Coadministration of Novel Analgesic Isovaline with Tramadol Reduces Inflammatory Pain Response in Rats

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Abstract

Aim: Isovaline is a new and promising analgesic with an antinociceptive effect and, unlike μ-opioid agonists, interacts with aminobutyric acid receptors without causing sedation or respiratory depression. In this study we aimed to investigate whether subcutaneous application of isovaline alone has anti-hyperalgesic, anti-allodynic, and anti-edema effects on inflammatory pain experimentally induced using carrageenan.

Methods: In this study, isovaline, tramadol, and the combination of isovaline and tramadol were subcutaneously administered to rats with carrageenan-induced inflammation of the hind paws. Hyperalgesia in response to thermal stimuli and allodynia in response to mechanic stimuli were assessed by using a thermal plantar test and a dynamic plantar aesthesiometer, respectively.

Results: The administration of subcutaneous isovaline 400 mg/kg and tramadol 4 mg/kg combination effect was higher than the other groups on latencies and thresholds ($p<0.001$, $p<0.001$, respectively). Additionally, isovaline 400 mg/kg administration caused a statistical difference in latencies when compared with carrageenan, isovaline 200 mg/kg, and tramadol 2 mg/kg groups (($p<0.001$, $p<0.001$, $p<0.001$, respectively) and a statistical difference in thresholds when compared with carrageenan, tramadol 2 mg/kg and tramadol 4 mg/kg groups (($p<0.001$, $p<0.001$, $p=0.008$, respectively). When isovaline 200 mg/kg was used in combination with tramadol 2mg/kg, the latencies and thresholds were significantly higher than either treatment alone tramadol 2 mg/kg and carrageenan groups ($p\leq 0.001$, $p<0.001$, respectively).

Conclusions: The results of this study demonstrated that the subcutaneous administration of isovaline had analgesic efficacy and was effective in combination with tramadol when used for the treatment of inflammatory pain.

Keywords: *Inflammatory pain, isovaline, tramadol, paw edema*

1. Introduction

Tissue infection, trauma, and injury, which can cause the release of inflammatory mediators, often result in inflammatory pain[1,2]. Inflammatory mediators, such as potassium, serotonin, substance P, nitric oxide, bradykinin, and prostaglandins are responsible for the development of nociceptive hypersensitivity[3,4]. Analgesic medications allow us to control pain via the central or peripheral mechanisms[5,6]. The opioids currently used in the treatment of pain may result in complications during acute or chronic administration, such as sedation and respiratory depression[7,8].

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Tramadol hydrochloride ((1RS, 2RS)-2-[(dimethylamino)methyl]-1-(3-methoxyphenyl)cyclohexanol HCl) is a weak μ-opioid receptor agonist, which has both analgesic and adjuvant efficacies and is used commonly as multimodal analgesia for the treatment of postoperative pain. It inhibits the pre-synaptic reuptake of noradrenaline (NA) and serotonin (5-HT) while stimulating the release of 5-HT[9,10]. Studies have reported that the peripheral administration of tramadol has antinociceptive and anti-inflammatory effects[11-13]. As tramadol does not cause respiratory depression, these medications are preferred in treatments; however, their analgesic efficacies are dose-dependent. This has led to several challenges, particularly during attempts to cope with pain, and encouraged the search for new analgesics.

Isovaline (2-amino-2-methyl butanoic acid) is a new and promising non-proteinogenic amino acid that displayed analgesic efficacy and no respiratory depression or effects on the central nervous system

in animal experiments[14,15]. Whitehead et al. reported that the administration of isovaline with propofol was observed to be like fentanyl with propofol for anesthesia in mice[16]. Studies have shown that isovaline has an antinociceptive effect and unlike μ-opioid agonists, interacts with aminobutyric acid (GABA) receptors without sedation and respiratory depression. As isovaline activates GABA and group II metabotropic glutamate receptors, the coadministration of isovaline with an opioid may provide a stronger analgesic effect[17,18].

This study aimed to investigate whether subcutaneous application of isovaline alone has anti-hyperalgesic, anti-allodynic, and anti-edema effects on inflammatory pain experimentally induced using carrageenan.

