Experimental Research

Systemic lidocaine administration influences NF-kβ gene expression, NF-kβ and TNF- α protein levels on BALB/c mice with musculoskeletal injury

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ABSTRACT

Background: The immune system can produce various inflammatory mediators to protect the body from stress and surgical trauma. However, this excessive inflammatory response will interfere with the body's immune system, causing systemic inflammatory response syndrome and multi-organ failure if allowed to continue. Lidocaine as an anti-inflammatory is used to treat surgical pain and pain arising from the disease process and treat ventricular arrhythmias. This study aims to prove the efficacy of systemic lidocaine injection as an anti-inflammatory drug in BALB/c mice with sterile musculoskeletal injuries.
Methods: This study used a prospective experimental laboratory study on experimental animals of BALB/c mice using a simple randomized design. Sixteen adult white BALB/c mice (male, healthy, 10–12 weeks old, 35–40 g body weight, and no disability) were selected and randomly divided into two groups: the group given lidocaine (2 mg/kg body weight) and a group that was given sterile distilled water. NF-kβ and TNF-α protein levels were detected by ELISA, while mRNA expression of NF-kβ was analyzed and determined by quantitative real-time PCR.
Results: Musculoskeletal injury significantly increased the expression of both mRNA and protein levels of NF-kβ and TNF-α protein level. In addition, the NF-kβ (protein and mRNA) and TNF-α (protein) levels in rats experiencing inflammation due to musculoskeletal injury were significantly decreased in the lidocaine group ($p < 0.001$).
Conclusions: The administration of systemic lidocaine injection was able to inhibit the expression of mRNA NF-kβ, the protein levels of NF-kβ, and protein levels of TNF-α in mice with musculoskeletal injuries.

1. Introduction

Fractures of the long bones associated with soft tissue injury contribute to the inflammatory response [1]. Excessive inflammatory response – if allowed to continue – will disrupt the body's immune system causing systemic inflammatory response syndrome and multi-organ failure [2].

The activation of NF-kβ signaling leads to the production of various inflammatory cytokines, chemokines, and transcription factors, which initiate and modulate inflammatory reactions and regulate the host response to tissue damage. NF-kβ also plays an essential role in regulating the survival and differentiation of immune cells and inflammatory T cells [3–6]. Nuclear factor-kappa β (NF-kβ) has long been considered a prototypical pro-inflammatory signaling pathway, mainly based on the

activation of NF-kβ by pro-inflammatory cytokines, such as interleukin 1 (IL-1) and tumor necrosis factor-α (TNF-α) [7]. TNF-α, IL-1, and IL-6 are the main cytokines in acute inflammatory reactions, which take 4–6 h to induce a reaction after the initial trigger and can last up to 24 h [8]. Increased levels of inflammatory cytokines, such as interleukins (IL-1, IL-6) and TNF-α, will mediate systemic responses and exacerbate organ failure [9]. Nuclear factor-kappa β (NF-kβ) regulates the inflammatory response and bone remodeling processes in bone-forming cells and bone resorption. *In vitro* and *in vivo* evidence suggest that NF-kβ is a significant potential therapeutic target for inflammation-related bone disorders by simultaneously modulating the inflammatory process and bone remodeling [6].

Local anesthetics modulate various steps of the inflammatory cascade and protect the endothelial barrier by reducing neutrophil adhesion and endothelial hyperpermeability, which can lead to major organ dysfunction [10,11]. In addition, local anesthetics can inhibit axonal transport and release some pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α [12]. Lidocaine is a traditional local anesthetic drug that can be safely administered intravenously [13,14]. Lidocaine is an amide local anesthetic that has long been used in clinical practice to treat surgical pain, pain arising from the disease process, and treat ventricular arrhythmias. Lidocaine is also known to have many other properties, including an anti-inflammatory property [15,16].

