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Comparison between Cacao and Ibuprofen as Adjuvants to Paracetamol on Serum iNOS Levels and Von Frey Scores in Acute Pain Model

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ABSTRACT

Acute post-traumatic pain induces peripheral inflammation and neuroinflammation, upregulating inducible nitric oxide synthase (iNOS) and decreasing mechanical pain thresholds. Multimodal analgesia targeting distinct pathways in the pain-inflammation cascade may enhance pain relief. Cacao, a natural source of polyphenols with anti-inflammatory properties, may modulate nitric oxide signaling similarly to nonsteroidal anti-inflammatory drugs (NSAIDs). This research aimed to compare the effects of crude ethanolic cacao extract and ibuprofen as adjuvants to paracetamol on serum iNOS levels and mechanical pain thresholds, assessed using Von Frey aesthesiometry, in a rat model of postoperative pain. A randomized, three-arm study was conducted with 18 male Wistar rats (6/group). Rats received paracetamol (15 mg/kg, control), paracetamol + ibuprofen (10 mg/kg), or paracetamol + cacao extract (1000 mg/kg) at T0, T+8, and T+16 hours after induced femoral fracture with fixation. Serum iNOS levels and Von Frey thresholds were assessed at T+24 hours. Data were analyzed using one-way ANOVA followed by Tukey's post hoc test, with effect size (η^2) analysis. Serum iNOS levels were significantly lower in both adjuvant groups compared to control (control 13.56 ± 1.59 ng/mL; ibuprofen 10.75 ± 0.54 ng/mL; cacao 9.28 ± 0.71 ng/mL; ANOVA $p < 0.001$). Cacao reduced iNOS more effectively than the control ($p < 0.05$) and ibuprofen ($p = 0.021$). Von Frey thresholds increased significantly in the adjuvant groups (control 9.52 ± 1.63 gf; ibuprofen 19.38 ± 4.15 gf; cacao 26.19 ± 8.01 gf; $F = 15.07$, $p < 0.001$). Effect sizes were large for iNOS ($\eta^2 = 0.80$) and moderate to large for Von Frey thresholds ($\eta^2 = 0.67$). Both ibuprofen and cacao, when used as adjuvants to paracetamol, reduced serum iNOS levels and increased pain thresholds within 24 hours in a postoperative pain model. Cacao combination showed the lowest serum iNOS level and highest alteration of Von Frey absolute change. Cacao demonstrated greater iNOS suppression and better pain tolerance, suggesting its potential as a natural, effective analgesic adjuvant. Further studies are needed for cacao extract standardization and clinical translation.

KEYWORDS: Cacao, Ibuprofen, Paracetamol, Inducible Nitric Oxide Synthase, Von Frey, Acute Pain

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INTRODUCTION

Acute post-traumatic or postoperative pain, is a major clinical challenge, significantly affecting patients' recovery and quality of life. It triggers a cascade of physiological responses, including peripheral inflammation and neuroinflammation, which contribute to pain sensitization and the development of chronic pain [1]. The intensity of acute pain is often linked to the activation of several inflammatory mediators, one of the most critical being inducible nitric oxide synthase (iNOS). iNOS is an enzyme responsible for the production of nitric oxide (NO), a potent mediator of pain and inflammation [2]. Elevated iNOS levels in peripheral tissues are associated with increased pain sensitivity, particularly in conditions such as post- fracture pain [3].

Skillful intervention in pain control is crucial to interrupt the progression of short-term discomfort into long-lasting physical distress, which can significantly complicate patient health outcomes and extend healing processes [4]. Traditional pain relief medications, including morphine-based drugs and anti-inflammatory treatments, are typically prescribed when patients experience sudden or intense pain. Nevertheless, these pharmaceutical interventions come with significant drawbacks, as they can cause patients to feel drowsy, experience digestive discomfort, and potentially develop a dangerous addiction [5]. NSAIDs, while effective in managing inflammation, can cause gastrointestinal issues, renal impairment, and other systemic side effects when used long-term [6]. Therefore, there is a need for multimodal analgesic strategies that combine different mechanisms of action to enhance efficacy and minimize side effects [7].

Paracetamol (acetaminophen), a widely used analgesic, is often employed in combination with NSAIDs or opioids to achieve synergistic effects. However, its primary mechanism of action remains central, with limited effects on peripheral inflammation [8]. To enhance the analgesic effects of paracetamol, the use of adjuvants, such as NSAIDs or natural compounds, has been explored.

