# ANALGESIA ENHANCEMENT AND PREVENTION OF TOLERANCE TO MORPHINE: BENEFICIAL EFFECTS OF COMBINED THERAPY WITH OMEGA 3 FATTY ACIDS

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**Abstract**

**Objectives**: Recent evidence associates omega-3 fatty acids (O3) with pain reduction. The aim of this work was to evaluate the antinociceptive effect of fish oil as a source of O3, either alone or in combination with morphine after acute and chronic administration in rats. As well, a new pharmaceutical mixture which allows the concomitant administration of fish oil and morphine as an oral solution was developed.

**Methods**: Animals were fed on a control or an experimental diet supplemented with O3. The animals were subjected to the hot-plate test in order to assess analgesic effect and tolerance to the analgesic effect of morphine. The open field test was carried out to determine if the differences in the response latency can be related to non-specific sedative effects.

**Key Findings:** O3 dietary supplementation for 16 or 30 days increased the response latency compared with the control group. Acute treatment with morphine in these groups resulted in an additive antinociceptive effect not related to locomotor activity. Chronic coadministration of morphine with O3 attenuated the development of tolerance to morphine. Oral administration of the new pharmaceutical mixture showed analgesic activity even with a subtherapeutic dose of morphine.

**Conclusion**: This finding suggests a possible role for omega-3 fatty acids as adjuncts to opioids in pain therapy and might contribute to the reduction of the occurrence of some morphine side-effects.

**Keywords:** omega-3 fatty acids; fish oil; morphine; hot-plate test; analgesia; tolerance

**Abbreviations:** Omega-3 fatty acids (O3), docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), alpha-linolenic acid (ALA), acid-sensing ion channels (ASICs), such as ASIC1a, ASIC1b and ASIC3 and the transient receptor potential vanilloid 1 (TRPV1), morphine (MOR), $NH_4OH$ (ammonium hydroxide), sterile saline solution (SAL), subcutaneously (SC), orally (OR), naloxone (NAL).

## INTRODUCTION

The treatment of acute and chronic severe pain remains a major but common challenge faced by clinicians working with the general population. Chronic pain is a factor that negatively affects the quality of life, especially among the elderly, for whom increased life expectancy also increases the risk of developing chronic pain secondary to degenerative diseases or various types of tumors. [1]

Morphine (MOR), as the hydrochloride or sulphate salts, is one of the most used opioids for acute and chronic control of moderate to severe pain. [2, 3] Adverse effects frequently observed in patients receiving MOR during chronic pain treatment are constipation, myoclonus, nausea, vomiting, sedation, body weight loss, drowsiness and pruritus. [4] It may also cause loss of analgesic efficacy (tolerance) or the activation of pronociceptive mechanisms leading to increased pain sensitivity (hyperalgesia) [5, 6] These effects may require the discontinuation of MOR treatment which results in inadequate pain control. Successful pain management with MOR requires that adequate analgesia be achieved with a minimum of adverse effects, but a substantial minority of patients (10% to 30%) suffers from excessive adverse effects, inadequate analgesia, or a combination of both events. The management of excessive adverse effects is still a major challenge for clinicians. [7]

Pain, in physiological and pathological conditions, may be managed with drug treatment, as well as with a nutritional strategy or using dietary supplements. [8] Omega-3 fatty acids (O3), in the form of fish oil, are probably the best example of how diet may be a therapeutic option for the treatment of a variety of diseases. [9, 10] The three types of O3 involved in human physiology are alpha-linolenic acid (ALA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).

O3 have anti-inflammatory properties [11, 12]; EPA and DHA, for example, are precursors of potent anti-inflammatory lipid mediators such as resolvins and protectins. [13] In clinical and preclinical studies, O3 intake contributed to the reduction of inflammatory pain associated with rheumatoid arthritis, [14] neuropathic injury, [15] musculoskeletal injury, [16] inflammatory bowel disease, [17] and dysmenorrhea. [18] Other preclinical studies revealed the antinociceptive effect of different types of O3. [19-21] A recent study suggests that the antinociceptive effects of O3 may involve an opioidergic system. [22]

Because of their antinociceptive properties, the use of MOR associated with O3 could offer a new avenue for the prevention and treatment of chronic pain disorders, allowing the improvement of the clinical effectiveness of MOR.

At present, no studies have been reported on the antinociceptive effects of the combination of MOR and O3.Therefore, The objective of this work was to assess: (1) the antinociceptive effect of O3, either alone or in combination with MOR acutely administered via a subcutaneous (SC) or oral (OR) route, (2) the influence of the pretreatment with the opiate antagonist naloxone (NAL) on the antinociceptive effect of O3, either alone or in combination with acutely administered MOR, and (3) the effect of O3 on MOR-induced tolerance to antinociception. Also, we developed a pharmaceutical mixture containing MOR: O3 that can be used as a base to obtain oral formulations.

