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Influence of ethanol and morphine on pain perception evoked by deep tissue injury

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The aim of this study was to evaluate the effect of ethanol and morphine on nociceptive behavioral responses evoked by the injection of formalin into the temporomandibular joint region of rats (the TMJ formalin test). In experiment 1, animals were given an ethanol solution (6.5%) or tap water to drink for 4 and 10 days, before the procedure for TMJ pain. In the group treated for 4 days, significant analgesia was observed in the TMJ formalin test, whereas the group treated for 10 days did not show this effect, revealing the development of tolerance to ethanol antinociceptive effects. In experiment 2, animals were submitted to chronic regimen of ethanol (6.5% for 10 days) and the control group was given tap water to drink. After this period, morphine (10 mg/kg i.p.) was administrated 30 minutes before the TMJ formalin test. Morphine had the same analgesic effect in both groups, showing that the treatment with ethanol was not able to alter the analgesic potency of morphine. The results showed that ethanol can affect nociceptive behavioral responses related to pain from deep tissues, like the TMJ, and the absence of interaction between ethanol and morphine suggest that ethanol-induced analgesia was mediated by nonopiate mechanisms.

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INTRODUCTION

Ethanol and opioids interfere diferentially with transmission processes in the central nervous system (CNS), affecting many of the known neurotransmitter systems (Vasconcelos *et al.*, 2003). Morphine, the main effective substance in opium, has been used as a potent analgesic in clinical practice and is still the primary analgesic for severe pain. Unfortunately, opioids have adverse side effects, including constipation, nausea and respiratory depression, which limit their use. Moreover, the users readily develop tolerance and dependence to opioids (Ikeda *et al.*, 2002).

Ethanol affects many functions of the CNS, resulting in analgesia, sedation, hypnosis, motor disturbance, memory disturbance, confusion, neurodegeneration, and/

or dependence (Deitrich *et al*., 1989; Fadda, Rossetti, 1998). Ethanol-induced analgesia has been reported in both humans (Cutter, O´Farrel, 1987; Woodrow, Eltherington, 1988) and animals (Bass *et al*., 1978; Brick *et al*., 1976; Pohorecky, Shah, 1987; Yirmiya, Taylor, 1989). Previous research evaluated nociception using typical cutaneous pain models such as tail-flick (Jorgensen, Hole, 1981) and foot-shock (Friedman *et al*., 1980). Our study shows the effects of ethanol and morphine on pain response evoked by deep stimuli, different from the one evoked by superficial stimuli. A particular feature of deep pain conditions is the considerable convergence of cutaneous, visceral, muscle, or joint afferents of spinal as well as trigeminal somatosensory neurons transmitting deep nociceptive information (Sessle, Hu, 1991). Moreover, studies in animals models of persistent pain at spinal levels indicate that peripheral tissue or nerve injury induces a state of hyperexcitability that participates in the development of persistent pain and hyperalgesia (Dubner, 1991; Woolf 1983). In relation to the orofacial region, Iwata *et al.* (1999) demonstrated that TMJ inflammation resulted in more robust changes in medullary dorsal horn nociceptive neurons as compared with perioral inflammation. Thus, considering that the nociceptive behavioral responses elicited by the injection of formalin into the TMJ represent a valid and reliable model of deep orofacial pain (Roveroni *et al*., 2001), the purpose of this study was to evaluate the effect of ethanol and morphine on the pain responses induced by TMJ formalin test. Information on the extent of ethanol-induced changes in nociceptive systems can be of clinical value, especially in individuals who regularly drink alcohol and suffer from some kind of chronic pain conditions, like temporomandibular disorders.

MATERIAL AND METHODS

Animals

Male Wistar rats (obtained from Centro Multi-institucional de Bioterismo-Cemib, Unicamp, Campinas, Brazil) weighing 200-300 g served as subjects. The rats were housed individually and maintained on a 12L:12 D cycle (light onset at 07:00 h) in a temperature-controlled room (23 ± 1 ºC). The study was conducted in accordance with the ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983).

