COMPARATIVE ANALGESIC ACTIVITY OF TROIS IN RAT FORMALIN TEST

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ABSTRACT

Several topical formulations are available in the market for the management of pain. With the aim of increasing efficacy and decreasing side effects, herbal drugs are being researched for various therapeutic applications. This study involved comparison of marketed formulations: Trois, Volini, Moov and Fast-Relief. Trois is a proprietary herbal product indicated for the management of painful states. The rat formalin test was utilized as the animal model for pain and observations of composite pain score, number of paw licks and pain response latency were recorded in the early and late phases. Results of the present study indicated that Trois was superior in management of acute and chronic pain states as indicated by pain response latency, number of paw licks and composite pain score. Pain scoring indicated that all formulations, except Volini, were significantly better than control in the early phase with Trois showing the highest efficacy. Late phase observations suggested that all formulations were significantly better, with Trois maintaining its higher efficacy. Trois reduced the number of licks and pain response latency to a significant extent in the early and late phases whereas the effect of Moov, Volini and Fast Relief, were significant only in the late phase. Hence, it can be concluded that Trois is a better herbal topical pain relief alternative as compared to other commercial products.

KEY WORDS: Trois, Pain, Gaultheria procumbens, Eucalyptus globulus, Menthol, Vitex negundo, Apium graveolens

INTRODUCTION

Acute and chronic inflammatory diseases and associated pain are responsible for causing significant debility and loss in productive time of individuals across the world. From minor sports/accidental injuries to Arthritis-associated pain involving joints and muscles which result in immobility are conditions that require immediate pain relief [1],[2]. This kind of mild-to-severe pain is usually abrogated with the help of topical analgesics. The topical analgesic therapy offers the advantages of ease of application with immediate relief from pain locally. Every individual has a different perception of pain and responds in a unique manner to analgesic drugs [3]. Different formulations in the form of sprays, ointments and gels are available commercially for the management of pain.

Current research on complementary and alternative medicine is focused on the use of indigenous herbs based product for increasing efficacy and decreasing side effects for various therapeutic applications. Trois is polyherbal formulation available in the market and indicated for anti-inflammatory analgesic purpose. The present study was designed to investigate the comparative topical analgesic activity of Trois with other commercially available formulations.

This study was performed using the rat formalin test as the model for nociception [4],[5],[6]. The principle of test method depends upon the fact that the injection of chemicals into subcutaneous or deep tissues induces pain and can be used to evaluate analgesia. Particular attention has been paid to the effects of bradykinin, a naturally
occurring plasma peptide which produces brief, intense pain near the injection site [7],[8]. The procedure consists of injection of a small amount of 5% formalin subcutaneously into the paw; the animal’s behavioral responses are then subjectively evaluated according to a predetermined set of pain intensity ratings. The pain generally lasts for at least 30 min, and the observations are made on animals which are restrained only lightly or not at all [5],[9].

An important feature of the formalin test in rodents is that the animals show two phases of nociceptive behaviour which seem to involve two distinctly different stimuli. The first phase starts immediately after injection of formalin and lasts for 3-5 min and represents acute pain. It is probably due to direct chemical stimulation of nociceptors, and experimental data indicate that formalin predominantly evokes activity in C fibres and not in Aδ afferents. Subsequently, there is a period of 10-15 min when the animals display very little behavior suggestive of nociception. The second phase starts approximately 15-20 min after formalin injection and lasts for 20-40 min [5],[6] and represents late phase. Opioid classes of analgesics are active in both phases while non-steroidal anti-inflammatory drugs (NSAIDs) reduce nociceptive behaviour during the second/late phase, while the first phase seems unaffected [10]. This lack of effect in the early phase persists even when a very low formalin concentration is used, suggesting that the two phases are qualitatively different and that the difference is not due to a difference in stimulus intensity or motor abnormalities.

MATERIALS AND METHODS

Materials

The formulations Trois, Volini, Moov and Fast Relief used for treatment were purchased from a local pharmacy. Formalin (AR grade) and normal saline were sterilized by filtration through a 0.22 μm nylon syringe filter.

Animals

Healthy adult Sprague Dawley rats weighing 150-250 g were taken for the experiment. The study was approved by the institutional animal ethics committee. Rats were maintained under 12 h light: dark cycle in a temperature (22°C) and humidity controlled room. Unless mentioned, food and water were available ad libitum. Animals were acclimatized in standard animal house environmental conditions for 5 days before the start of the experiment. On the last day of acclimatization, animals were randomized on the basis of their body weights and were allocated to 5 groups as per scheme mentioned in Table 1. G1 group served as the vehicle control and was treated with 220 microlitre of Normal Saline (NS) topically. G2, G3, G4 and G5 served as test groups and were treated topically with the dose equivalent to 200 mg of test substance.