## MATERIALS AND METHODS

### Materials

MOR hydrochloride (pharmaceutical grade) was purchased from Laboratorio Verardo, Buenos Aires, Argentina. NAL was purchased from Sigma (St Louis, MO, USA). MOR and NAL were dissolved in sterile saline solution (SAL). SAL was used as a control. Fish oil (Laboratorio Saporiti, Buenos Aires, Argentina) was used as the source of O3.

### Animals and diets

Adult male Wistar rats weighing 200–350 g at the beginning of the experiment were used. The animals were maintained on a 12 hour light (08:00–20:00 h) -12 hour dark cycle, with free access to food and water, except during testing. They were housed in groups of three, in individual polyethylene cages (55 x 38 x 30 cm). Rat weights were recorded before and at the end of the experiments. Animals were used only in one experiment. No inflammation or any pathological condition was observed during the experiments. All studies described were conducted in accordance with the Guide for Care and Use of Laboratory Animals provided by the National Institutes of Health, USA and AVMA Guidelines for the Euthanasia of Animals, 2013 Ed. The study obtained the clearance from the Ethics Committee for the care and use of Laboratory Animals of the Universidad Nacional de La Rioja, Argentina.

The doses of MOR and O3 used in this experiment were based on previous studies in rats. [23, 24]

Each morning the rats were fed with a control (20% casein) or an O3 diet. The O3 diet was prepared each day by adding 4% salmon oil to the control diet (12 mg of O3 per 1 g of the diet). Each 1000 mg of concentrated salmon oil contained approximately 30% w/w O3, of which 17% is EPA and 13% is DHA. Both diets were equivalent in terms of overall fat,

protein, carbohydrate and caloric content. Table 1 details the components of the diets and their content of fatty acids.

Daily intake of food per rat was calculated by subtracting the food remaining in each cage from the amount given and dividing it by the number of rats per cage.

Rats were given an average of 20 g of food each day regardless of their assigned diet. For the rats in the O3 condition, this amount of food contained a total of 240 mg of O3. Therefore, rats in the O3 group received a dose of approximately 720 mg/kg/day O3 (408 mg of EPA and 312 mg of DHA) at the time of testing.

**Preparation of a pharmaceutical mixture containing MOR:O3 for oral administration**

MOR was obtained by neutralizing MOR hydrochloride with $NH_4OH$ (proanalysis, Cicarelli ®). Pure MOR crystals were obtained as monohydrated zwitterions which were insoluble in salmon oil. MOR freely dissolved in salmon oil after processing it according to Argentine Patent Application P20120100854 [25]. A dose of this pharmaceutical mixture containing 12 mg of MOR/kg and 720 mg of O3/kg was orally administered to the rats as shown in the protocol for the experiment IV. The oral dose of MOR was selected because Iwamoto and Klaassen [23] showed that it results in sub-effective levels since only about 18% of MOR dose reached the plasma in rats. In the case of MOR, a therapeutic dose was defined as the dose required to produce the desired therapeutic effect (analgesia). The dose of O3 was selected from reference 26 which shows the doses that were effective in reducing pain in humans. The dose translation to animal was based on the recommendations of the International Union of Basic and Clinical Pharmacology. [27]

**Pharmacological treatments and experimental groups**

The following experiments were carried out:

*Experiment I:* The antinociceptive effect of chronic dietary O3 supplementation, either alone or in combination with acutely injected MOR.

*Experiment II:* The effect of NAL on antinociception produced by chronic dietary O3 supplementation, either alone or in combination with acutely injected MOR.

*Experiment III:* The effect of chronic dietary O3 supplementation on MOR-induced tolerance to antinociception.

*Experiment IV:* The antinociceptive effect of the orally administered pharmaceutical mixture containing MOR: O3, after chronic dietary O3 supplementation.

Table 2 describes the pharmacological treatments and the experimental groups, whose nomenclature is used throughout this article.

The subcutaneous doses of MOR (1, 2.5, 5, 6 mg/kg) and NAL (1 mg/kg) were chosen according to previous studies. [28, 29] The dose of 1 mg/kg of MOR was chosen to test the antinociceptive activity in the hot-plate test since it does not exert a significant antinociceptive effect when given alone [28]. Such dose seemed, therefore, to be the most suitable way to assess an eventual potentiation effect when coadministered with O3.