This study aims to prove the efficacy of systemic lidocaine injection as an anti-inflammatory drug in BALB/c mice with sterile musculoskeletal injuries related to the dynamics profile of the expression of mRNA NF-kβ, protein levels of NF-kβ, and protein levels of TNF-α.

2. Methods

This research is a prospective laboratory experimental study on experimental animals of BALB/c mice using a simple randomized design [17]. These experimental animals were fed standard chow. The cages were cleaned regularly, given lighting (12:12h light-dark cycle), and the temperature was kept at 24 ± 2 °C. The research was conducted in the Laboratory of Molecular Microbiology and Immunology, Faculty of Medicine, Hasanuddin University Makassar. The current study, including the protocol, was reviewed and approved by the animal ethics committee of the Hasanuddin University Faculty of Medicine with approval number: 329/UN4.6.4.5.31/PP36/2021. This research was conducted ethically according to the ARRIVE guidelines in reporting animal research [18].

2.1. Animals

This research used experimental animals with the following inclusion criteria: 1) white, male, adult, and healthy BALB/c mice; 2) 10–12 weeks of age; 3) weight, 35–40 g; and 4) no defects. The exclusion criteria in this study were allergic to injection of ketamine, lidocaine, and animals that died before all blood samples were taken. Mice were obtained from the Maintenance and Development Unit of the Laboratory of Molecular Microbiology and Immunology Experimental Animals, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

The number of mice was determined following the ethics of using experimental animals in health research, using the principles of replacement, reduction, and refinement [19,20]. Federer's formula was used to determine the number of mice required, i.e., (t-1) (n-1) $\geq$ 15, where t is the number of treatment groups and n is the number of mice required in this study, there were two treatment groups, and according to the above formula, at least eight mice were needed per group. Hence, the total number of mice required for the two groups was 16 mice. Mice were randomly assigned into two groups, the treatment and control groups.

2.2. Sample examination

All blood samples were examined at the Molecular Microbiology and Immunology Laboratory of the Faculty of Medicine, Hasanuddin University, Makassar. Protein levels of NF-kβ and TNF-α were detected by MPO Sandwich ELISA with Catalog No. Ls-F12149 and LSF12798 were purchased from Life Span Bio Sciences, Inc. (Seattle, USA) according to the manufacturer's instructions [21–24].

NF-kβ gene expression was measured with GAPDH as the housekeeping gene (Oligo, Macrogen, catalog number: OG280920). Forward primer: CAG CTC TTC TCA AAG GAGCA; reverse primer: TCC AGG TCA TAG AGA GGC TCA; GAPDH forward primer: GGT GCA TGG CCG TTC TTA; GAPDH reverse primer: TCG TTC GTT ATC GGA ATT AACC. RT-PCR was performed using PCR-Bio-Rad BR004129USA machine. A mixture of 22.5 μl PCR Mastermix and SYBR green QRT (Applied Biosystems, Warrington, UK) was prepared. DNA extract of 2.5 μl was added to the 22.5 μl mixture of PCR mix. First stage amplification was performed at 94 °C for 3 min and continued up to 40 cycles 60s at 54 °C, and 45s at 57 °C. Expression of mRNA were calculated using the $2^{-\Delta\Delta CT}$ method [14,25–27].

2.3. Research procedure

BALB/c mice were considered healthy if they were actively moving, had bright eyes, did not have dull hair, and had no defects. The dose of mg/kg weight of lidocaine was converted to volume in units of ml. The sterile distilled water solution was given in ml according to the volume of lidocaine. The expression of mRNA NF-kβ was assessed in units of fold change [28]. The protein levels of NF-kβ are expressed in picograms (pg/ml). The protein levels of TNF-α are expressed in nanograms (ng/ml).

The treatment group. After 4h of experiencing a musculoskeletal injury, they were given an intravenous injection of lidocaine (Kimia Farma Ltd, Jakarta, Indonesia) 2 mg/kg body weight through the tail vein, once every 2h, continuously for 24h. The milligram dose of lidocaine was converted to volume in milliliters (ml).