Procedure: Ethanol administration

Rats (*N*=6/group) were given either an ethanol solution, as their sole drinking solution, or tap water for 4 or

10 days (according to experiments). The ethanol drinking solution was mixed to a 6.5% concentration using 99.5% ethanol and tap water (Gatch, Lal, 1999; Bell *et al*., 1998). Pretreatment was carried out in the animals´ home cages. On day 4, one group was taken from the vivarium to the test room and submitted to the TMJ formalin test. In another group, the nociception assay was accomplished 10 days after ethanol or water consumption (control group) to evaluate the development of tolerance to antinociceptive effects of chronic ethanol. Blood ethanol concentrations remain elevated throughout chronic administration when this concentration of ethanol solution is used (Shah *et al*., 1997).

Morphine test

Animals were submitted to chronic regimem of ethanol (6.5% for 10 days) and the control group was given tap water to drink. After this period, morphine 10 mg/kg (Bell *et al*., 1998) was injected IP 30 minutes before the procedure for TMJ pain.

Testing procedure for TMJ pain

The design of this study follows the design used by Roveroni *et al*. (2001). Testing sessions took place between 08:00 and 13:00 h in a quiet room mantained at 23 ± 1 ºC. Each animal was first placed in a test chamber (30 X 30 X 30 cm mirrored-wood chamber with glass at the front side) for a 30 min habituation period to minimize stress (Abbott *et al*., 1986). After the period of adaptation, the animal was removed from the test chamber and lightly anesthetized by inhalation of halothane to allow the TMJ injection.

Rats received a 50 µL injection of diluted formalin (1,5 %) into the left TMJ region. The injections were performed via a 30-gauge needle introduced into the TMJ capsule. A cannula consisting of a polyethylene tube was connected to the needle and also to a Hamilton syringe (50 µL) previously filled with formalin 1.5%. The lower border of the zigomatic arch was palpated and the needle was introduced in direction to the lateral region of condyle (Figure 1).

Following the TMJ injection, the rat immediately recovered from the anesthesia and was returned to the test chamber for a 45 min observation period (15 blocks of 3 min). For each block of 3 min, the behavior characterized by rubbing the orofacial region was quantified by the amount of time that the animal exhibited it and the behavior characterized by flinching the head was quantified by its occurrence (number of head flinches). Considering that the flinching of the head behavior followed a uniform pattern

of 1 s in duration, each flinching was expressed as 1 s. The combination (sum) of both behaviors provides a better measure of pain intensity than any single behavior (Roveroni *et al.*, 2001). An investigator who was blind to the rat´s group assignment made the analysis of the behaviors.

After the conclusion of each experiment, Evans blue dye (1%, 5 mg/kg) was injected systemically in order to confirm the TMJ injection site at post-mortem, as previously described (Haas *et al.*, 1992) by the visual examination of formalin-induced plasma extravasation of Evans blue dye bond to plasma protein.

FIGURE 1 - Punction local for formalin administration.

Drugs

Ethanol (99.5%) was mixed with water for a concentration of 6.5% (v/v). Morphine sulfate was dissolved in isotonic saline and delivered in a volume of 1 mL/100 g of body weight. Evans blue dye was dissolved in isotonic saline for a concentration of 1%. All drugs were obtained from Sigma, São Paulo, Brazil.

Data Analysis

The sum of rubbing and flinching responses exhibited by each animal was computed. Parametric tests [*t*-test and ANOVA (analysis of variance)] were employed, as appropriate. All values are given as mean ± standard deviation (SD). A level of 5% was taken as evidence of statistical significance. Body weight, water and ethanol consumption were analyzed by repeated measures ANOVA and polynomial regression. Data were analyzed using SAS (version 8.2 for windows) by Institute Inc., Cary, NC, USA-licensed to Universidade Estadual de Campinas.

RESULTS

Body Weight and Fluid Consumption

Figure 2 presents body weight and fluid consumption over the course of 4– day access to 6.5 % ethanol. Although there was a significant increase in body weight over days [F=18.06, $p<0,0001$ (Figure 2A)], there was no effect of ethanol treatment on body weight (F=0.01, $p=0.9095$) and no significant interaction between days and treatment (F=1.73, $p=0.1825$). There was no effect of ethanol treatment on fluid consumption (F=2.29, $p=0.1613$) and no significant interaction between days and treatment [F=0.11, $p=0.9562$ (Figure 2B)].