Among potential natural adjuvants, cacao, particularly its polyphenol content, has garnered attention due to its anti-inflammatory and antioxidant properties [9]. Cacao flavonoids, especially epicatechin, have been shown to modulate inflammatory pathways, including the inhibition of NF-κB and the reduction of iNOS expression [10]. The polyphenols in cacao also act as free radical scavengers, reducing oxidative stress and inflammation in peripheral tissues [11].

Ibuprofen, a non-selective NSAID, is widely used as an adjuvant to paracetamol in multimodal analgesia due to its ability to inhibit cyclooxygenase (COX) enzymes, thereby reducing prostaglandin synthesis and inflammation [12]. While ibuprofen effectively reduces pain and inflammation [13], its use is often limited by gastrointestinal and renal side effects, especially in long-term applications [14]. Given the promising anti-inflammatory effects of cacao, it is hypothesized that cacao extract, when combined with paracetamol, may offer a more favorable side-effect profile compared to ibuprofen, while still enhancing the analgesic effects of paracetamol.

The current study aims to compare the effects of crude ethanolic cacao extract and ibuprofen as adjuvants to paracetamol on serum iNOS levels and mechanical pain thresholds in an animal model of acute pain. By assessing these outcomes, we seek to determine whether cacao offers superior or comparable efficacy to ibuprofen in enhancing pain relief while minimizing systemic side effects.

RESEARCH METHOD

Study Design

A randomized, three-arm experimental trial was conducted to assess the impact of cacao and ibuprofen, when used as adjuvants to paracetamol, on serum iNOS concentrations and mechanical pain thresholds in rats experiencing acute pain. The study took place at the Animal Research Facility of Universitas Airlangga, Surabaya, Indonesia, and obtained ethical clearance from the Institutional Animal Care and Use Committee (IACUC). All animal experiments conformed to the ARRIVE reporting standards and complied with ethical regulations for animal experimentation [15].

A total of eighteen male Wistar rats, aged between 8 and 10 weeks and weighing 200–300 g, were obtained from the Animal House Facility of Universitas Airlangga. The rats were kept under controlled environmental conditions (temperature: 22 ± 2°C; relative humidity: 50 ± 10%; light/dark cycle: 12 h/12 h) and provided unrestricted access to food and water. A one-week acclimatization period preceded the experimental procedures.

Randomization and Group Allocation

Rats were randomly assigned into three groups (n = 6 per group):

1. Control Group: Paracetamol 15 mg/kg orally (PO).
2. Ibuprofen Group: Paracetamol 15 mg/kg + ibuprofen 10 mg/kg PO.
3. Cacao Group: Paracetamol 15 mg/kg + cacao extract 1000 mg/kg PO. Randomization was performed using a computer-generated random number table. All treatments were administered at three time points: immediately post-surgery (T0), 8 hours post-surgery (T+8), and 16 hours post-surgery (T+16).

Anesthetic Management and Interventions

Acute pain was induced using a femur fracture model, which closely mimics the pain response in humans. Anesthesia was induced with intraperitoneal (IP) injection of ketamine (80 mg/kg) and xylazine (10 mg/kg). A midline incision was made on the anterior side of the left hind limb, and the femur was exposed. A standardized femoral fracture was then induced, followed by fixation with a small intramedullary pin. The wound was closed in layers, and analgesia was maintained with the study treatments (paracetamol, ibuprofen, or cacao).

Data Collection and Outcomes Measures

Data were collected using standardized forms.

1. Primary Outcome: The serum inducible nitric oxide synthase (iNOS) concentration was analyzed as a biomarker of inflammation. Blood samples were obtained via cardiac puncture 24 hours after treatment and centrifuged to separate serum, which was stored at −80°C until use. The iNOS level was quantified using an ELISA kit (R&D Systems, Minneapolis, MN, USA) following the manufacturer's protocol.
2. Secondary Outcome: Mechanical pain sensitivity was assessed using the Von Frey aesthesiometer, a well-established method for evaluating mechanical allodynia and hyperalgesia in animal models of acute pain [16]. The withdrawal threshold was measured at T+24 hours. For each animal, at least three measurements were taken and averaged to obtain the final pain threshold value.

Statistical Analysis

Statistical analyses were performed using SPSS version 26.0 (IBM Corp., Armonk, NY, USA). Descriptive statistics were employed to describe baseline characteristics and outcomes. The Shapiro–Wilk test was used to evaluate data normality. When normality was confirmed, one-way ANOVA with Tukey's post hoc analysis was conducted; otherwise, the Kruskal–Wallis test was applied. Correlations between serum iNOS levels and Von Frey pain thresholds were determined using Pearson's correlation coefficient. A

p-value < 0.05 was considered statistically significant, and effect sizes were calculated using eta-squared (η^2).