The control group. After 4h of suffering from sterile musculoskeletal injuries, they were given an intravenous injection of sterile distilled water through the tail vein instead of lidocaine injection.

Before inducing sterile musculoskeletal injuries, 0.3 ml of mice blood was taken from the tail vein and mixed with L6 buffer solution (first blood test). The mice were then anesthetized by injecting ketamine (Combiphar Ltd, Jakarta, Indonesia) 50 mg/kg intraperitoneally. After the mice were sedated, the left thigh was shaved clean. The musculoskeletal injury was performed in a sterile manner. The groin of the mice was clamped firmly with one needle holder for fixation, and the middle of the femur was also clamped with the other needle holders. The left thighbone of the mouse was broken by moving the needle holders located in the middle of the thigh up and down against the needle holders in the groin until a cracking sound was heard and the fracture of the bone in the thigh when moved by hand was felt [29]. It was stabilized with external fixation and the mice were then returned to their cages. Metamizole 50 mg/kg orally was given as an analgesic for two consecutive days. After 4h of suffering from sterile musculoskeletal injuries, 0.3 ml of blood was taken again through the tail vein (second blood test).

The lidocaine group mice were then given an injection of lidocaine at a dose of 2 mg/kg BW through the tail vein, once every 2h for 24h, while the control group was given an injection of sterile distilled water as a substitute for lidocaine. Two hours after the injection of lidocaine and sterile distilled water was completed, 0.3 ml of mice blood was taken again through the tail vein (third blood test). A fourth and final blood sample was drawn at 24h (fourth blood test). Mice were euthanized by cervical dislocation under anesthesia 2 days after musculoskeletal injuries.

2.4. Data analysis

Statistical analyses were done using IBM SPSS 22 with the following test methods:

1. The normality of the mRNA expression of NF-kβ, protein levels of NF-kβ, and protein levels of TNF-α in experimental animals were tested using the Saphiro-Wilks test.
2. Changes in the dynamics of expression mRNA NF-kβ, protein levels of NF-kβ, and protein levels of TNF-α among the experimental animal treatment groups were tested by repeated ANOVA.

3. Results

Based on Fig. 1, differences in mean mRNA expression of NF-kβ, protein levels of NF-kβ, and protein levels of TNF-α were seen 2h after treatment relative to before treatment in the lidocaine or placebo groups.

In the lidocaine group, the mean NF-kβ mRNA level was 4.69 fold changes before the injury, then increased to 11.267 fold changes at 4h after injury, and subsequently decreased to 9.171 fold changes at 2h after treatment, and 5.427 fold changes at 24h after treatment, respectively. In the placebo group, the NF-kβ level (mRNA) was 4.881 fold changes before the injury, then increased to 11.56 fold changes at 4h after injury, and continuously increased to 13.575 fold changes at 2h after treatment, and 15.12 fold changes at 24h after treatment, respectively (Fig. 2).

In the lidocaine group, the mean protein level of NF-kβ was 1.888 pg/ml before the injury, then increased to 8.024 pg/ml at 4h after injury, and decreased to 6.085 pg/ml following 2h after treatment, and continuously decreased to 2.668 pg/ml at 24h after treatment, respectively. In the placebo group, the protein levels of NF-kβ was 2.078 pg/ml before the injury, then increased to 8.267 pg/ml at 4h after injury, and remained to increase to 10.387 pg/ml at 2h after treatment, and 11.997 pg/ml at 24h after treatment, respectively (Fig. 3).

