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Melatonin synergizes with the antinociceptive effect of N-palmitoylethanolamide and paracetamol

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Melatonin has been shown to have an antinociceptive effect and its administration could enhance the antinociceptive effect of other drugs. This study assessed the antinociceptive effects of melatonin in combination with paracetamol and N-palmitoylethanolamide (PEA) using the formalin test in mice. Melatonin, paracetamol, and PEA were administered intraplantarly (paw) alone or combined to mice. A concentration-response curve was generated to determine the concentration needed to reach 30% of the maximal antinociceptive effect (EC_{30}). Melatonin, paracetamol and PEA induced a concentration-dependent antinociceptive effect in both phases of the formalin test, being PEA more potent (EC_{30} = 7.4±0.2 mg/paw) than melatonin (EC_{30} = 20.5±3.1 mg/paw) or paracetamol (EC_{30} = 41.8±2.6 mg/paw). Combinations of melatonin with paracetamol or PEA also induced a concentration-dependent antinociceptive effect in the formalin test. Isobolographic analysis showed that melatonin interacts synergistically with either paracetamol or PEA to reduce formalin-induced inflammatory pain. However, the experimental values of EC_{30} were significantly smaller than those calculated theoretically.

1. Introduction

Melatonin (5-methoxy-N-acetyltryptamine) is secreted from the pineal gland; this regulates body functions as a neuroprotector in a wide range of conditions affecting the central nervous system (Shu et al. 2018) and acts as an antioxidant and free radical scavenger. Melatonin decreases nociception through specific MT2 melatonin receptors (Lakin et al. 1981; Ambriz-Tututi et al. 2009). Melatonin has been co-administered with many drugs to enhance antinociception. For instance, melatonin induces a synergistic interaction with morphine or deltorphin I, a delta-opioid receptor agonist. In contrast, melatonin is not able to enhance the antinociceptive effect of endomorphin-1 (Pang et al. 2001; Li et al. 2005). Melatonin interacts synergistically with clonidine (an a_1 adrenoceptor agonist) and neostigmine (acetyl-cholinesterase inhibitor). These effects were produced by activating MT2 receptors at the spinal level in the formalin test (Ng et al. 2017). In addition, melatonin increases the antinociceptive effect of diazepam in mice (Pang et al. 2001). The effect of melatonin on paracetamol and N-palmitoylethanolamide (PEA) actions has not been explored so far.

Paracetamol is a widely used drug as an analgesic and antipyretic. It is also used to treat pain associated with osteoarthritis. Paracetamol exerts its analgesic actions by mechanisms such as cyclooxygenase inhibition and activation of descending inhibitory serotonergic and opioidergic pathways. Paracetamol activates TRPV1 (transient receptor potential vanilloid 1 TRPV1) and voltage-gated Kv7 potassium channels and inhibits T-type Cav3.2 calcium channels (Kerckhove et al. 2014; Ray et al. 2019; Przybyła et al. 2021). Moreover, paracetamol induces local peripheral antinociception through activation of CB1 and CB2 cannabinoid receptors and A1 adenosine receptors (Dani et al. 2007; Liu et al. 2013).

On the other hand, PEA is an endocannabinoid analogue that can interact with the cannabinoid system by the "entourage effect". PEA increases the endogenous levels of anandamide and 2-arachidonoylglycerol (2-AG), which activates CB2 receptors and TRPV1 channels by inhibiting the expression of the enzyme FAAH (fatty acid amide hydrolase). PEA also activates TRPV1 via PPARα (Costa et al. 2008; DÁngostino et al. 2009; Gabrielsson et al. 2017). There is evidence that local peripheral administration of PEA inhibits formalin-, acetic acid-, kaolin-induced nociception (Calignano et al. 1998, 2001).

Since these drugs induce antinociception by different mechanisms of action, we hypothesized that combinations of melatonin with either paracetamol or PEA would produce a synergic antinociceptive effect in the formalin test in mice.

2. Investigations and results

2.1. Formalin injection produced a typical pattern of flinching behavior

Values of AUC after formalin injection were 713.7±25.2 ua and 1400±49.5 ua for phase I and phase II, respectively. These values were considered 100% of nociception. Treatment with melatonin, paracetamol, and PEA induced a concentration-dependent antinociceptive effect (Fig. 1). Melatonin administration gradually decreased the number of flinches to 50.2 % as the maximum antinociceptive effect with 300 µ/paw (Fig. 1A). In phase II, melatonin 3 µ/paw did not produce any antinociceptive effect, whereas injection at 100 and 300 µ/paw induced similar effects, 73 % (378.33±32.8 ua) and 74.2 % (362.5±15 ua), respectively (Fig. 1B). Fig. 1C shows the antinociceptive effect of paracetamol on phase I; mice did not modify the nociceptive process in the neurogenic phase when they were treated with 3 and 10 µ/paw. However, the concentrations 30, 100, and 300 µ/paw induced 29.1 %, 35.6 %, and 36 %, respectively ($F_{(6,30)}$= 2.6). Paracetamol, at low concentrations, induced a limited antinociceptive effect on phase II; 3 (19.5 %) 10 (25.6 %), and 30 µ/paw (36.5%). In contrast, this drug, at high doses, induced a prominent antinociceptive effect. Paracetamol 100 and 177 µ/paw induced 48.6 % and 48.2 % of antinociception, respectively. In the case of PEA,