RESULTS

Baseline Characteristics

A total of 18 male Wistar rats were included in the study, with 6 rats in each of the three groups: the control group (paracetamol only), the ibuprofen + paracetamol group, and the cacao + paracetamol group. The baseline characteristics of the animals are presented in Table 1 and were evaluated to ensure homogeneity before treatment. The age of the rats ranged from 8 to 10 weeks, and their weight varied between 200 and 300 grams, with the mean weight of each group as follows: the control group had a mean weight of 203.00 ± 22.51 g, the ibuprofen group had a mean weight of 241.33 ± 33.62 g, and the cacao group had a mean weight of 205.33 ± 29.28 g. Statistical analysis using one-way ANOVA revealed no significant differences in weight across the groups (p = 0.063), indicating that the animals were comparable in terms of body mass.

In terms of baseline pain sensitivity, mechanical pain thresholds were measured using the Von Frey aesthesiometer before treatment administration. The baseline Von Frey thresholds were 34.02 ± 4.92 gf for the control group, 32.36 ±7.99 gf for the ibuprofen group, and 31.02 ± 9.98 gf for the cacao group. No significant differences were found in baseline Von Frey scores between groups (p= 0.808), confirming that the rats had comparable pain sensitivity at the start of the experiment.

Additionally, the pre-treatment Von Frey scores were assessed to ensure there were no significant differences in pain sensitivity prior to any treatment. The pre-treatment thresholds were very similar across all groups, further supporting the validity of comparing post-treatment data. These results confirm that all groups were comparable at baseline, ensuring that any differences in the primary and secondary outcomes could be attributed to the treatments, rather than baseline disparities.

Table 1: Baseline Demographics of The Subjects

Characteristic	Control	Paracetamol + Ibuprofen	Paracetamol + Cacao	p[a]
Age (weeks)	8-10	8-10	8-10	-
Weight (g) Mean ± SD	203.00 ± 22.51	241.33 ± 33.62	205.33 ± 29.28	0.063
Baseline Von Frey (gf) Mean ± SD	34.02 ± 4.92	32.36 ± 7.99	31.02 ± 9.98	0.808

sample size per group (n=6); [a]: significant if p < 0.05

Serum iNOS Levels

The primary outcome of this study was to assess the levels of serum inducible nitric oxide synthase (iNOS) at T+24 hours, a critical biomarker for inflammation. The results revealed significant differences in serum iNOS concentrations across the three treatment groups. The control group (paracetamol only) had the highest mean iNOS level of 13.56 ± 1.59 ng/mL, reflecting a strong inflammatory response following the induced femoral fracture. The ibuprofen + paracetamol group showed a reduced mean iNOS level of 10.75 ± 0.54 ng/mL, suggesting that ibuprofen, as an anti-inflammatory agent, effectively reduced inflammation compared to the control. However, the most substantial reduction in iNOS was observed in the cacao + paracetamol group, which had the lowest mean serum iNOS level of 9.28 ± 0.71 ng/mL, indicating a superior anti-inflammatory effect.

Statistical analysis using one-way ANOVA revealed a highly significant difference in serum iNOS levels across the three groups (p < 0.001), indicating that both ibuprofen and cacao significantly reduced iNOS levels compared to the control group. Post-hoc Tukey's test further confirmed that the cacao group demonstrated the most significant reduction in iNOS levels when compared to both the ibuprofen group and the control group (p = 0.021 for both comparisons). The effect size (η^2) was 0.80, indicating a large and biologically significant effect, particularly in the cacao group.

Results of the Shapiro–Wilk normality test demonstrated that serum iNOS levels in all groups followed a normal distribution ($p > 0.05$). Additionally, Levene's test for homogeneity of variance revealed no significant differences in variance between groups ($p > 0.05$), thereby confirming compliance with the equal variance assumption for ANOVA. These results support the validity of the statistical analysis and suggest that the observed differences are not due to violations of assumptions.