Figure 3 presents body weight and fluid consumption over the course of 10–day access to 6.5 % ethanol. Although there was a significant increase in body weight over days [F=79.06, $p<0,0001$ (Figure 3A)] there was no effect of ethanol treatment on body weight (F=0, $p=0.9533$) and no significant interaction between days and treatment (F=0.86, $p=0.5633$). There was no effect of ethanol treatment on fluid consumption (F=0.59, $p=04585$)
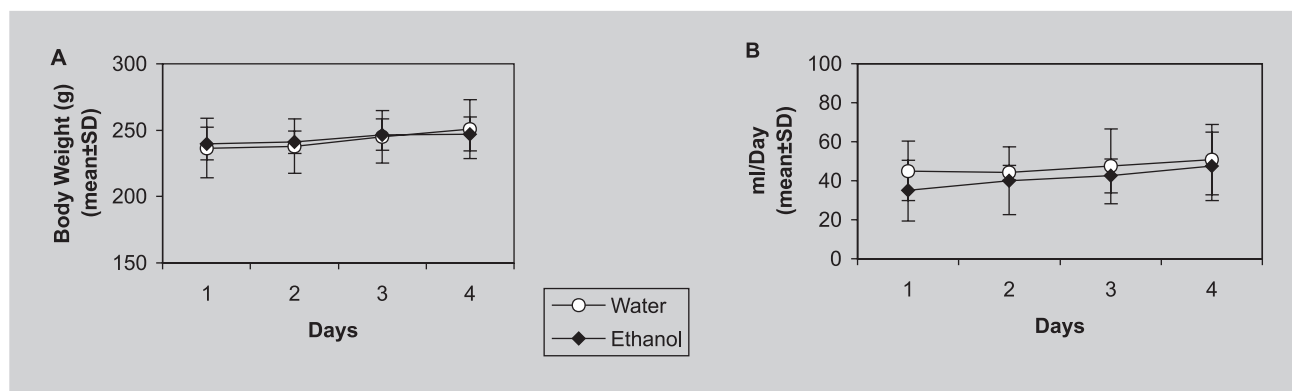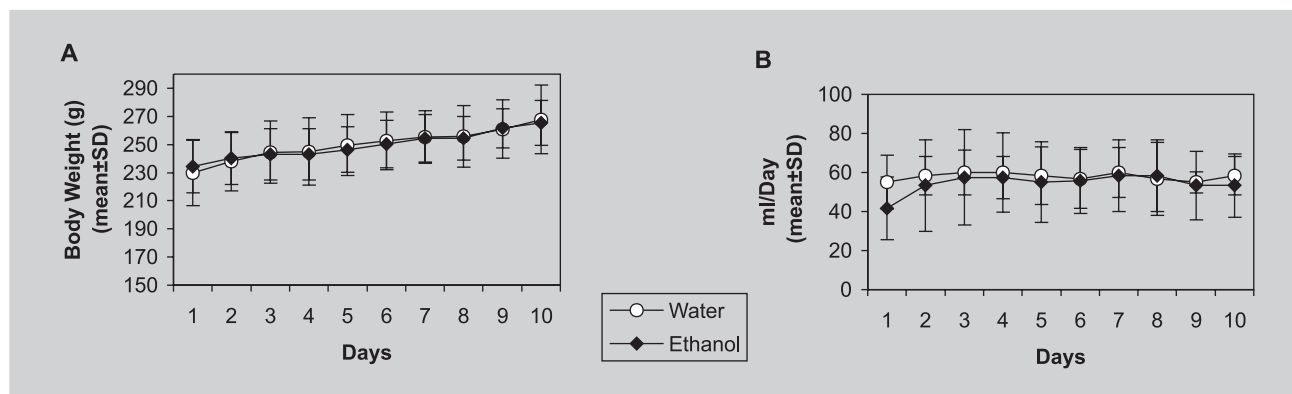

FIGURE 2 - The effect of ethanol drinking on body weight and fluid consumption. Rats had forced access (24 h/day) to water or ethanol (6.5 % v/v) for 4 days. **A** - mean ± SD daily body weight (*N*=6/group). **B** - the amount of water and ethanol consumption per day is presented as mL/day.

FIGURE 3 - The effect of ethanol drinking on body weight and fluid consumption. Rats had forced access (24 h/day) to water or ethanol (6.5 % v/v) for 10 days. **A** - mean ± SD daily body weight (N=6/group). **B** - the amount of water and ethanol consumption per day is presented as mL/day.

and no significant interaction between days and treatment [F=0.21, p=0.9930(Figure 3B)].