In the lidocaine group, the mean protein level of TNF-α was 171.838 ng/ml before the injury, then increased to 536.978 ng/ml at 4h after injury, and subsequently went down to 415.0473 ng/ml at 2h after treatment, and continuously declined to 215.909 ng/ml at 24h after treatment, respectively. In the placebo group, the protein levels of NF-kβ was 167.545 ng/ml before the injury, then rose to 506.136 ng/ml at 4h after injury, and kept increasing to 626.629 ng/ml at 2h after treatment, and 716.762 ng/ml at 24h after treatment, respectively (Fig. 4).
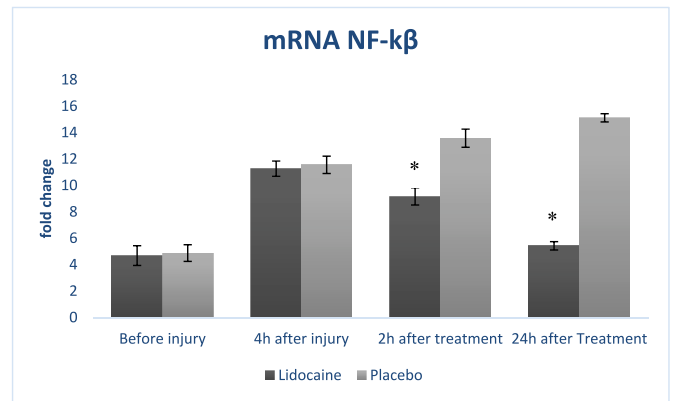

Fig. 2. The difference in mean NF-kβ mRNA expression before and after the treatment in the lidocaine and placebo groups (n = 8 per group). Data are presented as mean ± standard deviation. Mean difference between the lidocaine and placebo groups at 2h and 24h after treatment are statistically significant (*$p < 0.05$).

Fig. 3. The difference in mean protein levels of NF-kβ before and after the treatment in the lidocaine and placebo groups (n = 8 per group). Data are presented as mean ± standard deviation. Mean difference between the lidocaine and placebo groups at 2h and 24h after treatment are statistically significant (*$p < 0.05$).
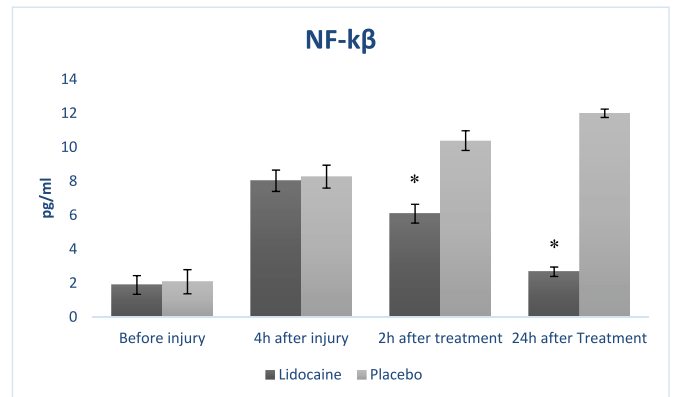
4. Discussion

Compared with after injury, in the lidocaine or placebo groups, differences in the mean NF-kβ mRNA expression began to be seen at 2h
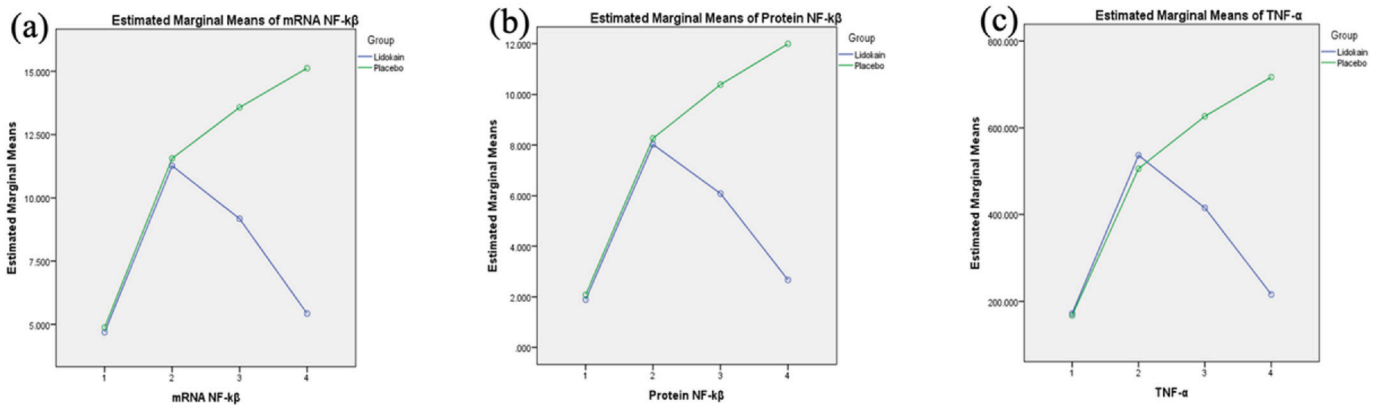