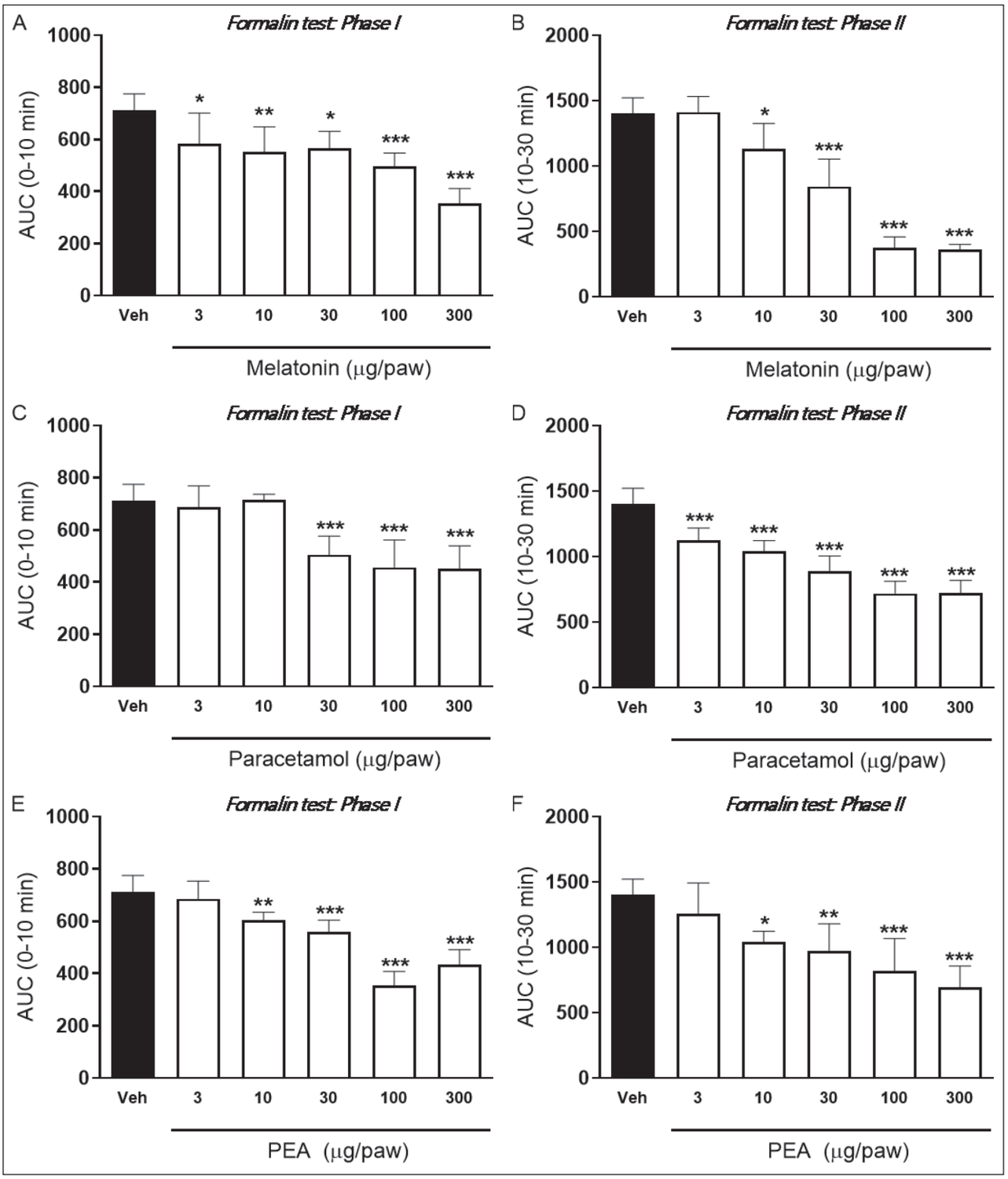

Fig. 1: Antinociceptive effect of the intraplantar administration of melatonin, paracetamol, and PEA in the formalin test; Phase 1 (Panels A, C and E) and Phase II (Panels B, D, and F). The antinociceptive effect is represented as the AUC of the temporal course (time vs. the number of flinches). Each measurement is represented as the mean ± SEM with at least six mice per group. Significantly different from vehicle value (Veh; saline solution (0.9%)). (*p<0.05: **p<0.01; ***p<0.001) was determined by one-way ANOVA followed by Dunnett's post hoc test.

it produced a gradual increment of antinociception parting to 10 μ/paw with 15.2 %. The maximum antinociceptive effect was obtained at a concentration of 17.7 μ/paw (50.43 %) and a greater concentration did not further increase the antinociceptive effect (39.4 %) ($F_{(6,30)}$=1.212) (Fig. 1E). In phase II, PEA produced a 3 μ/paw antinociceptive effect in 9.53 %. After this, the increments were 31.97 %, 41.01 %, 47.29 %, and 68 % with respect to the vehicle ($F_{(6,29)}$=1.340) (Fig. 1F).