In experiment III, MOR tolerance was induced using the protocol described in figure 1a and b in accordance with previous studies [30]

In experiment IV, 2.4 mg of salmon oil/kg (0.05 ml of fish oil equivalent to 39.3 mg of salmon oil) was administered orally to the O3 group. Therefore, the rats received a dose of 720 mg/kg/day. [24] All the solutions were administered via oral gavage in a volume up to 20 ml/kg.

For all the experiments, the animals were subjected to the hot-plate test to assess the antinociceptive effect which was calculated as the percentage of the response latency of each group with respect to that of the C–SAL group. Besides, it is known that MOR produces a dose-dependent decrease in locomotor activity due to non-specific sedative effects. [31] The open field test was carried out to determine if the differences in the response latency observed in the hot-plate test were related to changes in locomotor activity.

**Hot-plate test**

The hot plate consisted of an electrically heated surface (Socrel DS-35, Ugo Basile, Comerio, VA, Italy) kept at a constant temperature of 54 ± 0.8 $^{o}$C. In the test, the rats were kept inside a circular transparent plastic cage on the hot plate. Licking paws or jumping was considered as a sign of thermal nociception. The time to the first reaction (response latency) was recorded for each animal. If the animals did not respond within 45 s (cut-off time), they were removed from the plate to avoid tissue damage. All the assays were done 24 h after 16 or 30 days of treatment with diet (Control or O3), and antinociception was assessed 30 min after subcutaneous or 60 min after oral administration of MOR and MOR: O3 pharmaceutical mixture. [30, 32] The results were expressed as the percentage of response latency compared with the C-SAL group.

**Open field test**

Spontaneous motor activity (total locomotion) was assessed. The apparatus for the open field test consisted of a black, square open field (60 x 60 cm) with the floor divided into squares (15 × 15 cm) by means of white lines. Testing was performed between 14:00 and 17:00 h, illuminated with a 75 W electric bulb, hanging 75 cm above it, in a quiet room. During all the experiments, the laboratory room was dark. Fifteen minutes after administration of MOR, the animals were gently placed in the center of the open field arena, and allowed to explore freely; their locomotion was measured by the number of squares entered with all four paws (counts) during a period of 5 min. After each animal was removed, the open field was carefully cleaned with a damp cloth. The behavior was scored by an observer who was unaware of the experimental procedures previously performed on the animals and the results were expressed as mean ± SE.

**Statistical analyses**

Data were expressed as mean ± standard error (SEM). ANOVA models were used to evaluate the differences of hot plate and open field outcomes among treatments. Bonferroni tests were used to compare the means, considering a significance level of $\alpha=0.05$ ($p<0.05$), with different letters indicating significant differences. Analytical probes were performed using the InfoStat 2012 software.

## RESULTS

### 1. Combined treatment with O3 and MOR solution produced an additive antinociceptive effect

Antinociceptive effects observed in the hot-plate test after administration of MOR solution (1, 2.5, 5 or 6 mg/kg) and/or dietary supplementation with O3 are shown in figure 2. Consistent with previous observations, C-MOR$_{SC\ (2.5)}$, C-MOR$_{SC\ (5)}$ and C-MOR$_{SC\ (6)}$ groups produced a dose-related antinociception [$F(4, 69) = 46.19$; $P < 0.01$] 30 min after injection. [33, 34] In agreement with Hernández-Delgadillo (2003) [28], no analgesia was achieved at a dose of 1 mg/kg of MOR solution (C-MOR$_{SC\ (1)}$).

As can be observed in figure 2a, dietary supplementation with O3 for 16 days increased the response latency (56.25% analgesia) on day 17 compared with the C-SAL group [$F(1, 66) = 91.98$; $p < 0.01$]. Interestingly, acute treatment with MOR solution (1, 2.5, 5 and 6 mg/kg) following 16 days of dietary supplementation with O3 had a greater antinociceptive effect [$F(1, 66) = 91.98$; $p < 0.01$] than MOR solution alone at the same dose, rendering an additive effect (treatment x diet interaction: $F(1, 55) = 3.78$, $p < 0.05$).

Doses of 1, 5 and 6 mg/kg of MOR failed to modify locomotor activity in animals fed with both, control or O3 supplemented diets [$p > 0.05$]. However, locomotor activity increased at a dose of 2.5 mg/kg of MOR [$F(1, 45) = 4.57$; $p < 0.01$], but remained unaltered by the combined treatment with O3 (figure 2b).