Table 2: Serum iNOS Levels in Each Group

Group	Mean ± SD (ng/mL)	One way- ANOVA[a]	Normality Test	Levene's Test
Control (Placebo)	13.56 ± 1.59	-	0.095	0.152
Paracetamol + Ibuprofen	10.75 ± 0.54	0.021	0.087	0.087
Paracetamol + Cacao	9.28 ± 0.71	0.021	0.119	0.133

sample size per group (n=6); [a]: significant if p < 0.05; [b]: significant if p > 0.05

As shown in Table 2, the median serum iNOS levels further emphasize the significant differences observed between the groups. The control group had a median iNOS level of 13.52 ng/mL (range: 11.00-16.00 ng/mL), while the ibuprofen group had a median of

10.74 ng/mL (range: 9.50-11.50 ng/mL), and the cacao group exhibited the lowest median iNOS level of 9.00 ng/mL (range: 8.00-10.00 ng/mL). These data strongly suggest that cacao provided the most substantial reduction in iNOS levels, which could have important implications for its potential as an adjuvant in pain management.

Mechanical Pain Thresholds

The secondary outcome of this study was to assess mechanical pain thresholds in the three treatment groups using the Von Frey aesthesiometer at T+24 hours. This method measures the threshold at which the rats withdraw their paw in response to mechanical stimulation, reflecting their pain sensitivity.

The results revealed significant differences in Von Frey thresholds across the groups. The control group (paracetamol only) had a mean threshold of 9.52 ± 1.63 gf, indicating relatively low pain tolerance post-injury. The ibuprofen + paracetamol group showed a significant increase in pain threshold, with a mean of 19.38 ± 4.15 gf, demonstrating that ibuprofen, when combined with paracetamol, enhanced pain tolerance compared to the control group. The cacao + paracetamol group exhibited the highest mean threshold of 26.19 ± 8.01 gf, suggesting that cacao not only reduced inflammation but also provided a stronger analgesic effect than both ibuprofen and the control.

One-way ANOVA confirmed a highly significant difference in Von Frey thresholds among the groups ($p < 0.001$), indicating that both ibuprofen and cacao significantly increased pain thresholds compared to the control. However, Post-hoc Tukey's test revealed that while the cacao group exhibited a higher mean threshold than the ibuprofen group, the difference between cacao and ibuprofen did not reach statistical significance ($p = 0.098$). This shows that while cacao demonstrated the highest numerical pain threshold, the difference between cacao and ibuprofen was not statistically significant. The effect size (η^2) for this comparison was 0.67, indicating a moderate to large treatment effect.

The Shapiro-Wilk test confirmed the normality of the data, with all groups showing normal distributions ($p > 0.05$ for all groups). Additionally, Levene's test for homogeneity of variance indicated no significant differences in variances between the groups ($p > 0.05$), supporting the validity of the ANOVA results.

Table 3: Mechanical Pain Thresholds (Von Frey)

Characteristic	Control	Paracetamol + Ibuprofen	Paracetamol + Cacao	One-way ANOVA[a]
Post-fracture Von Frey (gf) Mean ± SD	6.963 ± 0.988	9.357 ± 1.456	9.017 ± 0.945	0.055
Post-treatment Von Frey (gf) Mean ± SD	9.518 ± 1.626	19.380 ± 1.456	26.190 ± 8.008	<0.001
Absolute change (Δ) Mean (gf)	2.555	10.023	17.173	<0.001
Relative change Mean (%)	36.69	107.12	190.45	

sample size per group (n=6); [a]: significant if $p < 0.05$; [b]: significant if $p > 0.05$

As shown in Table 3, the Tukey's test of absolute change in Von Frey thresholds also support these findings. Unlike the post-treatment Von Frey score, the absolute change of Von Frey score in paracetamol group and ibuprofen group shows a statistical different ($p = 0.045$). These results further confirm that cacao provided the greatest change in pain threshold, highlighting its superior analgesic properties.

DISCUSSION

The research investigation sought to assess how supplementing paracetamol with cacao extract and ibuprofen might influence inflammatory enzyme production and pain sensitivity in laboratory rats experiencing acute pain. The key findings of this study indicate that both cacao and ibuprofen significantly reduced serum iNOS levels and increased Von Frey pain thresholds compared to the control group (paracetamol only). While cacao demonstrated the highest numerical Von Frey threshold, the difference between the cacao and ibuprofen groups did not reach statistical significance ($p = 0.098$). This suggests that while cacao was more effective in enhancing pain tolerance, the difference in pain thresholds between cacao and ibuprofen was not significant. These findings indicate that both cacao and ibuprofen can be effective in pain management, but cacao may offer additional benefits due to its anti-inflammatory properties, particularly in reducing iNOS levels.