2. Materials and methods

2.1. Animals

The Medical Sciences Experimental Research Centre of KSU provided male Wistar rats (240–260 g); six rats were used in each experimental group and handled for a minimum of 2 weeks before the experiments. The study was approved by the animal research committee of KSU (2016/06-04). All procedures were performed by the guidelines of the IASP (International Association for the Study of Pain) Committee for Research and Ethical Issues. Assessment of thermal latency and mechanic allodynia were accepted for primary outcomes.

The rats were housed in a sound-proofed room in regulated conditions (temperature, 22–24 °C; relative humidity, 40%–60%; 12 h (06:00–18:00) of light and 12 h of darkness) and fed with ad libitum water and food pellets. The air was changed 8 to 12 times every hour for the duration of the study. The number of animals used was kept to a minimum and each animal was used once.

2.2. Carrageenan-induced paw inflammation

Inflammation was induced by intraplantar injections of carrageenan (lambda carrageenan, Sigma-Aldrich Chemie GmbH, Munich, Germany) into the hind paws of rats. Carrageenan is ideal for the investigation of the indicators of inflammatory pain and anti-inflammatory factors and is often used as a medium for assaying inflammation during the development of new therapies. After collection of the baseline measurements, the rats were administered sevoflurane (1%–2% in oxygen) anesthetic. The right rear paw was administered an intraplantar injection of 0.1 mL of 2% (w/v) carrageenan by using a 26-gauge needle on a 1 mL syringe; for the control group, an equal volume of saline solution was administered via the same method.

2.3. Experimental Procedures and Drugs

As stress can skew measurements of the nociceptive threshold, the experiments were conducted by the same researcher in a quiet testing room close to the rat colony room to minimize the stress caused by the laboratory conditions. The rats were placed in the colony room 2 weeks before the experiments and were acclimatized to the experimental conditions for 1 week. The animals were handled by institutional guidelines. The researcher accustomed the rats to being handled by holding them a minimum of three times a day, for 20–30 s, for 3 days before the experiment so that on the day of the experiment, the rats did not react adversely to handling. The rats were acclimated to the experimental setup by the introduction of the rats to the experimental apparatus for a minimum period of 30 min, three times per day, for 3 days before the experiment. Once the rats were settled, baseline measurements were collected from all animals 1 h before the injection of the hind paw to allow sensor calibration.

As the experiment was conducted under blinded conditions, the measurements were all collected without the researchers' knowledge of the animal treatments.

2.4. Drugs

S-isovaline monohydrates were purchased from ACROS Organics (Geel, Belgium). The other drugs used in the experiments were purchased from Sigma-Aldrich (Munich, Germany). Isovaline or saline, in a total volume of 1 mL for subcutaneous (SC) was administered at 60 min after the carrageenan or saline injection. For the vehicle-only control groups, equal volumes of medium were injected subcutaneously. The pilot studies were used to determine the appropriate doses of drugs; S-isovaline monohydrates (200 mg/kg and 400 mg/kg SC) or tramadol (2 mg/kg and 4 mg/kg SC) were injected into the rats.

2.5. Sensory Testing Procedure

Sensory abnormalities, such as hyperalgesia in response to thermal stimuli and allodynia in response to mechanical stimuli, were assessed by using a thermal plantar test and a dynamic plantar aesthesiometer, respectively. For the daylight phase of the cycle, the tests were conducted on groups of six animals each in a quiet room kept at 23°C–25°C between 09:00 and 13:00.

2.6. Assessment of Thermal Latency

A system to measure delayed paw withdrawal in response to thermal stimulation (Commat, Ankara, Turkey) was used to detect if thermal hyperalgesia was present [19]. The rats were placed in separate boxes made of plexiglass measuring 10×20×24 cm. The boxes were set on a transparent glass base. After 15 min of acclimation, a radiant heat source attached to a moveable arm underneath a pane of glass was maneuvered into place to apply heat to the mid-plantar region of the rear paws. The idle intensity of the heat source was set to 1% of the maximum intensity. The purpose of the heat source was to concentrate the thermal stimulus on the correct part of the rear paw. Before any baseline latencies could be set, the light intensity was standardized, and it was maintained for the duration of the experiment. When set to deliver infrared stimulus at 25% of maximum intensity, the apparatus induced withdrawal of the paw after 10–12 s.