**2. Naloxone was able to antagonize the antinociceptive effect of O3 when given alone or in combination with MOR**

The opioid receptor antagonist NAL was used to assess if O3 have an opiod receptor-mediated pain control activity. The influence of NAL treatment on the antinociceptive effects produced by the administration of MOR solution or O3 dietary supplementation (16 days) is shown in figure 3a. The presence of NAL significantly prevented the antinociceptive effect on the C-NAL-$MOR_{SC\ (5)}$ and O3-NAL groups [$F(1, 57) = 21.39$; $p < 0.01$]. A similar result was obtained in the O3-NAL-$MOR_{SC\ (5)}$ group [$F(3, 57) = 40.65$; $p < 0.01$]. None of the treatments affected motor activity levels in the open field chamber (figure 3b).

**3. O3 prevent MOR tolerance**

In experiment III, when the rats were challenged with 5 mg/kg MOR in the hot-plate test, the analgesia observed in C-$MOR_{chronic}$ group was 138.0% compared to C-SAL group. A significant reduction in the response latency was observed in the rats treated chronically with MOR, which became tolerant and exhibited a response latency of only 37.6% (figure 4a). In contrast, the response latency increased to 90.62% and 183.98 % in O3-$MOR_{chronic}$ group at 16 and 30 days, respectively (figures 4a and 5a). These results demonstrated that O3 attenuated the development of tolerance to MOR analgesia in a time supplementation-dependent manner.

None of these treatments affected the activity levels in the open field chamber (figures 4b and 5b).

At the end of the treatment period, the C-SAL group gained 2.37% of body weight compared to its initial value. The C-$MOR_{chronic}$ group showed a decrease in body weight of 3.1% but the O3-$MOR_{chronic}$ group lost only 1.78% (16 days) (figure 4c). Interestingly, the O3-$MOR_{chronic}$ group (30 days) showed a slight increase in body weight [$F(1, 12) = 5.51$, $p < 0.01$] (figure 5c).

**4. Pharmaceutical mixture containing MOR:O3 at sub-therapeutic doses produced analgesia**

Analgesic activity results are shown in Figure 6a. The O3-MOR: $O3_{OR}$ group showed a significantly greater antinociceptive effect [$F(1, 66) = 91.98$; $p < 0.01$] than the O3-SAL and C-$MOR_{OR}$ group at the same doses (720 mg O3/kg and 12 mg MOR/kg). On the other hand, the C-MOR: $O3_{OR}$ group failed to augment the latency period. None of the treatments affected motor activity levels in the open field chamber (figure 6b).

## DISCUSSION

The main findings in this study are as follows. First, we have demonstrated that O3 by themselves produced analgesia after 16 days of dietary supplementation in rats. Second, O3 increased the analgesic effect of acute MOR. Third, these fatty acids attenuated the development of tolerance to MOR analgesia.

In this study, we used the hot-plate test, one of the oldest [35] and most widely used experimental methods to assess nociception in rats and mice. [35] It measures the complex response to a non-inflammatory, acute nociceptive input and is considered a valid model for the study of central antinociceptive activity. [29]

Dietary manipulation may influence pain perception of acute and chronic nociceptive stimuli. [36-40] The O3 perform a variety of functions in the body, including the regulation of immune and inflammatory responses. There is evidence that the levels of these fatty acids may be involved in human diseases such as arthritis and other inflammatory conditions. [41] Yehuda and Carasso [37] have reported that some O3 such as ALA can have an effect on analgesic response.

To analyze the O3 effects on analgesia, the diet composition, the O3 dose and the duration of the treatment should also be considered. There has been much research on the role of the acute administration of O3 before the antinociceptive test [20, 22, 42]; however, there is sparse evidence about the effectiveness of chronic administration of O3 in attenuating pain in experimental animals. [21, 37] For instance, the treatment of rats for 2-4 weeks with ALA, the precursor of O3, had an antinociceptive effect in the hot-plate test. [37] Besides, Veigas *et al.* [21] demonstrated that there was an increased response to thermal nociception in mice fed on concentrated fish oil (46.5% EPA and 37.5% DHA) for 6 months. Contrary to expectations, no analgesic effects (18% EPA and 12% DHA) were observed in mice fed on regular fish oil. The results of the present study show that the supplementation with O3 salmon oil (17% EPA and 13% DHA) had a significant antinociceptive effect after 16 days of administration. In addition, the increased response to thermal nociception through this treatment with O3 can be considered specific since it is not attributable to changes in locomotor activity. To our

knowledge, this is the first report about the antinociceptive effect of O3 after 16 days of supplementation in male rats using salmon oil (EPA plus DHA).