The individual EC_{30} values obtained from each drug were used to determine the experimental values for the five concentrations of each combination at a 1:1 fixed ratio (Table). The effective concentration of 30 was determined because none of the drugs evaluated individually achieved a more than 70 % antinociceptive effect. The EC_{30} was obtained by logarithmic linear analysis; PEA was more potent (EC_{30} = 7.4±0.2 μ/paw) than melatonin (EC_{30} = 20.5±3.1 μ/paw) or paracetamol (EC_{30} = 41.8±2.6 μ/paw).

Table: Concentrations employed in the combination curve concentration response

	Individual concentration (μ/paw)		
	Melatonin	Paracetamol	PEA
Individual	3, 10, 30, 100, 300	3, 10, 30, 100, 177	3, 5.6, 10, 17.7, 30
	Concentration in combination (μ/paw)		
Melatonin	Paracetamol	Total dose	Melatonin	PEA	Total dose
0.6	1.3	1.9	0.6	0.2	0.8
1.3	2.6	3.9	1.3	0.4	1.7
2.5	5.2	7.8	2.6	0.9	3.5
5.1	10.4	15.6	5.1	1.8	6.9
10.2	20.9	31.1	10.2	3.6	13.9

2.2. Pharmacological antinociceptive interaction of melatonin, paracetamol, and PEA

Coadministration of melatonin with paracetamol or PEA produced a gradual antinociceptive effect in both phases (Fig. 2). The melatonin and paracetamol combination at a 3.9 μ/paw concentration produces antinociceptive effects in phase I (21.3%) and phase II (15 %) (Fig. 2A and 2B). Increasing the concentration combination produced a better antinociceptive effect. For example, the 15.6 μ/paw combination produces 33.2 % and 45.6 % in phases I and II, respectively. This combination is formed by 5.12 and 10.4 μ of melatonin and paracetamol, respectively. To produce a similar effect by individual injection, both require 30 μ of melatonin or paracetamol to produce 30% of antinociception on phase I, 25 μ more than melatonin and 20 μ more in the case of paracetamol. In addition, 300 μ of melatonin produces 50 % antinociception, and 100 μ of paracetamol produces 48 %. However, in combination, only 5.1 μ is necessary to generate 45.6 % antinociceptive activity. Fig. 2C and 2D show the antinociceptive effects generated by melatonin combined with PEA in a concentration-dependent manner in both phases. Low-concentration combinations can produce high antinociception: concentrations of 7.8, 15.6, and 31.1 μ/paw generated an antinociceptive effect between 30 and 50 % of respondents in both phase I (ANOVA one way, $p<0.05$, $F_{(6,30)}= 0.2334$) and 30 and 75 % in phase II (ANOVA one way, $p<0.05$, $F_{(6,30)}= 0.278$). The 13.9 μ/paw concentration generated the maximum antinociceptive effect found in this study (50.3 % phase I and 75.3 % phase II). This combination is formed by 10.2 and 3.6 μ/paw melatonin and PEA. With 10 μ/paw injection of melatonin, we observed 22.6 % antinociceptive effects in phase I. In contrast, the same concentration increase was 2.2 times more effective. This melatonin concentration produces 19.5 % injected individually in the inflammatory phase, but it produces 75.3 % (3.8 times) in combination. A combination of 3 μ/paw did not produce antinociception on the neurogenic phase and presented 19 % antinociception on the inflammatory phase. A relative concentration of 3.6 μ/paw evaluated in combination increased the antinociceptive activity to 50.3 % in phase I and 70 % in phase II (3.8 times). These data showed that fewer concentrations of individual drugs present more antinociceptive effects when combined.