The mechanism was triggered by the withdrawal of the paw by the rat when the pain was felt. As the paw was pulled back, a beam of light was broken; this switched off the photocell and the infrared generator, and the timer, which measured the delay in withdrawal. This method for the measurement of paw withdrawal was accurate to 0.1 s. The thermal source automatically switched off after 25 s (cut-off delay) if the paw was not withdrawn to minimize animal suffering. Each rat had both rear paws tested three times for baseline determination and three times during an hour-long testing period. The tests on each rear paw were conducted 5 min apart and the average of three results was calculated. The values refer to the time before the paw was withdrawn.

2.7. Assessment of Mechanical Allodynia

The threshold before the rat withdrew its paw in response to mechanical stimulation was measured to determine mechanical allodynia. To measure the rat's sensitivity to benign, light touches of its paws, a dynamic plantar anesthesiometer was used. This is a mechanical version of the von Frey hair test (Ugo Basile, Comerio, Italy). Mechanical allodynia was considered present if there was a significant reduction in the threshold at which the rat withdrew its paw in response to mechanical stimulation [19].

The used rats were in separate plexiglass boxes measuring 10×20×24 cm placed on a steel mesh surface. Using a metal rod measuring 0.5 mm in diameter, force (increasing at 2.5 g/s) was constantly applied to the plantar region of the rear paw until the rat lifted its paw away, at which point the threshold for paw withdrawal (in grams) was recorded digitally. The mechanical stimulus automatically cut off at 50 g to prevent excessive tissue damage. Each paw was tested at least three times to establish a baseline and again over the 1-hour test period with a 5 min interval between each test.

The average for these tests was used for analysis. The values presented refer to the threshold for withdrawal of the paw.

2.8. Assessment of Paw Edema

After the sensor tests were completed, the mass of each paw was measured, and tissue samples were collected 210 min after the first injection was administered. The rats were then anesthetized with a light dose of sevoflurane and killed by decapitation. The paws were amputated at the ankle and measured to determine the mass in grams.

2.9. Statistical Analysis

The statistical analysis was conducted with SPSS 19 for Mac (IBM SPSS Statistics, Chicago, IL). The data are reported as mean group values ± SD (standard deviation). The results were assessed for normality with the Shapiro-Wilk test and equality of variance with the Mauchly test of sphericity and the Levene test. The data were analyzed by a repeated measure ANOVA followed by the Bonferroni test for between-group comparisons. We analyzed the interaction between 2 factors (the effects of time after drug administration and time by group interaction). When the Mauchly test was significant, normality and equality of variance were not violated in groups and the Greenhouse-Geisser adjustment was performed to determine the statistical significance of the factors (time after treatment and time group interaction). A p-value of <0.05 was considered statistically significant.

2.10. Sample Size

A priori sample size was estimated from paw edema. The pilot study results were analyzed by the One-Way ANOVA test. The sample size was determined based on the difference between paw edema in the carrageenan-induced inflammatory pain model. At one time point (240 minutes), the change in edema (Mean 45-77, SD: 12) in the number of 6 animals per group provided sufficient statistical power (1 - β ≥ 0.95). Study statistical power was confirmed via a sensitivity analysis performed with G*Power 3.1.9.2 software (University of Dusseldorf; http://www.gpower.hhu.de/en.html).

3. Results

3.1. Effects of Subcutaneous Administration of Isovaline on Latencies and Thresholds in a dose-dependent manner

Before the injections (Saline, Isovaline 200 mg/kg, and 400 mg/kg), no statistically significant differences were found between the basal latencies and thresholds. (p>0.05) We used a repeated measure ANOVA followed by Bonferroni's correction to analyze the significance of the data. There are statistically significant differences produced by time and by the time*group interactions (p<0.001) on latencies. Isovaline 200 mg/kg and 400mg/kg affected latencies in the first hour when compared with saline group latencies (p=0.003, p=0.001, respectively) (Figure.1a).