Methods

The study design is given in Figure 1. The animals were treated with normal saline, or with test substance as designed in Table 1. After 15 min of treatment, 50 µL of sterile 5% formalin was injected subcutaneously into the dorsal surface of the left hind paw of the animal with the use of a 1 mL syringe with a 26-gauge needle. Immediately after formalin injection, each animal was placed in a glass chamber for pain-scoring and the stop clock was started simultaneously. The total time (sec) spent in each scoring profile given below was measured as an indicator of pain. Periods of 0–5 min immediately after formalin injection and 20-30 min were considered as the early and late phases respectively. No observations were recorded during the inter phase.

Pain scoring and rating was done as per the method described below [11],[12]

<table>
<thead>
<tr>
<th>Group</th>
<th>Number of Animals</th>
<th>Treatment</th>
<th>Dose (mg) / Regimen</th>
<th>Route of Administration</th>
</tr>
</thead>
<tbody>
<tr>
<td>G-1*</td>
<td>3</td>
<td>Control</td>
<td>----</td>
<td>Topical</td>
</tr>
<tr>
<td>G-2</td>
<td>3</td>
<td>Trois</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>G-3</td>
<td>3</td>
<td>Volini</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>G-4</td>
<td>3</td>
<td>Moov</td>
<td>200</td>
<td></td>
</tr>
<tr>
<td>G-5</td>
<td>3</td>
<td>Fast Relief</td>
<td>200</td>
<td></td>
</tr>
</tbody>
</table>

*G-1 was treated with 220 microlitre of normal saline applied topically. Doses are reported in mg/kg.

Both forepaws are placed on the floor and weight is evenly distributed. During locomotion, there is no discernible favoring of the injected paw.

i. Both forepaws are placed on the floor and weight is evenly distributed. During locomotion, there is no discernible favoring of the injected paw.

ii. The injected paw rests lightly on the floor or on another part of the animal’s body and little or no weight is placed upon it. During locomotion, there is an obvious limp. The rating is also given to normal grooming, during which both paws are elevated and "washed" and to rearing where neither forepaw is in contact with the floor, although either may be in contact with the wall. Attempts to sleep by curling up with both paws off the floor are also given this rating.

iii. The injected paw is elevated, and not in contact with any surface. The un injected paw is placed firmly on the floor. Attempts to sleep by curling up with only the injected paw off the floor, even when it is tucked under the body, are given this rating.
iv. The injected paw is licked, bitten, or shaken, while the un injected paw is not. This behavior is quite distinct from normal grooming (rating 1), although transitions between the two are common.

![Figure 1: Experimental set up for the rat formalin test](image)

Pain rating was calculated using the formula given below. Where $T_1$, $T_2$, and $T_3$ are the durations (in sec), spent in categories 1, 2 or 3 respectively during the early and late phases. Animals were observed for early phase (300 sec post formalin injection) and late phase (600 sec post formalin injection).

Total number of paw licks in each phase was calculated by an individual blinded to the treatments. In each phase, latency in the initiation of responses were also recorded as a measure of pain response latency.

**RESULTS**

**Pain Rating (Composite Pain Score, CPS)**

CPS was calculated using the above mentioned formula.

**Early Phase**

Treatment groups G-2 (treated with Trois) showed significant ($p<0.001$) reduction in CPS / Pain during early phase of formalin test whereas G-3 (treated with volini), did not show significant reduction in CPS during early phase. The activity found with G-4 (treated with Moov) was comparable to that of Trois and reduction in pain score observed in G-5 (treated with Fast relief) was also found to be statistically significant. Trois showed highest pain relieving activity during early phase with 53.77 % reduction in pain as compared to control group; whereas Moov, Fast Relief and Volini showed only 37.29 %, 22.87 % and 18.76 % reduction in pain score.

**Late Phase**

All treatment groups G-2, G-3, G-4 and G-5 treated with Trois, Volini, Moov and Fast Relief showed significant ($p<0.001$) reduction in number of paw licking during early phase of formalin test. Trois showed highest Pain Relieving activity during early phase with 77.25 % reduction in pain; whereas Volini, Moov and Fast Relief showed 49.78 %, 68.44 % and 37.57 % reduction in pain score.

**Number of Paw Lickings**

Paw licking was counted and is represented below (Figure 3).

**Early Phase**

Treatment group G-2 (treated with Trois) showed significant ($p<0.001$) reduction in number of paw licking during early phase of formalin test in comparison to control group (which indicates significant reduction in pain intensity). None of any other treated groups showed any significant reduction in number of paw licking (indicating high pain intensity). Trois showed the highest Pain intensity reducing activity during early phase with 84.08 % reduction in pain intensity whereas Volini, Moov and Fast Relief showed only 31.83 %, 38.20 % and 13.53 % respectively reduction in pain intensity during early phase.