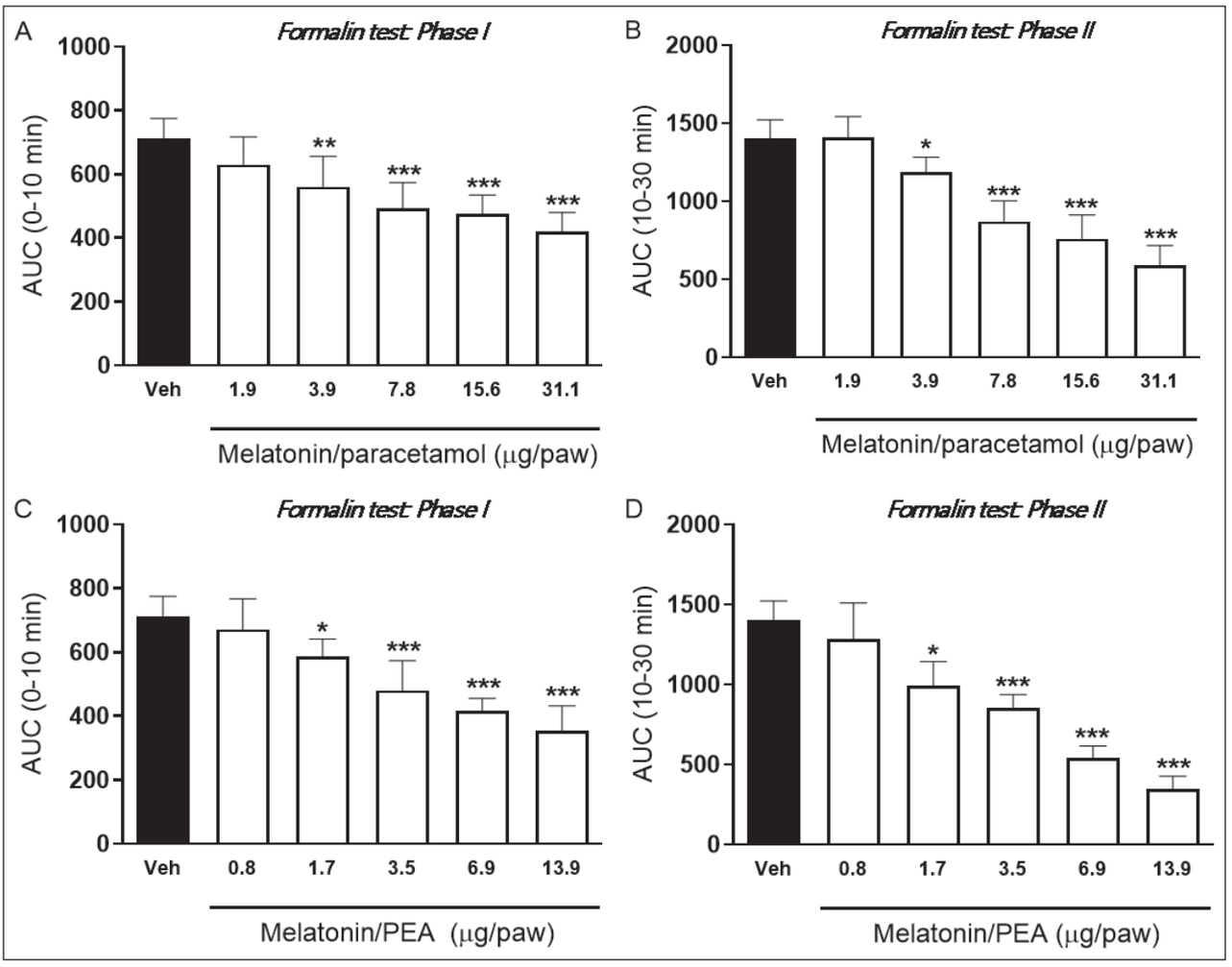

Fig. 2: Antinociceptive effect of the intraplantar injection of melatonin in combination with paracetamol or PEA in the formalin test; Phase 1 (Panel A and C) and Phase II (Panel B and D). The antinociceptive effect is represented as the AUC of the temporal course (time vs. the number of flinches). Each measurement is represented as the mean ± SEM with at least six mice per group. Significantly different from vehicle value (Veh; saline solution (0.9%)) (*p<0.05: **p<0.01; ***p<0.001) was determined by one-way ANOVA followed by Dunnett's post hoc test.