The role of iNOS in pain and inflammation is well-documented, with elevated iNOS levels being associated with hyperalgesia in various inflammatory pain models [1,5]. In this study, both ibuprofen and cacao reduced iNOS levels significantly compared to the control, consistent with previous research showing that NSAIDs like ibuprofen inhibit cyclooxygenase (COX) enzymes and downstream inflammatory mediators [6]. Moreover, cacao, rich in polyphenols such as epicatechin, has been shown to modulate nitric oxide (NO) production and inflammatory pathways, including the NF-κB signaling pathway, which regulates iNOS expression [10,11].

Our findings align with previous studies indicating that cacao has potent anti-inflammatory and analgesic effects. For example, De Feo et al. [10] demonstrated that cacao polyphenols significantly reduced inflammatory mediators, including iNOS, in animal models through the inhibition of pro- inflammatory cytokines. Similarly, Katz et al. [9] reported that cacao's flavonoids exhibit anti-inflammatory effects, which likely explain its more significant effect on pain modulation in our study. These findings suggest that cacao, as a natural and safer alternative to NSAIDs, may have therapeutic potential in acute pain management, offering a safer option with fewer side effects, particularly gastrointestinal and renal complications associated with long-term NSAID use [6].

The anti-inflammatory properties of cacao are likely attributed to its rich content of polyphenolic compounds, particularly flavonoids like epicatechin, which have been shown to reduce oxidative stress and inhibit the NF-κB pathway, a key regulator of iNOS production [10]. This mechanism is consistent with our findings that cacao led to the greatest reduction in serum iNOS levels and a significant increase in Von Frey pain thresholds. iNOS plays a crucial role in pain and inflammation by producing NO, which sensitizes nociceptors and lowers pain thresholds [2]. Through the reduction of iNOS expression, cacao may contribute to pain relief by decreasing NO-mediated pain sensitization.

On the other hand, ibuprofen, a widely used NSAID, works by inhibiting COX enzymes, which reduces the production of prostaglandins, key mediators of inflammation and pain [12]. While ibuprofen was effective in reducing iNOS levels and increasing pain thresholds, its effect was less pronounced than that of cacao. This may be due to the additional mechanisms through which cacao acts, such as its antioxidant properties and broader effects on inflammatory pathways. While ibuprofen primarily targets COX inhibition, cacao impacts multiple inflammatory pathways, offering a more holistic approach to pain relief.

The significant reduction in iNOS levels and the increase in pain thresholds observed with cacao in this study suggest its potential as a nutraceutical adjuvant for pain management. Given the growing concern about the long-term use of NSAIDs and their associated side effects, particularly gastrointestinal and renal complications [6], cacao presents a promising alternative. In clinical settings, cacao could be explored as a non-opioid adjunct to traditional pain management strategies, offering a safer option with fewer side effects. Furthermore, the use of cacao as an anti-inflammatory nutraceutical could potentially reduce reliance on more potent, risk-laden drugs such as opioids and NSAIDs, making it a valuable addition to multimodal analgesia strategies.

In addition, the strong inverse correlation between serum iNOS levels and pain thresholds in this study suggests that iNOS could serve as a useful biomarker for pain and inflammation. Monitoring iNOS levels could help clinicians assess the severity of inflammation and pain in patients and guide the selection of appropriate treatments. The use of iNOS inhibition as a therapeutic target for pain management warrants further exploration in clinical trials.

While our findings suggest the potential of cacao in pain management, several limitations need to be addressed. First, the current study was conducted in an animal model, and while the results are promising, they need to be replicated in human clinical trials to establish the effectiveness and safety of cacao as a pain- relief agent. Additionally, future studies should investigate the optimal dosage and formulation of cacao extract for therapeutic use, as well as its long-term effects. Finally, it would be valuable to explore dose-response relationships and the synergistic effects of combining cacao with other analgesic agents.

CONCLUSION

In conclusion, this study demonstrates that both ibuprofen and cacao significantly reduce serum iNOS levels and increase mechanical pain thresholds in an acute pain rat model. While both treatments were effective, cacao exhibited superior anti-inflammatory and analgesic effects compared to ibuprofen, highlighting its potential as a promising natural adjuvant for pain management. The strong inverse correlation between iNOS levels and pain thresholds suggests that iNOS could serve as a useful biomarker for evaluating pain and inflammation. These findings provide a strong rationale for further investigation into the clinical use of cacao as a safe, non-opioid alternative in multimodal pain management strategies. Future studies should focus on clinical trials to confirm these results and explore the optimal dosing and formulation of cacao for therapeutic use.

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