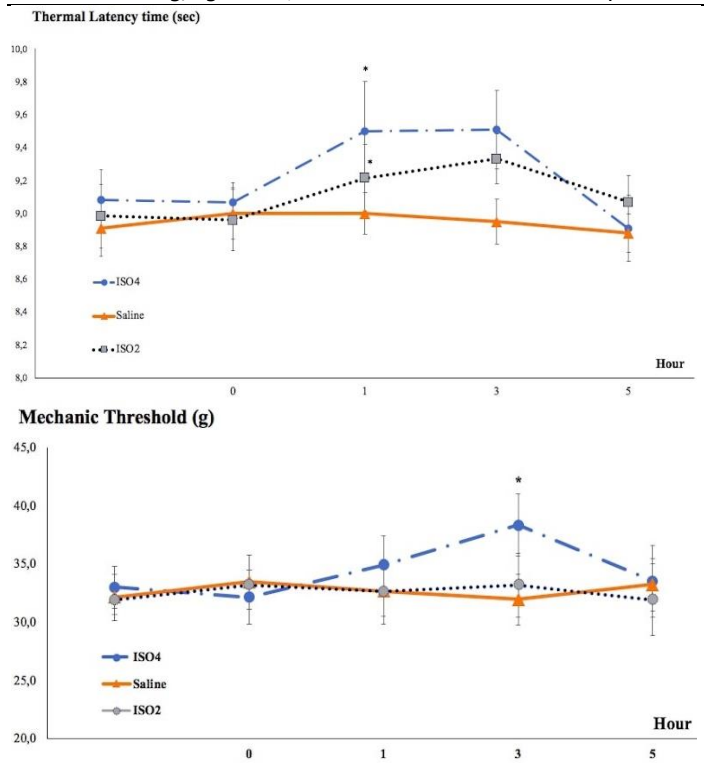
No significant differences were produced by time (p=0.116) however by time*group interaction is analyzed a significant difference was found (p=0.025) on thresholds. Isovaline 400mg/kg had a transient effect on the threshold at the third hour (p=0.010) (Figure.1b).

3.2. Effects of Subcutaneous Administration of Isovaline and Tramadol Combinations on Latencies in Carrageenan-Induced Inflammatory Pain

We used a repeated measure ANOVA test followed by Bonferroni's correction to analyze the significance of the data. Statistically significant differences were found in latencies produced by time and time*group interaction (p<0.001). When the post hoc test was used, the administration of isovaline 400 mg/kg and tramadol 4 mg/ kg combination effect was higher than the other groups (CARR, ISO4, TRA4, TRA2, TRA2ISO4) on latencies ((at 1. hour p<0.001, p<0.001, p<0.001, p<0.001, p<0.001, respectively; at 3. hour: p<0.001, p<0.001, p<0.001, p<0.001, p<0.001, respectively; at 5. hour: p<0.001, p=0.001, p<0.001, p<0.001, p<0.001, respectively).

Figure 1a-1b

Comparison of subcutaneous isovaline 200mg/kg (ISO2), isovaline 400mg/kg (ISO4), and Saline efficacies in healthy rats.



Dose-dependent changes in paw withdrawal latencies (a) and mechanical thresholds (b) Each point represents the mean value of six rats, and vertical bars indicate SD Statistical evaluation was performed by repeated measure ANOVA with Turkey multiple comparison post hoc test. The results were assessed for normality with the Shapiro-Wilk test and equality of variance with the Mauchly test of sphericity and Levene test. Statistical significance (P < 0.001 repeated measure ANOVA followed by Tukey HSD test) followed as determined by comparison with the curve of the control group*