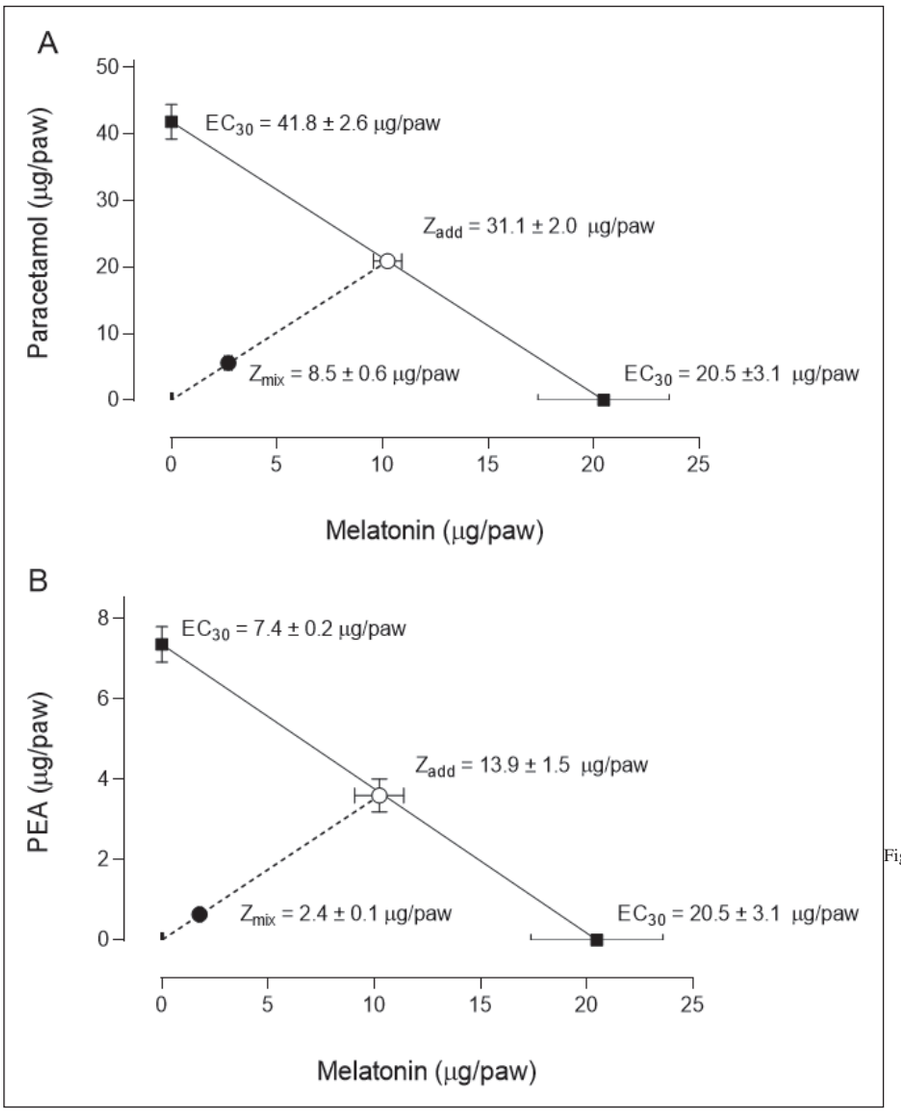

Fig. 3: In the mouse formalin test, isobolograms describe the synergistic interaction between melatonin combined with paracetamol (Panel A) or PEA (Panel B). Horizontal and vertical bars indicate SEM. The oblique line that connects the individual EC30 values in each combination (■) is the theoretical additive line. The point in this line is the theoretical additive point (Zadd, ○) calculated from the individual drug EC30 values. The experimental EC30 value (Zmix, ●) is far below the additive lines, indicating a significant synergism *p<0.05 with respect to the Zadd value, Student's t-test.

To analyze the interaction of melatonin with paracetamol or PEA, we used isobologram analysis. Fig. 3 depicts the isobologram of melatonin in combination with paracetamol or PEA. In both cases, the interaction was synergistic, with Z_{mix} being higher than Z_{add}. Local peripheral injection of melatonin (EC_{30} = 20.5±3.1 µ/paw), paracetamol (EC_{30} = 41.8±2.6µ/paw), and PEA (EC_{30} = 7.4±0.2 µ/paw) decreased nociception-induced formalin in a concentration-dependent manner, as shown in Fig. 1. In addition, melatonin in combination with paracetamol (EC_{30} = 8.5±0.6 µ/paw) and PEA (EC_{30} = 2.4±0.1 µ/paw) also decreased nociception (Fig. 2). The isobologram demonstrates that the combinations investigated in this study produced a synergistic interaction. Experimental values for melatonin and paracetamol (Z_{mix} = 8.5±0.6 µ/paw) were significantly smaller than those calculated theoretically (Z_{add} = 31.1±2 µ/paw). Additionally, the experimental values (Z_{mix} = 2.4±0.1 µ/paw) in the melatonin and PEA combination were significantly smaller than those calculated theoretically (Z_{add} = 13.9±1.5 µ/paw). These findings indicate that melatonin coadministered with PEA or paracetamol can synergistically reduce inflammatory pain.

3. Discussion

The results obtained in the present study suggest that combination of melatonin with paracetamol or PEA is useful in formalin-induced nociception in mice. Inflammatory pain has a high incidence worldwide and is difficult to control due to its variable pathophysiological mechanism. In this study, we used the formalin test to demonstrate this synergistic interaction. Formalin-induced nociception involves a biphasic response wherein phase I nociception results from the direct activation of primary nociceptive afferents (neurogenic phase), whereas phase II pain involves, at least in part, central sensitization in the dorsal horn of the spinal cord (Dickenson and Sullivan 1987; McNamara et al. 2007). Mediators such as neurokinin 1 (NK1), cytokines, vasoactive intestinal peptide (VIP), substance P (SP), calcitonin gene-related peptide (CGRP), TRPV1 receptors, and cytokins such as IL-1β, TNF-α, and IL-6 contribute to neurogenic pain. Phase II involves prostaglandins (PGs), pro-inflammatory cytokines, TRPA1 channels, NMDA, and neurokinin-1 receptors, which also participate in central sensitization (Vanegas 2004).

