared to each other (Table 2).

Table 2: Comparison of mean and SD values of writhing response (in minutes) by writhing method in all groups under study.

Group	Drug	Writhing Response Mean ± SD		
		Onset of writhe (In min)	Duration (In sec)	No. of Writhes in 45 min
A	Normal saline (10 ml/kg)	5.17 ±0.98	6.97 ±0.55	21.67 ±4.84
B	Diclofenac (15 mg/kg)	7.67 ± 1.21	4.33 ±0.51	7.33 ±1.63 (66.17)
C	Morphine (1 mg /kg)	6.50 ±1.05	6.26 ±0.88	17.67 ±2.16 (23.07)
D	Calcitriol (15 µg/kg)	4.00 ±01.41	7.84 ±0.46	23.67 ±6.65 (-9.23)
E	Calcitriol (30 µg/kg)	5.67 ±1.03	8.17 ±0.76	21.33 ± 1.51 (1.56)
F	Calcitriol (60 µg/kg)	6.67 ±1.21	6.95 ±0.57	18.83 ± 2.32 (13.11)

(Numbers in parenthesis indicate percentage inhibition in number of writhes in 45 mins when compared with control)

The mean values of no. of writhes in 45 min is significantly less in group B, followed by group C, group F, group E, group A and group D when all groups compared to each other. The percent inhibition of writhes or percent protection was found to be maximum in group-B (66.17%) whereas only group-F of test drug showed some percentage inhibition (13.11%) which was not comparable with standard Diclofenac. Group C also showed some percentage inhibition (23.07%). Thus by using writhing method group B showed highest analgesic activity.

In writhing method Diclofenac sodium **(**Group B) showed highest analgesic activity than other groups whereas Morphine (Group C) which proved to be best for thermal and mechanical pain, had weak analgesic efficacy in inflammation induced pains. The weak analgesia shown by Morphine may be attributed to its decrease in peripheral and central afferent nociceptive transmission.[7] The test doses (15 and 30µg/Kg) of Calcitriol failed to show analgesic efficacy in writhing model of inflammatory pain while test dose of 60µg/Kg showed some analgesic activity which was not comparable to standard.

The battery of experiments for evaluation of analgesic effect of Calcitriol in animal models revealed no analgesic activity at the dose of 15-30µg/kg but some analgesic activity at the dose of 60µg/kg. At higher dose of 60µg/kg the minimal analgesic activity demonstrated may be due to its ability of Calcitriol to inhibit PG synthesis responsible for PG induced pains.[3]

DISCUSSION

This study was initiated with an aim of comparing analgesic action of Calcitriol for pain similar to the other nonclassic actions of Vitamin D demonstrated by Daniel bikle in his review stating its utility in inflammatory disorders.[11] The same was reported in the Vitamin D report by Stewart B that the role of Vitamin D extends beyond bone and muscle involvement in chronic pain syndromes supported by some studies in the literature suggesting benefits of Vitamin D supplementation in musculoskeletal pain.[12]

Anti-inflammatory properties of Vitamin D were demonstrated in animal experiments, and various clinical studies indicates that Vitamin D supplementation modulates or decreases pro-inflammatory cytokines (e.g., C-reactive protein, interleukin 6 and 12, and tumour necrosis factor-alpha) while increasing anti-inflammatory cytokines such as interleukin-10.[13-16]

Although the literature suggest that Calcitriol inhibits the synthesis and biological actions of pro inflammatory PGs by various mechanisms. These mechanisms may not be involved in the acute mechanical and inflammatory pains which may be the reason for inability of Calcitriol to produce analgesia in acute pains.

With the plethora of the evidences obtained from this study on Calcitriol, it can be concluded that there is some analgesic utility of Calcitriol in the above experimental models of mechanical and inflammatory pain but these evidences need to be further evaluated in terms of mechanisms, safety and efficacy by planning extensive animal experimentation.

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