The results show that NAL blocked the analgesia produced by O3. These observations are in agreement with Nakamoto *et al.* (2010), [42] who reported that the antinociceptive effect of DHA is inhibited by NAL. Furthermore, the analgesia produced by the combination of O3 and MOR is inhibited by NAL, suggesting that the analgesic activity of both might be exerted via a mechanism related to the opioid system. Because NAL is a non-selective opiate antagonist, more studies using peripheral and mu- or delta-selective antagonist administration are necessary to better understand the mechanism of action of O3 and its combination with MOR. The data obtained in this experiment suggest that NAL blocks the actions of endogenous opioid peptides. Further experiments aimed to determine the endogenous opioid levels are needed.

In this study, we demonstrated that the combination of O3 (720 mg/kg) with any of the MOR doses produced dose-related antinociception, and that chronic O3 supplementation in combination with acute MOR doses (1, 2.5, 5 and 6 mg/kg) had significantly higher antinociceptive effects than individual treatments, showing an additive effect.

Opioid receptor stimulation with MOR treatment affects locomotor activity, producing an inverted U-shaped dose-response curve. Locomotor activity was significantly increased in animals treated with a single dose of MOR (2.5 mg/kg), which is consistent with previous observations. Specifically, Halladay et al observed that 2.5 mg/kg of MOR increased locomotor activity, while 10.0 mg/kg MOR decreased it. [43]

However, no locomotor effects were observed after O3 treatment alone or in combination with an acute MOR dose. Thus, locomotor effects cannot account for the analgesia observed in the hot plate for the O3-$MOR_{SC}$ groups. To the best of our understanding, this is the first report on the additive effect of the combination of O3 supplementation with acute MOR.

The use of opioids is associated with a number of side-effects [4-6]. Co-administration of a non-opioid substance has been proposed as a method for reducing opioid intake and minimizing side-effects. Such a reduction might result in fewer opioid-related side-effects, but the potential for additional side-effects due to the adjunct drugs must also be considered. Results from clinical studies demonstrated that many different classes of drugs can serve as effective adjuncts to opioids for the treatment of pain. These include non-steroidal antiinflammatory drugs (NSAIDs such as ibuprofen), [44] acetaminophen, [45] clonidine, [46] antidepressants (such as desipramine), [47] and anticonvulsivants. [48] For many patients, the proper use of adjuncts will improve analgesia and could also allow a reduction of the dose of

opioids. This would therefore attenuate opioid-induced adverse reactions such as nausea, vomiting, constipation, pruritus, sedation, and respiratory depression. [49] While some of the most common adjuvant analgesics can have unwanted side-effects and cause drug interactions, O3 are free from any significant adverse effects. [26]

The novel finding regarding O3 (EPA plus DHA) is that after 16 and 30 days of treatment, O3 attenuated or blocked the development of tolerance to MOR in male rats. Also, with the treatment with O3 (16 or 30 days), we did not observe any locomotor effects on the development of tolerance seen with chronic MOR administration.

With respect to body weight measures, we observed weight loss in animals treated with chronic MOR compared to the control group, which matches previous observations. [50]

In contrast, the lower decrease in body weight observed in animals treated with MOR in combination with O3 could also represent an advantage if used in combined treatment.

The new pharmaceutical mixture obtained by dissolving MOR in salmon oil allowed the concomitant oral administration of both components. The mentioned mixture has an important advantage since it showed analgesic activity after acute oral administration with a dose of MOR previously described as ineffective in rats (12 mg/kg). [23] In fact, the pharmaceutical mixture has a significantly higher analgesic effect than O3 alone, suggesting that the O3 analgesic effect can be enhanced by inactive doses of MOR.

To sum up, this study provides evidence to support the hypothesis that this pharmaceutical mixture may help reduce the doses of MOR, and in consequence the magnitude of their side-effects. Interestingly, processed MOR [25] is freely soluble in salmon oil and no precipitation was observed after 3 months at room temperature (data not shown). Besides, because salmon oil is the main component, it can be used as a base to obtain soft capsules for oral administration.

Despite many reports on the analgesic effects of O3, the mechanisms are not fully understood. Some reports about the mechanisms underlying the antinociceptive effect of O3 suggest:

1) inhibition of the production of pro-inflammatory eicosanoids and cytokines via the suppression of the arachidonic acid cascade; [51-54]

2) analgesic action of the O3-derived mediators. Recent studies have demonstrated that EPA and DHA act/serve as precursors for E-series (RvE1, RvE2) and D- series (RvD1, RvD2) resolvins respectively, which are potent analgesics; [55-59]

3) regulatory action on both peripheral and central transient receptor potential vanilloid 1 (TRPV1) for O3. [60] The acid-sensing ion channels (ASICs) such as ASIC1a, ASIC1b and

ASIC3 and the TRPV1 have been implicated in pain perception.[61-63] However, recent studies have demonstrated that fish oil decreased the mRNA expression of ASIC1a, ASIC3 and TRPV1, suggesting a reduced inflammatory status, which may be the reason for the increased pain threshold; [21]

4) Increasing the release of β-endorphin. Nakamoto *et al.* (2011) [22] demonstrated that the DHA facilitated the release of β-endorphin and that the antinociception produced is mediated by the stimulation of μ- and δ-opioid receptors.