Fig. 1. The mean of (a) NF-kβ mRNA expression (fold change), (b) NF-kβ protein levels (pg/ml), and (c) TNF-α protein levels (ng/ml) in the lidocaine and placebo groups (n = 8 per group) before the injury (1), 4h after the injury (2), 2h after treatment (3), and 24h after treatment (4). Data are presented as mean ± standard deviation. Mean difference between lidocaine and placebo groups at 2h and 24h after treatment are statistically significant ($p < 0.05$).

Fig. 4. The difference in mean protein levels of TNF-α before and after the treatment in the lidocaine and placebo groups (n = 8 per group). Data are presented as mean ± standard deviation. Data are presented as mean ± standard deviation. Mean difference between lidocaine and placebo groups at 2h and 24h after treatment are statistically significant (*$p < 0.05$).
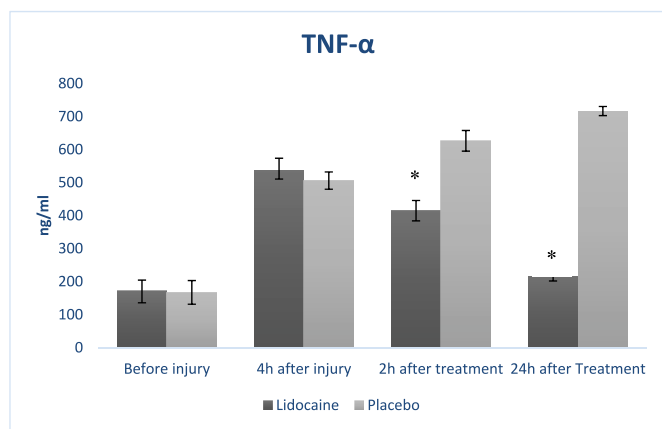
after treatment. There was a decrease in the average mRNA expression of NF-kβ at 2h and 24h after lidocaine treatment. There was an increase in the average mRNA expression of NF-kβ 4h after injury compared to before injury in the placebo group. The mRNA expression of NF-kβ continued to increase at 2h and 24h after treatment.

Administration of systemic lidocaine has an anti-inflammatory effect because it can inhibit the release of pro-inflammatory cytokines, the metabolic activity of leukocytes, and the release of histamine [16,30]. In addition, the anti-inflammatory effect of lidocaine may be mediated by inhibition of NF-kβ activation and cytokine release [31].

Another study found that lidocaine attenuates LPS-induced lung injury through mechanisms involving inhibition of NF-kβ activation and cytokine release, which implies that lidocaine may be a potential anti-inflammatory agent in endotoxemia [32].

In the lidocaine or placebo groups, compared with the mean NF-kβ protein levels after the injury, differences started to take place at 2h after treatment. After 2h of treatment, there was a decrease in the average protein levels of NF-kβ and remained to decrease at 24h after treatment. There was an increase in the average protein levels of NF-kβ 4h after injury compared to before injury in the placebo group. The average protein levels of NF-kβ continued to increase at 2h and 24h after treatment.