Effects of Chronic Ethanol Administration

The antinociceptive effects of ethanol (6.5% in drink solutions) were apparent by the fourth day of exposure to ethanol solution. The reduction of pain responses was statistically significant (p< 0.001, t-test; Figure 4) when the control group (219.48 ± 40.148) was compared with the experimental group (122.36 ± 45.48).

By day 10, the sum of nociceptive behaviors in the TMJ formalin test was not significantly different between control X test group, revealing that tolerance to the antinociceptive effects of ethanol has been developed

(p=0.98, t-test, Figure 4). The values of control group (171,07 ± 39,35) were comparable with the test group (170,71± 43,15; Figure 5).

Morphine test

Analysis of simple main effects of baseline behavior (formalin) vs. postdrug behavior (formalin + morphine) revealed that morphine produced significant analgesia in both groups [water pretreatment: baseline (171.07 ± 39.24) vs. postdrug (13.96 ± 19.68); ethanol pretreatment: baseline (170.71 ± 43.146) vs. postdrug (14.29 ±10.79) – p<0.001 Oneway ANOVA + Tukey, Figure 6]. No significant differences were found between postmorphine behaviors, indicating that the rats did not develop cross-tolerance
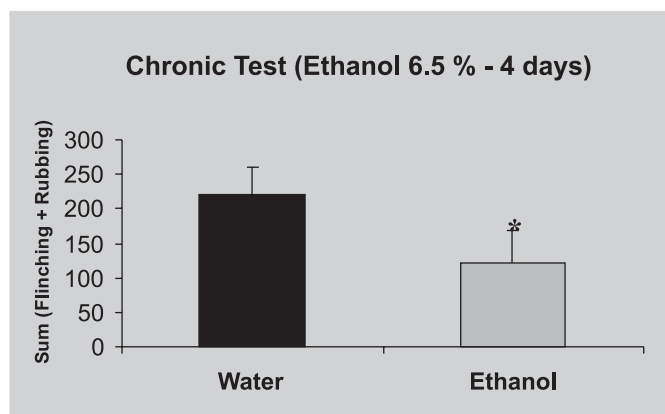

FIGURE 4 - Sum of nociceptive behaviors in the TMJ formalin test after 4 days of drinking an ethanol solution (N=6) or water (N=6). Each column represents the mean. Errors bars indicate the SD. Single asterisk indicates significant difference between the experimental and water control group (p< 0.001, t-test).
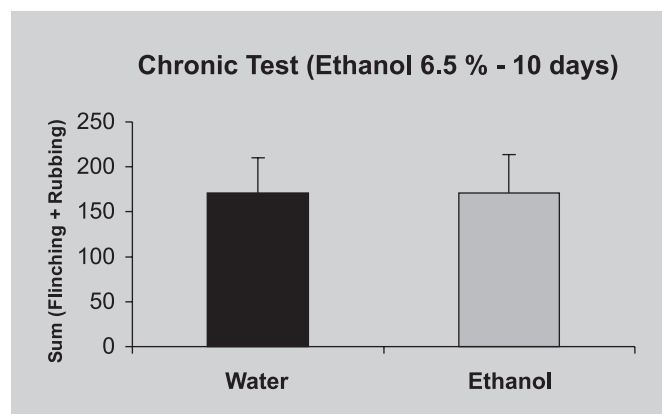