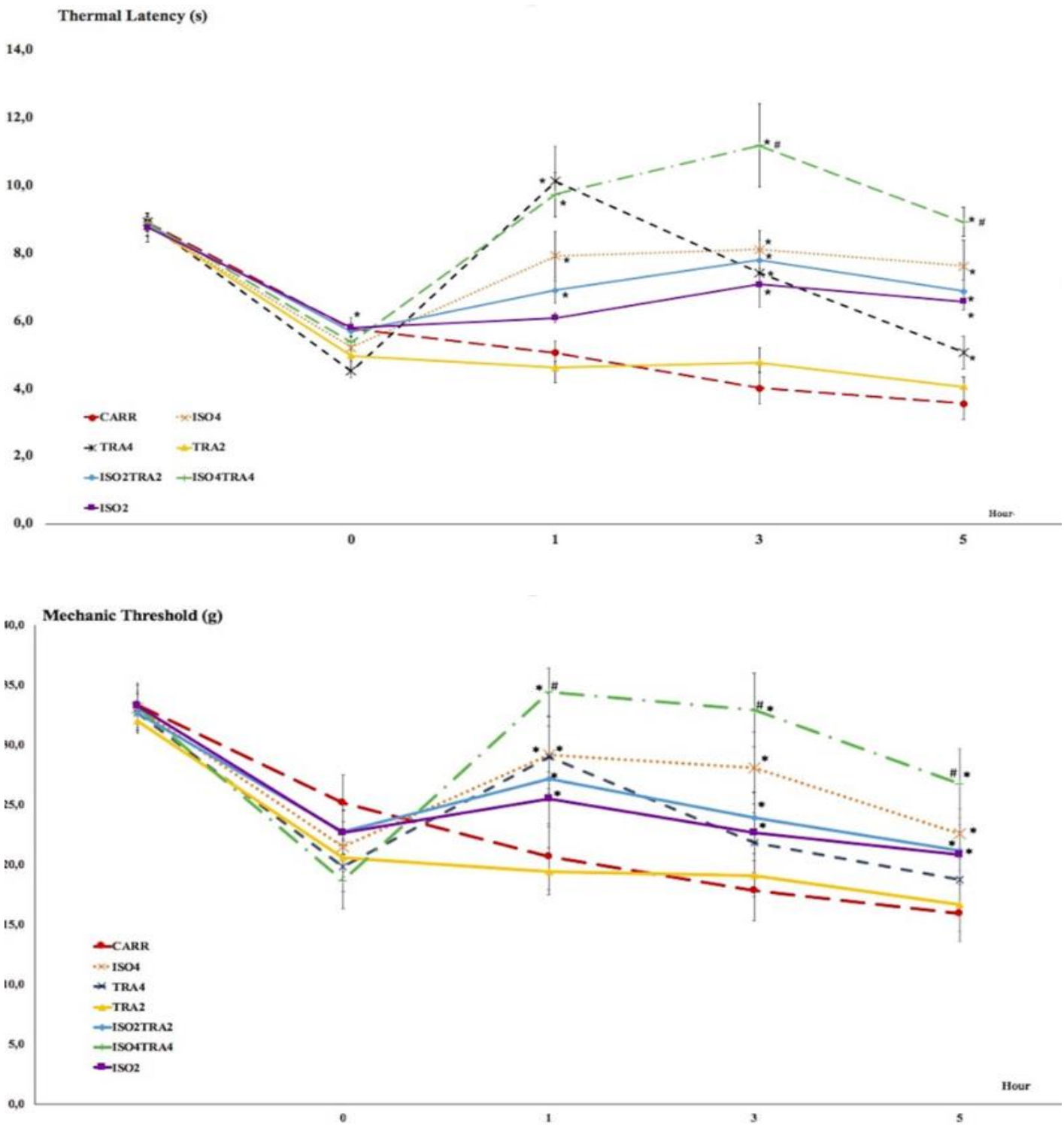
Additionally, isovaline 400 mg/kg administration caused a statistically significant difference in latencies when compared with carrageenan, isovaline 200 mg/kg, and tramadol 2 mg/kg groups. (p<0.001, p<0.001, p<0.001, respectively). When isovaline 200 mg/kg was used in combination with tramadol 2mg/kg, the latencies were significantly higher than either treatment alone tramadol 2mg/kg and carrageenan groups (p<0.001, p<0.001, respectively). There is no significant difference in the latencies between the administration of isovaline 200 mg/kg and tramadol 2 mg/kg combination and tramadol 4mg/kg alone or isovaline 400 mg/kg alone (p>0.05) (Figure.2a).

