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ORIGINAL ARTICLE

ANALGESIC ED_{50} DICLOFENAC-EUGENOL COMBINATION EFFECT ON PROINFLAMMATORY CYTOKINES – IL-1β, IL-6, TNFα AND MAPKs – IN RAT MUSCLE

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Abstract

Inflammation is a typical response to a wide variety of stressors, including xenobiotics, which can develop a prolonged, severe, and inappropriate inflammatory response that can lead to events at the molecular level degenerating into adverse effects. The objective of the study was to evaluate the effect of ED_{50} diclofenac-eugenol (DFC+EUG) combination on proinflammatory cytokines (IL-1β, IL-6, TNFα and MAPKs) in rat muscle. The treatments as well as the control solutions were administered in the gastrocnemius muscle and the expression of cytokines and MAPKs was evaluated through an end-point PCR. There were no significant changes in the expression of IL-6, TNFα and MAPKs in healthy rats. In contrast, a statistically significant increase was shown in the relative expression of IL-1β when DFC+EUG was administered *vs.* the control group. The expression of IL-1β decreased in the group DFC+EUG by the addition of *Aloe vera*. The obtained data suggested that the DFC+EUG combination causes a moderate increase in the expression of the proinflammatory cytokines IL-1β, but not for the expression of IL-6, TNFα and MAPKs in healthy rats.

Rezumat

Inflamația este un răspuns tipic la o mare varietate de factori de stres, inclusiv la xenobiotice, care pot dezvolta un răspuns inflamator prelungit, acesta generând efecte moleculare nedorite. Obiectivul studiului a fost evaluarea efectului combinației DE_{50} diclofenac-eugenol (DFC+EUG) asupra citokinelor proinflamatorii (IL-1β, IL-6, TNFα și MAPK-uri) în mușchiul de șobolan. Tratamentele, precum și soluțiile de control, au fost administrate în mușchiul gastrocnemius și expresia citokinelor și MAPK-urilor a fost evaluată prin tehnica PCR. Nu au existat modificări semnificative în expresia IL-6, TNFα și MAPK-urilor la șobolanii sănătoși. S-a constatat o creștere semnificativă din punct de vedere statistic a expresiei relative a IL-1β atunci când s-a administrat DFC+EUG față de grupul control. Expresia IL-1β a scăzut în grupul DFC+EUG după adăugarea de *Aloe vera*. Datele obținute au sugerat că DFC+EUG determină o creștere moderată a expresiei citokinelor proinflamatorii IL-1β, dar nu și a expresiei IL-6, TNFα și MAPK-urilor la șobolanii sănătoși.

Keywords: diclofenac-eugenol, inflammation, IL-1β, IL-6, TNFα, MAPKs, PCR

Introduction

Studies of new drugs must verify their efficacy and safety in experimental models, thinking about future clinical trials and commercialization. For this aim, toxicity tests are used to reduce and ideally, eliminate the likelihood of dangerous effects on humans [18]. In fact, *in vivo* toxicity models use a complete organism with physiological reactions and biochemical interactions. It is also the only model that provides information about the drug's distribution in the organism and its possible interactions with organs different from its target. This model is the one that provides the most representative information on drug toxicity tests [26]. The interaction between drugs and the whole organism can generate pharmacological and adverse effects at the cellular level. On the other hand, inflammation is a typical result of a wide variety of stressors, including xenobiotics, which can develop a prolonged, severe, and inappropriate inflammatory response that can lead to molecular level events and adverse effects [34]. During the acute phase, leukocytes migrate toward a chemotactic gradient to act at the inflammatory process's damage site. The cytokines and acute-phase

proteins intend to remove the inflammatory stimulus or damaged cells and initiate healing [11]. The beneficial physiological effect is a function of endogenous suppressors available in the pro-inflammatory signalling pathways. However, when suppressor factors decrease, it can cause acute or chronic apoptosis, necrosis, fibrosis, and finally, organ destruction [15]. This response develops in tissues lesioned by bacteria, trauma, toxins, heat, or some external agent to the physiological content [6]. The drugs under study may themselves be agents that trigger these processes, so it is crucial to verify their safety and efficacy regarding the body's response.