FIGURE 5 - Sum of nociceptive behaviors in the TMJ formalin test after 10 days of drinking an ethanol solution (N=6) or water (N=6). Each column represents the mean. Errors bars indicate the SD. No significant differences were found in pain responses for animals pretreated with ethanol vs. water (p=0.98, t-test).
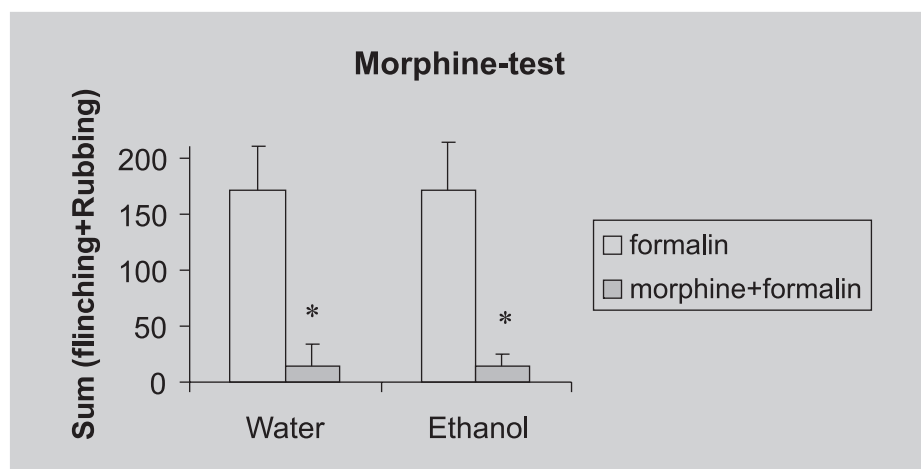
FIGURE 6 - Analgesic effect of morphine (10 mg/kg, IP) in the TMJ formalin test after ten days of drinking an ethanol solution (*N*=6) or tap water (*N*=6). Data are expressed as the mean of the sum of nociceptive behaviors SD. * indicates significant differences from baseline (formalin) nociceptive behaviors.

between ethanol-induced analgesia (EIA) and morphine-induced analgesia (MIA).

DISCUSSION

Alteration in the perception of pain can be produced by either environmental or pharmacological manipulations. In an early study, ethanol was found to attenuate naloxone-induced hyperalgesia (Bass *et al*., 1978), suggesting a possible interaction between EIA and the endogenous opioid systems. Ethanol-opiate interactions have also been addressed by examining cross-tolerance between EIA and MIA. Jorgensen and Hole (1984) found that neither tolerance to EIA nor cross-tolerance to MIA developed in rats injected with 2.5 g/kg ethanol for 8 days. However, tolerance to EIA accompanied by cross-tolerance to MIA was found if the animal were exposed to the analgesic test (i.e., tail-flick) during tolerance induction (Jorgensen *et al*., 1986). This tolerance is called "associative" or "environment-dependent" tolerance. Although some studies have shown that tolerance to ethanol's effects can be reduced or eliminated if animals are tested in an environment that is different from that which was used to induce tolerance (Jorgensen, Hole, 1984; Gatch, Lal, 1999; Jorgensen *et al*., 1986), Bell *et al*. (1998) showed that tolerance to antinociceptive effect of ethanol can be induced in rats using both non-associative (self-administration) and associative (IP injections) paradigms. They also support that EIA was nonopioid in nature, as neither form of ethanol tolerance was accompanied by cross-tolerance to MIA. Inversely, Shah *et al*. (1997) demonstrated that ethanol consumption decreases the analgesic potency of opioids in mice through a mechanism that is unrelated to pharmacokinetics or opioid receptor changes in brain and cord. The study of Duttaroy *et al*. (1998) showed that chronic forced ethanol drinking can decrease the analgesic potency of opioid agonists in mice, and this effect may be produced via alterations in G-protein-coupled intracellular mechanisms. The reason for these discrepancies should be related to a number of factors including species-related differences (Boada *et al*., 1981; Fidecka *et al*., 1986; Jorgensen, Hole, 1981), and the test used to measure opiate-ethanol interactions (Khanna, Lê, 1996). Our data show that tolerance to EIA was induced by pharmacological rather than behavioral factors, because the tolerance induction procedure was carried out in the rat's cages and the TMJ formalin test in another room, after a habituation period to minimize stress (Abbott *et al*., 1986). Furthermore, the results of this study do not support an ethanol-opiate interaction for EIA, as ethanol tolerance was not accompanied by cross-tolerance to MIA, indicating that ethanol's analgesic effects on TMJ formalin test is mediated by nonopiate mechanisms.

The mechanism though which ethanol produces antinociception is not clear; in fact, more than one mechanism could be involved. Ethanol has NMDA antagonist properties as supported by electrophysiological (Lovinger *et al*., 1989), biochemical (Hoffman *et al*., 1989), and behavioral studies (Kosten, Bombace, 2001). Grant (1999) reported that ethanol can also act as positive modulator of $GABA_A$ receptors, enhancing GABA-activated flow of Cl^-; lower training doses of ethanol can enhance the 5HT-effect on 5HT1B/2c receptors, which are situated basically in inhibitory interneurones. Gruss *et*

al. (2001) suggested that the activation of large-conductance calcium-activated K+ channels (BK (Ca) channels) induced by clinically relevant concentrations of ethanol might contribute to ethanol-induced analgesia. Ethanol inhibits current responses mediated by ATP receptor channels designated as P2X receptors (Li *et al*., 1994; Weight *et al*., 1994). Among the various targets of ethanol, G protein-activated inwardly rectifying potassium (GIRK) channels are the only molecules whose involvement in ethanol-induced analgesia has been demonstrated in vivo (Ikeda *et al*., 2001).