3.3. Effects of Subcutaneous Isovaline and Tramadol Combinations on Thresholds in Carrageenan-Induced Inflammatory Pain

We used a repeated measure ANOVA test followed by Bonferroni's correction to analyze the significance of the data. Statistically significant differences were found on thresholds produced by time and time*group interaction (p<0.001). When the post hoc test was used, the administration of isovaline 400 mg/kg and tramadol 4 mg/kg combination effect was higher than the other groups (CARR, ISO4, TRA4, TRA2, TRA2ISO4) on thresholds (at 1. hour p<0.001, p=0.011, p=0.007, p<0.001, p<0.001, respectively; at 3. hour: p<0.001, p=0.035, p<0.001, p<0.001, p<0.001, respectively; at 5. hour: p<0.001, p=0.046, p<0.001, p<0.001, p=0.002, respectively).

Figure 2a-2b

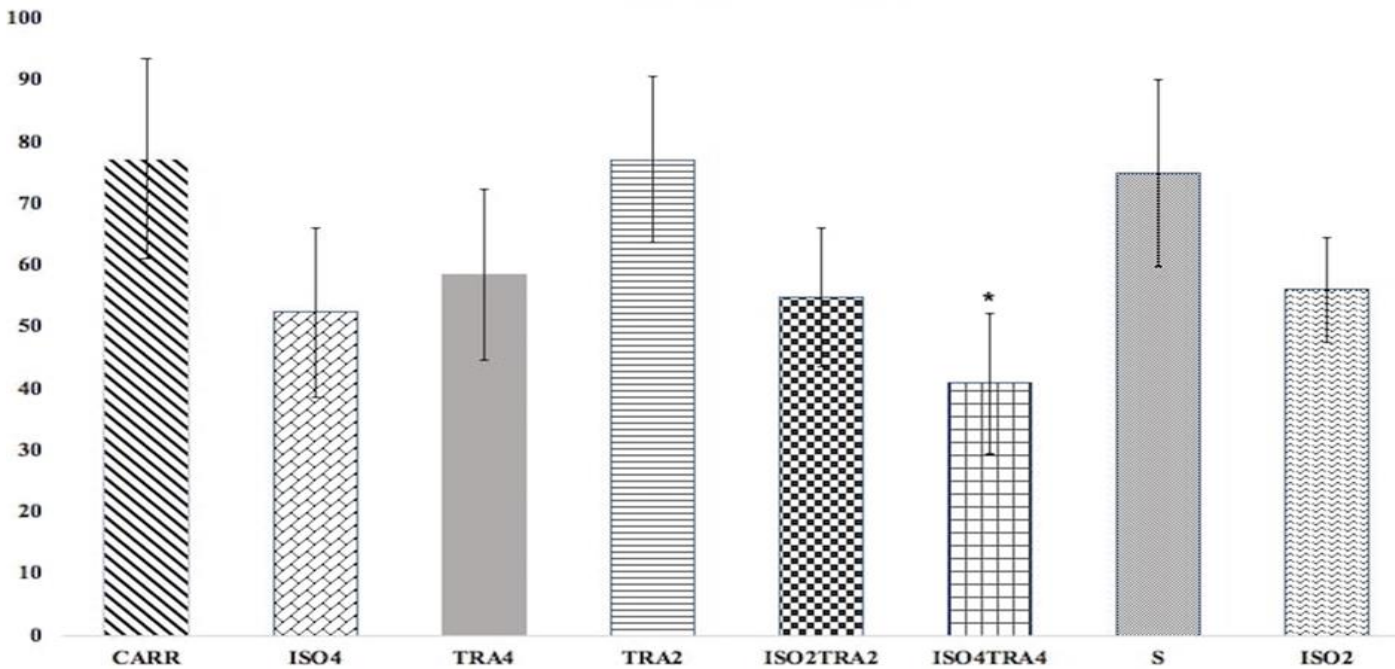
Time course of the paw withdrawal latencies