In this study, it was not possible to establish the pharmacological mechanism of this synergistic interaction. However, when two drugs are in combination, the synergistic antinociceptive effect could be due to the sum of the different mechanisms of action of melatonin, paracetamol, and PEA. This interaction mechanism needs to be established considering the different receptors involved in the mechanism of action of each drug. For example, it is well known that melatonin in the antinociceptive process can exert its downstream effects by binding to specific receptors MT1 and MT2, related to the physiological process of pain, or by direct association with its substrates. In many tissues, MT1 receptor activation seems to mediate melatonin antinociception by stimulating

the G proteins Giα2, Giα3, and Gαq, with inhibitory effects on the cAMP signaling pathways (Brydon et al. 1999). In contrast, MT2 receptors are coupled to phosphoinositide signal transduction pathways and inhibit adenylyl cyclase and guanylyl cyclase pathways (Boutin et al. 2005).

Furthermore, an interplay between melatonin, alpha-1 adrenergic, and 5HT2 and 5HT3 serotoninergic receptors may also contribute to antinociception. It has been demonstrated that melatonin decreases the nociceptive process in a different nociceptive animal test using capsaicin or acetic acid as algesic inflammatory substances, and the central and peripheral opioid receptors are related to antinociception-induced melatonin, also in neuropathic pain (Boutin et al. 2005; Ambriz-Tututi et al. 2009). In addition, intrathecal coadministration of melatonin at low doses of morphine attenuated mechanical and thermal hyperalgesia in an acute postoperative pain model in rats, suggesting that melatonin acts as a neuromodulator in the spinal cord (Mukherjee et al. 2015).

In the case of paracetamol, antinociception is located at the spinal and supraspinal levels. Spinal antinociception of paracetamol was reverted by the $5HT_{1A}$ antagonist WAY-100635, suggesting serotonin pathway participation (Bonnefont et al. 2005). Additionally, the antinociceptive effect implicates endogenous opioids (endorphins, enkephalins, and dynorphins) (Raffa et al. 2004). In this sense, melatonin and paracetamol could modulate the opioid and serotoninergic pathways together to synergize with antinociception. In the case of PEA, the opioid pathway is involved in antinociception (Erfanparast et al. 2015). Interestingly, PEA has been shown to inhibit opioid tolerance (Di Cesare et al. 2015). It is not known precisely how this process of analgesic tolerance occurs. However, neuronal mechanisms of adaptation and sensitization are involved in causing changes in microglia and astrocytes, causing a better production of many substances, such as free radicals, NO, cytokines and proinflammatory chemokines, PG, complement proteins, neurotoxins, neurotrophic factors, and excitatory amino acids, that actively oppose the analgesic effects of morphine and contribute to the development of tolerance (Williams et al. 2013; Jokinen et al. 2018). PEA protects nervous tissue under neuropathic conditions, prevents neurotoxicity and neurodegeneration, and inhibits peripheral inflammation and mast cell degranulation. Furthermore, PEA reduces the activation of microglia and astrocytes in inflammation-induced formalin, preventing an increase in glial cell numbers by prolonging the efficacy of morphine until day 10, suggesting a relationship between glial inhibition and the attenuation of tolerance (Di Cesare et al. 2015).

Another mechanism of melatonin is through modulation of Ca^{++}-activated calmodulin (CaM). Melatonin binds to CaM with a high affinity and has been shown to act as a CaM antagonist. Among the CaM-dependent enzymes, Ca^{++}/calmodulin-dependent protein kinase II (CaM-kinase II) is a particularly abundant enzyme in the nervous system (Boutin et al. 2005). These interactions are related to regulating enzymes such as cAMP phosphodiesterase, CaM-kinase II, and nitric oxide synthase (Ulugol et al. 2006). It has been established that a low dose of kelatorphan, an inhibitor of multiple endogenous opioid peptide-degrading enzymes, stabilizes endogenous opioid agonists released by cAMP-PDE inhibitors, resulting in the conversion of hyperalgesia to analgesia without requiring selective blockade of excitatory opioid receptor signaling (Crain et al. 2008).

Paracetamol is used in acute and chronic pain; it has been established that the antinociceptive effect is due to the metabolite of p-aminophenol, which crosses the blood-brain barrier and is metabolized by fatty acid amide hydrolase (FAAH) to yield N-acylphenolamine (AM404) (Högestätt et al. 2005). AM404 acts on the TRPV1 and CB1 receptors in the central nervous system, which are colocalized mediators of nociceptive modulation. Therefore, paracetamol induces analgesia *via* direct action on the brain (Bannwarth et al. 1992; Gelgor et al. 1992), and these receptor sites on the brain are the primary mediators of acetaminophen-induced analgesia.