Research conducted by Lahat et al. concluded that the anti-inflammatory effect of lidocaine might be mediated by a drug-induced abrogation of T cell proliferation and cytokine secretion independent of cell death. These effects are mediated at least partly by inhibition of NF-kβ signaling [33].

In another study, lidocaine treatment exerts its protective effect on cecal ligation and puncture (CLP)-induced septic rats. The mechanism was related to the inhibitory effect of lidocaine on the mRNA expression level of high mobility group box 1 (HMGB1) in multiple organs, the release of HMGB1 to plasma, and activation of NF-kβ [31]. In addition, Lang et al. reported that lidocaine could inhibit the secretion of IL-8 and IL-10 from intestinal cells through inhibition of NF-kβ activation and decreased Ikβ phosphorylation [34].

TNF-α protein levels increased at 4h after the injury both in the lidocaine or placebo groups, and difference of their levels in those groups began to appear at 2h after treatment. There was a decrease in protein levels of TNF-α at 2h after treatment and 24h after lidocaine treatment. In contrast, the mean protein levels of TNF-α continuously increased at 2h and 24h after treatment in the placebo group.

This study's findings are consistent with those reported by Garutti et al. (2014), who found that, compared to a control group, a pig that underwent lung resection surgery and received intravenous lidocaine produced a significant reduction in TNF-α levels in plasma and bronchoalveolar lavage samples. Moreover, inflammatory and apoptotic changes were observed in the control group [14].

It has been reported that ropivacaine and lidocaine effectively block TNF-α signaling inflammation in endothelial cells by attenuating the recruitment of p85 to the TNF-α receptor. The resulting decrease in Akt, endothelial nitric oxide synthase, and Src phosphorylation reduced neutrophil adhesion and endothelial hyperpermeability. These new anti-inflammatory "side effects" of ropivacaine and lidocaine might provide therapeutic benefits in acute inflammatory disease [11].

The limitations of this study is that we need to inject the lidocaine every 2h for 24 h as it works only for 2h. We may need to see whether the anti-inflammatory effect of lidocaine last longer than 2h in a time course study. We did not examine vital signs of the mice for excluding allergic reaction to ketamine or lidocaine. But we did not observe any sign of severe allergic reaction like anaphylactic shock and all of the mice were in good condition until the end of the experiment.

At this trial, confounding factors, such as infection, were excluded. Use of medication was in accordance with anti-inflammatory dose and treatment to experimental animals were equal. However, further preclinical studies need to be carried out with longer durations and using other pro-inflammatory cytokine markers such as IL-1, IL-6, and IFN-γ to investigate further the anti-inflammatory benefits of systemic lidocaine in musculoskeletal injury.

Our data indicate that lidocaine had an anti-inflammatory effect in rats after experiencing a musculoskeletal injury; this should be further tested in clinical trials to determine its protective effects in humans.

5. Conclusion

Administration of 2 mg/kg lidocaine effectively inhibited the inflammatory process in BALB/c mice with musculoskeletal injury by suppressing the mRNA expression of NF-kβ, protein levels of NF-kβ, and protein levels of TNF-α.

Provenance and peer review

Not commissioned, externally peer-reviewed.

Please state any conflicts of interest

The authors declare that they have no conflict of interests.

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Ethical approval

The study was conducted after obtaining approval from the Ethics Commission of Hasanuddin University, number: 329/UN4.6.4.5.31/PP36/2021.

Consent

This manuscript does not involve human participants, human data, or human tissue.

Author contribution

RK, SKA, MH, AGB, RDN, HSB, IJP and CK wrote the manuscript and participated in the study design. RK, SKA, MH, AGB, RDN, HSB, IJP and CK drafted and revised the manuscript. RK and MH performed the musculoskeletal injury model. RK and MH performed bioinformatics analyses and revised the manuscript. All authors read and approved the

final manuscript.

Registration of research studies

None.

Guarantor

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.amsu.2021.102660.

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