*Time course of the paw withdrawal latencies (a) and mechanical thresholds (b) of groups were administrated subcutaneous Isovaline 400 mg/kg (ISO4), TR4 (Tramadol 4mg/kg), TR2 (Tramadol 2mg/kg), Isovaline 200 mg/kg (ISO2), ISO2TR2 (Isovaline 200mg/kg coadministration with Tramadol 2mg/kg), ISO4TR4 (Isovaline 400mg/kg coadministration with Tramadol 4mg/kg) in Carrageenan (CARR) induced rats. Each point represents the mean value of six rats, and vertical bars indicate SD. Statistical evaluation was performed by repeated measure ANOVA with Turkey multiple comparison post hoc test. The results were assessed for normality with the Shapiro-Wilk test and equality of variance with the Mauchly test of sphericity and Levene test. Statistical significance (*P <0.05, repeated measure ANOVA followed by Tukey HSD test) followed as determined by comparison with the curve of the CARR group. #P <0.05 indicates significant differences as compared to all groups.*

Figure 3

Change in paw mass(%)



*One time point (240 minute after carrageenan injection) of the paw edema of groups were subcutaneous Isovaline 400 mg/kg (ISO4), TR4 (Tramadol 4mg/kg), TR2 (Tramadol 2mg/kg), Isovaline 200 mg/kg(ISO2), ISO2TR2 (Isovaline 200mg/kg coadministration with Tramadol 2mg/kg), ISO4TR4 (Isovaline 400mg/kg coadministration with Tramadol 4mg/kg), Saline (S) in Carrageenan (CARR) induced rats. Each point represents the mean value of six rats, and vertical bars indicate SD. Statistical significance (*P ≤ 0.001 one-way ANOVA followed by Tukey HSD test) followed as determined by comparison with the curve of the CARR group.*

Additionally, isovaline 400 mg/kg administration caused a statistically significant difference in thresholds when compared with carrageenan, tramadol 2 mg/kg, and tramadol 4 mg/kg groups (p<0.001, p<0.001, p=0.008, respectively). When isovaline 200mg/kg was used in combination with tramadol 2 mg/kg, the thresholds were significantly greater than treatment alone tramadol 2 mg/kg and carrageenan groups (p≤ 0.001, p<0.001, respectively). No significant difference was found on the threshold between the administration of isovaline 200 mg/kg tramadol 2 mg/kg combination and tramadol 4 mg/kg alone or isovaline 400 mg/kg and isovaline 200 mg/kg alone groups (p>0.05) (Figure.2b).

3.4. Effect of the Combination of Subcutaneous Isovaline and Tramadol on Carrageenan-Induced Paw Edema

Carrageenan injection caused paw edema in rats. One Way ANOVA test detected a significant difference in the carrageenan-injected paw mass after 240 min induction of inflammation between groups (p<0.001). Isovaline 400 mg/kg and tramadol 4 mg/kg combination produced a significant reduction of edema when compared with tramadol 2 mg/kg and saline treatment groups (the Tukey post hoc p=0.001; Figure 3).

4. Discussion

In this study, we found administration of isovaline 400 mg/kg alone and tramadol 4mg/kg alone effective on latencies and thresholds. The highest effect on latencies and thresholds was found when isovaline 400 mg/kg and tramadol 4mg/kg combination was administered. When isovaline 200 mg/kg was used in combination with tramadol 2 mg/kg, the latencies and thresholds were significantly higher than either treatment alone tramadol 2 mg/kg. The combined use of subcutaneous isovaline and tramadol enhanced the anti-hyperalgesic and anti-allodynic effects compared with the administration of the individual treatments.

Previous studies have suggested that isovaline may be a novel promising analgesic for anesthesia[20]. In an investigation of the anesthetic and analgesic efficacy of isovaline, the intravenous (IV) 50% effective dose (ED) was found to be 76 mg/kg and 500 mg/kg IV isovaline was found to significantly reduce total licking in the formalin test[15]. Whitehead et al. reported that the use of isovaline in combination with propofol for general anesthesia and “awake” sedation was safer than the propofol-fentanyl combination. This study concluded that isovaline was a suitable agent for total intravenous anesthesia and awake sedation. Isovaline alone or in combination with propofol did not cause respiratory suppression and propofol did not augment the effects on the central nervous system[16].