In this context, the assessment of analgesic efficacy through isobolographic analysis and formalin testing of a new drug combination, diclofenac-eugenol (DFC+EUG), was reported in a previous studies [12, 13]. The formalin test showed an effective dose fifty (ED_{50}) for phase 1 and 2 and the local co-administration of DFC+EUG gave a theoretical effective dose (Zadd) 2,936.27 ± 155.33 µg/kg bw significantly higher as compared to the effective experimental doses (Zmix) of 866.89 ± 0.02 µg/kg bw in phase 1 ($p \leq 0.05$) and 292.88 ± 0.05 µg/kg bw in phase 2 ($p \leq 0.05$), with an interaction index of 0.29 and 0.09, respectively [12].

The combination resulted from applying the principle of multimodal analgesia [20]. DFC is commonly used to treat long-term inflammatory disorders; however, due to its short half-life and some reports of toxicity, its use has been reduced [10, 20]. Besides, EUG is a monoterpene extracted from clove (*Syzygium aromaticum*) that proved an antinociceptive effect in several pain models [14, 17, 19, 24]. Because of its versatility, it is often used alone or in combination with non-steroidal anti-inflammatory drugs such as acetylsalicylic acid, piroxicam, ketorolac and DFC [3, 8, 13, 22].

Therefore, we aimed to evaluate the effect of the analgesic DFC+EUG combination on the expression of pro-inflammatory cytokines (IL-6, IL-1B), TNFα, and protein kinases in rat muscle. Since the mentioned doses are effective against acute and inflammatory pain, it is crucial to investigate safety *versus* proven efficacy.

Materials and Methods

Experimental units

Three rats Female Wistar Kyoto per group weighing between 200 g and 300 g, aged between 1 and 1.5 months, were kept in biotherium conditions (25°C, with 12-hour light-dark cycles), water, and food *ad libitum*. The treatment was administered intramuscularly in the gastrocnemius muscle, after which the tissue was extracted, washed with saline solution and placed in a solution of Trizol reagent to proceed with the extraction of total RNA. The animals were sacrificed under the ethics guides' alignments for pain research in experimental animals from the International Association for the Research of Pain, IASP, and the attachment to the Principles of Laboratory Animal Care (NIH N° 85-23, 1985) [23].

Experimental design

A double dose was used, administered at zero and eighteen hours, to the experimental units for the formalin test. The treatment consisted of doses corresponding to the ED_{50} of EUG (5735.5 µg/kg bw), DFC (136.99 µg/kg bw), DFC+EUG (292.88 µg/kg bw), DFC+EUG+AV (292.88 µg/kg bw), Tween (1%), AV (60%), Saline solution (SS; 0.9%) negative nociception control and Formaline (5%) [12].

Gene expression analysis

Approximately 0.5 g of muscle tissue was homogenized with the reagent Trizol® (Invitrogen) to perform RNA extraction according to the manufacturer's instructions. RNA extracted was resuspended in water-free RNase, and the genetic material concentration was determined considering the absorbance at 260 nm and the 260 nm/280 nm index in the NanoDrop spectrophotometer.

Synthesis of cDNA

The complementary DNA synthesis employed two µg of RNA and high-capacity reverse transcriptase (Applied Biosystems). It was incubated for one cycle at 37°C for 1 hour and at 95°C for 5 minutes.

PCR amplification products

Gene amplification performed individually was using primer pairs (direct; Fw and reverse; Rv) of the genes encoding IL-6, IL-1β, TNF-α, MAPK and GAPDH proteins (Table II). The method used polymerase chain reaction (PCR) with 12.5 µL of Master Mix of the enzyme Gotaq Green (Promega), 250 ng of cDNA, and 0.5 µM of primers corresponding to the gene in amplification. The samples were submitted to 42 cycles, where each cycle had the following conditions: 15 seconds at 95°C, 45 seconds at 55°C, 30 seconds at 72°C, and 10 seconds at 72°C.

Electrophoresis

Electrophoresis in a 1% agarose gel was performed at 90 Volts for 1 hour and then analysed in a photo-documentator (Quantity One BioRad; BioRad Lab., Hercules, CA, USA). Each sample's relative gene expression was compared to the control group (0.9% saline solution), and the reference used was the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH).