However, a new antinociceptive mechanism of acetaminophen was recently suggested according to behavioral measures, and *in vivo* and *in vitro* whole-cell patch-clamp recordings with rats, wherein the paracetamol metabolite AM404 directly induces analgesia via TRPV1 receptors on the spinal dorsal horn (Ohashi et al. 2017). Similar to the brain, the spinal cord, especially *Substantia gelatinosa* (SG, lamina II of Rexed), is also critical to pain pathways and modulates nociceptive transmission *via* primary afferent Aδ- and C-fibers (Ohashi et al. 2017; Kohno et al. 1999). Furthermore, TRPV1 receptors are abundant in the spinal cord dorsal horn (Yang et al. 1999, 2000).

Paracetamol does not possess any anti-inflammatory activity because it is a very weak inhibitor of COX and does not inhibit neutrophil activation (Hanel et al. 1982). Therefore, even though NSAIDs have always been discussed in terms of pharmacological mechanisms, paracetamol is not regarded as an NSAID and is not appropriate for treating inflammatory pain conditions. However, we also revealed that its metabolite AM404 induces analgesia *via* TRPV1 receptors on the spinal dorsal horn in a rat model of inflammatory pain. These antinociceptive effects were more potent in the inflammatory pain model than in naïve rats (Ohashi et al. 2017).

The combination of melatonin with paracetamol has a synergistic effect due to its individual mechanism of action. A further advantage of this combination is that melatonin could protect the mouse liver against severe damage induced by paracetamol (Matsura et al. 2006). Thus, the use of a melatonin-paracetamol combination could be helpful in chronic patients; the doses of synergism are low, and the adverse effects of paracetamol could decrease.

In the case of the combination of PEA with melatonin, there must be an interaction with the endocannabinoid system since it is known that PEA, as an endocannabinoid, can potentiate the effect of anandamide through indirect activation by inhibiting FAAH. PEA is an endogenous fatty acid mediator that is synthesized from membrane phospholipids by N-acyl phosphatidylethanolamine phospholipase D (Rinne et al. 2018). It is naturally produced in many plant and animal food sources, as well as in cells and tissues of mammals. It has central and peripheral effects with anti-inflammatory and analgesic properties; it is very well tolerated in humans (Petrosino et al. 2017; Rankin et al. 2020). PEA may indirectly stimulate the effects of both phyto- or endocannabinoids, either by its role as an agonist of TRPV1 channels, peroxisome proliferator-activated receptor α or cannabinoid receptors. PEA plays an essential role in the suppression of inflammation by reducing the activity of pro-inflammatory enzymes such as COX, eNOS, and iNOS and by reducing mast cell activation (Berker et al. 2017). In addition, PEA, through inhibiting the expression of fatty acid amide hydrolase, the enzyme responsible for the degradation of the endogenous cannabinoid receptor ligand anandamide, may indirectly activate CB1 and CB2 receptors (Rankin et al. 2020). PEA shows efficacy in various pain models, including carrageenan and prostaglandin-induced hyperalgesia, the formalin test of persistent pain, visceral hyperalgesia produced by instillation of nerve growth factor into the bladder, and the sciatic nerve ligature model of neuropathic pain (Gabrielsson et al. 2016). In cultured cell lines, human PEA shows anti-inflammatory activity (Couch et al. 2017), and PEA reduces pain intensity and the number of attacks per month in pediatric patients with migraine and low back pain (Crucu et al. 2019).

A previous study showed that PEA, combined with tramadol, produced synergistic antinociceptive interactions in the mouse formalin test (Deciga-Campos et al. 2015a). PEA, in combination with paracetamol, produced synergistic interactions in diabetic rats (Deciga-Campos et al. 2015b).

In summary, this study supports the potential use of melatonin and paracetamol or PEA in combination. These results provide evidence that this combination significantly decreases inflammatory nociception in mice. Future studies are necessary to demonstrate their mechanism of antinociceptive actions and their possible adverse effects, and it is essential to consider clinical studies to establish adequate doses in humans.

4. Experimental

4.1. Animals

This study used female ICR mice weighing 25-30 g from Cinvestav, South Campus (Mexico City). Before experiments, the animals were maintained with complimentary water and food in a controlled environment at 25 °C with 12-hour light/dark cycles (6:00 AM-6:00 PM). All experiments were conducted according to the ethical local ethics committee (Cinvestav, protocol 042-13), the Scientific Procedures Established by the Mexican Official Norm for Animal Care and Handing NOM-062-ZOO-1999 (NOM-062-ZOO-1999), and the EU Directive 2010/63/EU for animal experiments. Animals were randomized to each treatment group (n = 6). All efforts were made to minimize animal suffering and to reduce the number of animals used. Before administering any treatment, animals were placed in open Plexiglas observation chambers for 40 min at a controlled temperature of 25 °C for conditioning to their surroundings. The animals were humanely killed in a CO_2 chamber.