Both the R and S isomers of isovaline have anti-allodynic effects and neither shows acute toxicity[15]. It has been observed that no central nervous system depression was seen after the peripheral application of isovaline at a dose 10-fold higher than that applied for allodynia; even when a 20-fold higher dose was applied, no change in body temperature was observed. It was found to be safer than baclofen, which affects the GABAB receptor[21]. The tolerability of high doses of isovaline is an advantage, as it has a low potency; however, studies have been conducted on R-isovaline, S-isovaline, aminoisobutyric acid (AIB), the 1-amino-1-cyclobutane carboxylic acid (ACBC), which are isovaline molecules with different structures. Fung et al.[22] reported the antinociceptive effects of S-isovaline, the R-stereoenantiomer, and its cyclized isomer (ACBC) following

systemic administration. Both R-isovaline, S-isovaline, and ACBC decreased the response in phase II of the formalin foot assay without a loss of efficiency on the rotarod in mice.

As the potency of isovaline is low and because of the low side-effects such as respiratory depression and nausea and vomiting, it was thought that it could be more effective with an opioid, so in this study, the efficacy was evaluated of the combined use of isovaline and tramadol[16]. In addition to the efficacy of isovaline compared to the control group, when it was used together with tramadol, it was found to be more effective than either tramadol or isovaline used alone.

A previous study investigating the combined use of tramadol and baclofen found that baclofen enhanced tramadol's antinociceptive effect.[23] Like isovaline, baclofen affects the GABAB receptor[24]. Isovaline provides its analgesic efficacy through action on the GABAB receptors in the cutaneous tissue containing keratinocytes and nerve endings [21,25]. GABAB receptors on small primary afferent neurons with Aδ and C fibers regulate nociceptive transmission in the peripheral tissues and the spinal cord. GABAB receptors are G-protein coupled receptors and they have the capacity for presynaptic and post-synaptic inhibition[26]. Through the G-proteins, isovaline either increased the permeability to K^+ or suppressed voltage-gated Ca^{2+} channels [27].

Whitehead et al.[21] have reported that the subcutaneous application of isovaline was effective in the osteoarthritis (OA) model; however, the OA model was not inflammatory but had a degenerative status. The improvement in the degenerative OA model was attributable to the activation of the GABAB receptors present in the synovial fluid in the knee joint[28]. In this study, we prefer subcutaneous administration of isovaline and tramadol because subcutaneously administered substances are usually absorbed in a slower rate compared to other parenteral routes, and this provides a long-lasting effect. Although the exact absorption mechanism is not fully understood, it is considered that minimal lymphatic absorption occurs via the penetration of macromolecules to small capillaries in the subcutaneous tissue[29]. In addition, the effect of isovaline on GABAB receptors that are considered to be present in the subcutaneous tissue may explain the subcutaneous administration of isovaline effects[28].

The antinociceptive mechanism of tramadol results in μ-opioid receptor activation and inhibition of the reuptake of serotonin/noradrenalin via a non-opioid route. In studies made with tramadol, μ-opioid receptor activation and the inhibition of the reuptake of serotonin and noradrenalin resulted in a reduction in the development of hyperalgesia in carrageenan-induced inflammation[30,31].

In this study, the combination of isovaline and tramadol significantly reduced paw edema induced by carrageenan. The combination of the two treatments resulted in a greater reduction in paw edema than the use of either treatment individually. Therefore, it can be considered that the combination of isovaline and tramadol exerted anti-inflammatory efficacy through a different mechanism.

Further studies are planned to examine the combined use of isovaline with other analgesics. The current study may be limited as it was not possible to combine isovaline with different analgesic drugs such as opioids or NSAID.

5. Conclusion

In conclusion, the results obtained in this study demonstrated that the subcutaneous administration of isovaline alone had anti-hyperalgesic, anti-allodynic, and anti-edematous efficacy on inflammatory pain induced experimentally by using carrageenan. Furthermore, when isovaline was used together with tramadol, the anti-hyperalgesic and anti-allodynic effects were enhanced. Isovaline has been reported to have analgesic efficacy without causing respiratory depression; as a new drug, it may be effective in combination with tramadol, which is a weak opioid with few side effects when used for the treatment of inflammatory pain.

Statement of ethics

The study was approved by the animal research committee of KSU (2016/06-04).

Conflict of interest statement

The authors declare that they have no financial conflict of interest with regard to the content of this report.

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Author Contributions

All authors contributed equally to the article. All authors read and approved the final manuscript.

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