5) Analgesic action of the epoxidized metabolites derived from O3. DHA and EPA are metabolized by cytochrome P450. The metabolites produced, epoxy docosapentaenoic acid (EpDPE) and epoxy eicosatetraenoic acid (EpETE), have a direct antinociceptive role [64].

Although the present studies were designed only to determine if O3 and MOR produce beneficial analgesic effects in behavioral studies, this work opens the door to more detailed neurobiological and molecular studies that can identify and characterize their mechanisms of action.

This study demonstrated the existence of a significant additive effect of the combination of chronic O3 supplementation with acute MOR dosage. Additionally, O3 prevented the development of MOR tolerance.

The present findings regarding O3 dietary supplementation and its combination with chronic MOR are of clinical interest since opioids are still a mainstay for the treatment of moderate to severe pain despite their significant side-effects. The combination of O3 with MOR is expected to decrease these side-effects and minimize the development of tolerance. Overall, our data may lead to important consequences in medical practice and in the development of new treatment strategies for pain relief.

## CONCLUSIONS

This study demonstrated the existence of a significant additive effect of the combination of chronic O3 supplementation with acute MOR dosage. O3 could also reduce the possibility of developing tolerance to MOR. The new pharmaceutical mixture obtained can be used to develop soft capsules containing MOR and salmon oil for oral administration.

These data might contribute to new therapeutic approaches and may mean higher response rates and lower side-effects associated with MOR treatment.

More studies are required to understand the action mechanism underlying the use of the combination of MOR and O3.

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551

## FIGURE LEGENDS

**Figure 1**: Experimental protocols to evaluate MOR tolerance after 16 days (a) or 30 days (b) of O3 fatty acids supplementation.

**Figure 2**. Antinociceptive effect (in the hot-plate test) and locomotor activity (in the open-field test) after 16 days of O3 dietary supplementation, either alone or in combination with acutely injected MOR. (a) The mean response latency is higher in the O3-SAL, O3-$MOR_{SC\ (1)}$, O3-$MOR_{SC\ (2.5)}$, O3-$MOR_{SC\ (5)}$, O3-$MOR_{SC\ (6)}$ groups than in the C-SAL, C-$MOR_{SC\ (1)}$, C-$MOR_{SC\ (2.5)}$, C-$MOR_{SC\ (5)}$, C-$MOR_{SC\ (6)}$ groups, showing an additive effect. Only in O3-$MOR_{SC\ (5)}$ O3-$MOR_{SC\ (6)}$ such differences were significant. (b) Locomotor activity was not affected in any group except the C-$MOR_{SC\ (2.5)}$ group, in which the number of ambulatory counts was increased. Bars represent the mean ±S.E.M. ANOVA models were used to evaluate differences among treatments. Bonferroni tests were used to compare the means, considering a significance level of $\alpha=0.05$ ($p<0.05$), with different letters indicating significant differences.

**Figure 3**. Effects of NAL on the response latency (in the hot-plate test) and locomotor activity (in the open-field test) after administration of MOR, O3 and their combined treatments. (a) NAL significantly prevented the antinociceptive effect in the C-NAL-$MOR_{SC\ (5)}$, O3-NAL and O3-NAL-$MOR_{SC\ (5)}$ groups. (b) Locomotor activity was not affected in any group. Bars represent the mean ± S.E.M. ANOVA models were used to evaluate the differences between treatments. Bonferroni tests were used to compare the means, considering a significance level of $\alpha=0.05$ ($p<0.05$), with different letters indicating significant differences.

**Figure 4.** Antinociceptive effect (in the hot-plate test), locomotor activity (in the open-field test) and body weight changes after tolerance induction protocol (16 days). (a) The mean response latency is higher (non-significant) in the O3-$MOR_{chronic}$ group than in the C-$MOR_{chronic}$ group. (b) Locomotor activity was not affected in any group. (c) The body weight loss was lower (non-significant) in the O3-$MOR_{chronic}$ group than in the C-$MOR_{chronic}$ group. Bars represent the mean ± S.E.M. ANOVA models were used to evaluate the differences between treatments., Bonferroni tests were used to compare the means, considering a significance level of $\alpha=0.05$ ($p<0.05$), with different letters indicating significant differences.