4.2. Reagents

Paracetamol, melatonin, and N-palmitoylethanolamide (PEA) were purchased from Sigma-Aldrich (St. Louis, MO). Formalin was purchased from J.T. Baker (Mexico City). All reagents were dissolved in a vehicle (saline solution).

4.3. Pharmacological evaluation

The formalin test was used to determine the antinociceptive effects of all drugs and combinations. For this, 20 µ of 1% formalin was injected into the subcutaneous dorsal region of the mouse right paw. An acrylic cylinder chamber with mirrors was used to observe nocifensive behavior immediately after formalin paw injection. Mice produce flinches and linking behavior with formalin injection. In this case, the nocifensive behavior was quantified as the number of flinches of the injected paw during 5-min periods up to 30 min after injection. The formalin-induced flinching behavior was biphasic: the initial acute phase (first phase, 0-10 min), followed by a quiescent period, and then by a prolonged tonic response (second phase, 15-30 min) (Dubuisson et al. 1997).

4.3. Isobolographic analysis

To determine the pharmacological interaction between melatonin and paracetamol or PEA, we used isobole analysis to select a particular effect level considering the maximum response of individual curve-concentration responses. First, time courses were built, plotting the time course of the number of flinches for each group. The area under the curve (AUC) of each time course was used to build the concentration-response curve (CRC) of individual drugs. According to the maximum responses, we used the effective concentration to reach 30 % of the maximal possible effect (EC_{30}), determined by the logarithmic analysis in a 1:1 ratio to generate the theoretical combinations according to the isobolographic analysis. An isobologram was built with the EC_{30} of paracetamol and melatonin or PEA combinations. In this ploy, the theoretical additive concentration (Z_{add}) with the SEM for each combination was computed from the EC_{30} of the single drugs, according to the method previously described by Tallarida et al. (1989) to satisfy the following equation:

$Z_{add} = fA + (1 - f)B$

where A is the EC_{30} of melatonin and B is the EC_{30} of PEA or paracetamol. For a 1:1 fixed ratio, f is 0.5 and $(1-f)$ is 0.5. Z_{add} represents the total additive concentration of the drugs, theoretically providing a 30% reduction in the flinching response relative to the vehicle. The experimental concentration (Z_{mix}) is the total concentration of the mixture that was experimentally determined by the two-component drugs administered at a 1:1 fixed-ratio combination sufficient to reduce the time of flinching by 30% concerning the vehicle. The Z_{mix} values (with their 95% confidence limits) were determined from the CRC of the combined drugs by a standard logarithmic, linear regression analysis of the CRC (five concentrations). Subsequently, using the 95% confidence limits, the experimentally determined Z_{mix} was statistically compared to the theoretically calculated Z_{add} concentrations by Student's t-test, according to the procedures previously described by Tallarida (1992). Each isobologram was constructed with EC_{30} individual paracetamol, melatonin or PEA to obtain the respective additivity line; the Z_{add} lies on a line connecting the EC_{30} values of the individual drugs. Z_{mix} was plotted in the same graph if this value was below this additive line and was considered synergistic or superadditive. In contrast, values that lie above and to the right of the line demonstrate an attenuated or sub additive interaction (Tallarida et al. 1989).

4.4. Experimental design

Mice received a local injection of the vehicle and logarithmic concentrations of melatonin (3, 10, 30, 100, 300 µ/paw), paracetamol (3, 10, 30, 100, 177 µ/paw) or PEA (3, 5.6, 10, 17.7, 30 µ/paw) 15 min before 1% formalin injection. The flinches were counted on the paw injected with formalin. According to the isobolographic method, five concentrations of each combination melatonin-paracetamol or melatonin-PEA were analyzed for possible synergistic interactions

4.5. Data analyses

Concentration-response curves were built with the AUC obtained from the time courses by the trapezoidal rule. Each point of this graphic was expressed as the mean of six animals±SEM. Graphic concentration vs AUC was obtained in both phases of the formalin test. However, to determine the EC_{30}, the total AUC antinociceptive percentage was considered (phase I + phase II). The AUC values derived from the antinociceptive effects produced by melatonin, paracetamol or PEA (assayed separately or in combination) were compared with the AUC value obtained from the vehicle. One-way ANOVA and Dunnett's post hoc tests were used for these comparisons. The synergism between melatonin and paracetamol or PEA was calculated with isobologram analysis (Tallarida 1992). The AUC experimental values (Z_{mix}) for drug combinations were compared with the expected value (Z_{add}) using Student's t-test. The graphic program was GraphPad Prism software (version 6.0; GraphPad Inc., La Jolla, CA).

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