**Late Phase**

All treatment groups except G-5 (treated with Fast Relief) showed significant reduction in number of paw lickings (pain intensity) during late phase of formalin test. Trois showed highest Pain intensity reducing activity ($p<0.001$) during late phase with 91.84 % reduction in number of paw lickings as compared to control whereas Volini ($p<0.05$) and Moov ($p<0.001$) showed 32.81 % and 86.19 % reduction in number of paw lickings (Pain intensity). Fast Relief showed no significant lowering in number of paw lickings (pain intensity) with 14.76 % activity only.

![Figure 3: Number of paw lickings in the early and late phases of the rat formalin test](image)
ns indicates non-significant difference, all as compared to control group in the respective phases

Pain Response Latency

Pain response latency indicates the time (in sec.) taken by animal after the initiation of early phase and late phase to show first pain indication. Mean Pain Response Latency during Early and Late Phase is represented in Figure 4. Pain Response Latency indicates the onset of pain during Early and Late Phase in Formalin Test. Treatment groups G-2 (treated with Trois) showed highest and significant (p<0.001) Pain Response Latency during Early as well as Late Phase of formalin test (which indicates fastest onset of action). Trois showed mean Pain Response Latency of 139.06 ± 9.72 sec and 293.02 ± 39.99 in Early Phase and Late Phase respectively. Whereas Volini, Moov and Fast Relief showed mean Pain Response Latency of 59.85 ± 11.96 sec, 63.09 ± 14.48 sec & 58.46 ± 14.41 sec respectively during Early Phase and 98.96 ± 8.06 sec, 214.46 ± 16.09 sec & 146.26 ± 35.98 sec respectively during Late Phase. Trois was active in both phases of formalin mediated pain responses whereas other comparators showed significant pain response latency only in the late phase.

Figure 4: Pain response latency in the early and late phases. Data is represented as Mean ± SEM (n = 3). *** indicates P<0.001, * indicates P<0.05 and ns indicates non-significant difference, all as compared to control group in the respective phases
Results from the late phase of study support the analgesic activity of all the tested products. This suggests that all these formulations may act through prevention of release of inflammatory mediators like prostaglandins, serotonin and histamine, known to mediate pain [16],[17]. This phase involves nociceptive transmission from the unmyelinated Aδ fibres. Most of the NSAIDs are known to be active in this manner. Trois showed superior efficacy as compared to Volini and Fast Relief in the late phase of nociception. The effect of Moov was found to be comparable to that of Trois and statistical analysis revealed that the effect of both the formulations, Trois and Moov, were significantly better than control and superior to Volini and Fast Relief. Volini showed significant data for all the three parameters suggesting that it is active in the late phase of nociception. Fast Relief showed a reduced of pain score which was comparable to Trois and Moov, but the paw lick results revealed that Fast Relief was not able to aggravate severe pain and its results were not significantly different from the control.

Previous studies have shown that constituents like pinene, eucalyptol and methyl salicylate show good activities in the early and late phases observed with the rat formalin test [18],[19],[20],[21]. As Trois contains more than one active ingredient, it can act through both the central and peripheral mechanisms [22]. The peripheral activity can be attributed to the presence of methyl salicylate, while other constituents like eucalyptol and pinene are responsible for its central action. Additionally, the nanotechnology based micro emulsion may be responsible for improved product penetration, quick onset of action and improved retention of product for both early and late phase pain & inflammation management.

CONCLUSION

Results from all the commercial products studied led to the conclusion that Trois showed positive analgesic activity in the early and late phases of pain. Other commercial formulations (Volini, Moov and Fast Relief) were found to be active only in the late phase of nociception. Since the early phase of pain is associated with obnoxious/noxious stimuli-mediated pain transmitted through central mechanisms it may be concluded that Trois is active in this phase. The late phase corresponds to inflammatory pain and all the formulations showed analgesic activity in this phase, suggesting that these formulations might mediate their analgesic activity through modification of inflammatory mediators. Furthermore, from the experimental outcome it can be concluded that only Trois is effective in reducing the pain intensity in centrally as well as inflammatory pain, as it showed significant reduction in pain intensity during early phase as well as late phase of formalin test. Since pain response latency indicates the onset of pain during early and late phase in formalin test it was concluded that Trois showed highest pain response latency during early as well as late phases of formalin test. Higher pain response latency as compared to other test items i.e. Moov, Volini and Fast Relief may be attributed to fastest onset of action of Trois during early and late Phases. Hence, Trois shall be preferred for treatment of acute pain and inflammation than other marketed formulations.

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