**Figure 5**. Antinociceptive effect (in the hot-plate test), locomotor activity (in the open-field test) and body weight changes after tolerance induction protocol (30 days). (a) The mean response latency is significantly higher in the O3-$MOR_{chronic}$ group than in the C-$MOR_{chronic}$. (b) Locomotor activity was not affected in any group. (c) A body weight loss was observed in the C-$MOR_{chronic}$ group while a body weight increase was observed in the O3-$MOR_{chronic}$ group. Bars represent the mean ± S.E.M. ANOVA models were used to evaluate the differences between treatments. Bonferroni tests were used to compare the means, considering a significance level of $\alpha=0.05$ ($p<0.05$), with different letters indicating significant differences.

**Figure 6**. Antinociceptive effect (in the hot-plate test) and locomotor activity (in the open-field test) after oral administration of MOR or the pharmaceutical mixture containing MOR:O3. (a) The mean response latency is significantly higher in the $O3_{OR}$ and $O3_{OR}$-MOR:$O3_{OR}$ groups. (b) Locomotor activity was not affected in any group. Bars represent the mean ±S.E.M. ANOVA models were used to evaluate the differences betweentreatments. Bonferroni tests were used to compare the means, considering a significance level of $\alpha=0.05$ ($p<0.05$), with different letters indicating significant differences.

**Table 1**. Diet components and their content of fatty acids [a]

| | | control diet | O3 diet |
|---|---|---|---|
| **Diet components (g/kg of diet)** | Calcium caseinate | 200 | 200 |
| | Corn oil | 50 | 50 |
| | Choline chlorhydrate | 1.5 | 1.5 |
| | Vitamin mixture [b] | 10 | 10 |
| | Mineral mixture [c] | 35 | 35 |
| | Maltodextrin | 696.9 | 696.9 |
| | Salmon oil | ----- | 11.93 |
| **Content of fatty acids (as a percentage of total)[d]** | Myristic | 0.11 | 7.46 |
| | Palmitoleic | 0.16 | 9.74 |
| | C 16:0 palmitic | 6.75 | 16.25 |
| | C 18:0 stearic | 3.04 | 4.10 |
| | C 18:1 O9 oleic | 29.2 | 13.05 |
| | C18:2 O6 linoleic | 58.8 | 1.67 |
| | C 18:3 O3 alpha-linolenic | 0.11 | 0.69 |
| | C20:4 O6 arachidonic | 0.24 | 1.15 |
| | C20:5 O3 eicosapentaenoic | NC | 17.24 |
| | C22:6 O3 docosahexaenoic | NC | 12.21 |

[a] The average daily intake of food was 20 g in both groups providing 81 or 83.16 kCal/rat/day for control or O3 diet, respectively. Statistical evaluation of the calories consumed by both groups showed no differences ($p < 0.01$).

[b] Composition of vitamin supplement triturated in sucrose (g/kg of diet): D-calcium panthotenate, 1.60; nicotinic acid, 3.00; D-biotin, 0.02; menadione, 0.029; thiamine HCl, 0.60; riboflavin, 0.60; folic acid, 0,20; dl-alpha-tocopherol acetate (500 u/gr), 15.00; retinyl palmitate, (400 uI/gr), 0.228; pyridoxine HCl, 0.70; cyanocobalamin 0.1 % (triturated in mannitol 1:1000), 2.50; cholecalciferol, (250000 U/g), 0.40; sucrose, 975.123.

[c] Composition (g/kg of diet) as follows: $K_2HPO_4$, 322.5; $CaCO_3$, 357; NaCl, 74; MgO, 0.8; $MgSO_4$ $7H_2O$, 146.9 ; $ZnSO_4 \cdot 5\ H_2O$, 0.63; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 0.008; KI, 0.0078; $Na_2SeO_3$ $5H_2O$, 0.1025; iron and ammonium citrate, 6.06; ZnCl, 1.79; sucrose 91.