Since most of the molecules mediating ethanol effects are the same associated with the modulation of pain arising from deep tissues, for example, $GABA_A$-R (Kenji *et al*., 2001), GIRK (Ikeda *et al*., 2001), NMDA receptors (Yu *et al*., 1996), 5HT-receptors (Garraway, Hochman, 2001) and ATP-R (Hu *et al*., 2002), its possible that ethanol can influence nociceptive mechanisms related to deep tissue injuries, which have characteristics different from those related to cutaneous injuries (Sessle, Hu, 1991).

In the present study, we also used systemic morphine administration to evaluate the interaction between ethanol and opioids on the pain perception evoked by TMJ formalin test. Morphine significantly reduced the formalin-induced rubbing and flinching responses at a concentration of 10 mg/kg, the same used in the study of Bell *et al*. (1998). The analgesic potency of morphine was the same in animals pretreated either with water or ethanol, revealing that ethanol was not able to decrease the effectiveness of morphine. The absence of cross-tolerance between EIA and MIA suggest that the mechanisms of ethanol's analgesic effects are nonopiate in nature on the TMJ formalin test.

The formalin test (Roveroni *et al*., 2001; Dubuisson, Dennis, 1977) is a commonly used model of tonic and inflammatory pain. Because it models naturally-occuring painful stimuli, it is more relevant to clinical pain and more sensitive to the effects of analgesic drugs than phasic pain models such as the hot-plate or tail-flick (Hammond, 1989). One of the characteristics of the formalin response is its biphasic pattern (Abbott *et al*., 1995; Clavelou *et al*., 1995). Whilst the first phase formalin response is mediated by direct afferent stimulation, the second phase behavioral response is dependant on a central hyperexcitability of the recipient second-order dorsal horn neurons (Martindale *et al*., 2001). Thus, considering that the TMJ formalin test evaluate the later response (Roveroni *et al*., 2001), we suggest that ethanol's effects on this test are centrally-mediated.

In conclusion, our results show that ethanol can affect nociceptive behavioral responses related to pain from deep tissues, like the TMJ, and the absence of interaction between ethanol and morphine suggest that ethanol's analgesic effect on the TMJ formalin test is mediated by nonopiate mechanisms.

RESUMO

A influência do etanol e da morfina sobre a percepção dolorosa provocada por injúria tecidual profunda

O objetivo deste estudo foi avaliar o efeito do etanol e da morfina sobre as respostas comportamentais nociceptivas provocadas pelo teste da formalina na ATM de ratos (Teste da formalina na ATM). No experimento 1, os animais receberam uma solução de etanol 6,5 % ou água comum para beber durante 4 e 10 dias, antes da realização do teste da formalina na ATM. No grupo tratado por 4 dias, observou-se analgesia significativa ao teste da formalina, enquanto que no grupo tratado por 10 dias esse efeito não ocorreu, demonstrando o desenvolvimento de tolerância aos efeitos antinociceptivos do etanol. No experimento 2, os animais foram submetidos ao regime crônico de etanol (6,5% por 10 dias) e o grupo controle recebeu água comum para beber. Após esse período, foi administrado morfina (10 mg/kg i.p.) 30 minutos antes da realização do teste da formalina na ATM. A morfina teve o mesmo efeito analgésico nos 2 grupos, demonstrando que o tratamento com etanol não foi capaz de alterar a potência analgésica da morfina. Os resultados mostraram que o etanol é capaz de alterar as respostas nociceptivas relacionadas à dor proveniente de tecidos profundos, como a ATM, e a ausência de interação entre o etanol e a morfina indica que a analgesia induzida pelo etanol é mediada por mecanismos não-opióides.

UNITERMOS: Etanol. Morfina. Teste da formalina. Articulação temporomandibular.

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