[d] Determined by gas chromatography [24]

620 **Table 2**. Pharmacological Treatments and Experimental Groups

| Experiments | Groups [a] | Diets (1st -16th days) | Treatment on the 17th day |
|---|---|---|---|
| **Experiment I: The antinociceptive effect of chronic O3 dietary supplementation either alone or in combination with acutely injected MOR.** | C-SAL (N= 12) | Control | SAL administered subcutaneously |
| | C- $MOR_{SC\ (1)}$ (N= 12) | Control | MOR administered subcutaneously (1 mg/kg) |
| | C- $MOR_{SC\ (2.5)}$ (N= 12) | Control | MOR administered subcutaneously (2.5 mg/kg) |
| | C- $MOR_{SC\ (5)}$ (N= 12) | Control | MOR administered subcutaneously (5 mg/kg) |
| | C- $MOR_{SC\ (6)}$ (N= 12) | Control | MOR administered subcutaneously (6 mg/kg) |
| | O3-SAL (N= 12) | Omega 3 | SAL administered subcutaneously |
| | O3-$MOR_{SC\ (1)}$ (N= 12) | Omega 3 | MOR administered subcutaneously (1 mg/kg) |
| | O3-$MOR_{SC\ (2.5)}$ (N= 12) | Omega 3 | MOR administered subcutaneously (2.5 mg/kg) |
| | O3-$MOR_{SC\ (5)}$ (N= 12) | Omega 3 | MOR administered subcutaneously (5 mg/kg) |
| | O3-$MOR_{SC\ (6)}$ (N= 12) | Omega 3 | MOR administered subcutaneously (6 mg/kg) |
| **Experiment II: The effect of NAL on antinociception produced by chronic O3 dietary supplementation either alone or in combination with acutely injected MOR.** | C-SAL (N= 7) | Control | SAL administered subcutaneously |
| | C-NAL (N= 7) | Control | NAL administered subcutaneously (1 mg/kg) |
| | C- $MOR_{SC\ (5)}$ (N= 7) | Control | MOR administered subcutaneously (5 mg/kg) |
| | C-NAL-$MOR_{SC\ (5)}$ (N= 7) | Control | NAL (1 mg/kg) administered 10 min before MOR (5 mg/kg), both subcutaneously |
| | O3-SAL (N= 7) | Omega 3 | SAL administered subcutaneously |
| | O3-NAL (N= 7) | Omega 3 | NAL administered subcutaneously (1 mg/kg) |
| | O3- $MOR_{SC\ (5)}$ (N= 7) | Omega 3 | MOR administered subcutaneously (5 mg/kg) |
| | O3-NAL-$MOR_{SC\ (5)}$ (N= 7) | Omega 3 | NAL (1 mg/kg) administered 10 min before MOR (5 mg/kg), both subcutaneously |
| **Experiment III: The effect of chronic O3 dietary supplementation on MOR-induced tolerance to antinociception [b]** | C-SAL (N=10) | Control | SAL administered subcutaneously |
| | O3-SAL (N=10) | Omega 3 | SAL administered subcutaneously |
| | C-$MOR_{SC\ (5)}$ (N=10) | Control | MOR administered subcutaneously (5 mg/kg) |
| | O3-$MOR_{SC\ (5)}$ (N=10) | Omega 3 | MOR administered subcutaneously (5 mg/kg) |
| | C-$MOR_{chronic}$ (N=10) | Control | Tolerance induction protocol: MOR administered subcutaneously (10, 15, 20 and 30 mg/kg) on days 13th -16th, respectively.[c] Treatment: MOR administered subcutaneously (5 mg/kg on day 17th). |
| | O3- $MOR_{chronic}$ (N=10) | Omega 3 | |
| **Experiment IV: The antinociceptive effect of pharmaceutical mixture containing MOR:O3 after chronic O3 dietary supplementation.** | C-$SAL_{OR}$ (N= 7) | Control | SAL administered orally |
| | C-$MOR_{OR}$ (N= 7) | Control | MOR administered orally (12 mg/kg) |
| | C-MOR:$O3_{OR}$ (N= 7) | Control | MOR:O3 administered orally (12:720 mg/kg) |
| | $O3_{OR}$ (N= 7) | Omega 3 | SAL administered OR |
| | $O3_{OR}$- MOR:$O3_{OR}$ (N= 7) | Omega 3 | MOR:O3 administered orally (12:720 mg/kg) |

621 Control: Control diet; Omega 3: diet supplemented with fish oil as a source of O3 (equivalent to a consumption of 720 mg O3/kg); SAL: sterile saline solution; MOR:
622 morphine; MOR:O3: pharmaceutical mixture of MOR:O3 (12:720 mg/kg). [a] Subscripts indicate administration routes and doses used. [b] This trial was also carried out for 30
623 days using the same doses of MOR. [c] The daily dose of MOR solution was given in two parts at 10:00 a. m. and 8:00 p. m. for 4 consecutive days in agreement with the
624 protocol described in figure 1.The tolerance induction protocol was done on days 27-30 and the treatment on day 31st).