Statistical analysis

Densitometric analysis of the bands was performed and the results were analysed applying one-way ANOVA and Tukey post-test for multiple comparisons using Origin 2018 software. A $p < 0.05$ was considered as statistical significance.

Table I

Primers used in the evaluation of gene expression

Gene	Primer sequences (5'-3')	Temperature sequence alignment (°C)	PCR product size (bp)
IL-6	Fw: CCACTTCACAAGTCGGGAGGCTTA Rv: ACTAGGTTTGCCGAGTAGACCTCA	58.7	497
IL-1β	Fw: CGATGCACCTGTACGATCACTGAA Rv: CAACACGCAGGACAGGTACAGATT	56.9	223
TNF-α	Fw: TGGTCATGAGTCCTTCCACGAT Rv: TTCCTGAATCCCAGGTTTCGAAGTG	59.2	387
MAPK	Fw: CAGGAGTCCTTTGGGGCATTTT Rv: ACTTGGCAACAGCTTCGTGA	57.1	487
GAPDH	Fw: CCATCAATGACCCCTTCATTGACC Rv: TGGTCATGAGTCCTTCCACGAT	65.2	435

Results and Discussion

The relative expression of the inflammatory mediators was evaluated in gastrocnemius muscle in 6 experimental and two control groups. The tissue exposure to EUG showed no statistically significant change in the expression of IL-1β, IL-6, TNFα and MAPKs genes (Figures 1, 2, 3 and 4). In the same sense, the DFC administration did not cause changes in the expression of IL-6, TNFα and MAPKs (Figures 2, 3 and 4); however, IL-1β presented a significantly higher level of expression compared to the controls ($p < 0.05$ *vs.* SS group and FMN group, Figure 1).

The combination DFC+EUG did not change the expression of IL-6, TNFα and MAPKs (Figures 2, 3 and 4) compared to SS and FMN groups. In contrast, the level of expression of IL-1β was ten units higher than the SS group ($p < 0.05$), and seven units higher concerning the FMN group ($p < 0.05$) (Figure 1). This effect in IL-1β decreased by the addition of AV (60%) as was observed in the combination of (DFC+EUG)+AV compared to SS and FMN groups.

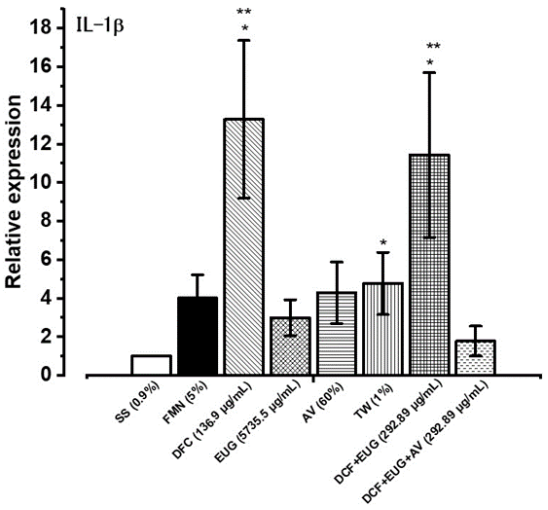

Figure 1.

Relative expression of IL-1β in muscle tissue after localized administration of the treatments

*p < 0.05 *vs.* SS, **p < 0.05 *vs.* FMN

Cytokines, among other functions in the body, are responsible for the inflammatory response. This process seeks to eradicate external elements or respond to deleterious tissue damage to maintain tissues, organs, or the entire organism's integrity. In this respect, the administration of pharmacological formulations (DFC, EUG and DFC+EUG) could trigger the production of pro-inflammatory cytokines by recognizing them as external substances. In general, after a tissue injury, the innate immune system is activated, generating cytokines [36], such as interleukins (IL), tumour necrosis factors (TNF), interferons (IFN), colony-stimulating factors (CSF), and chemokines. These act as systemic regulators and local growth factors (autocrine, paracrine, juxtacrine, or retrocrine). Their biological activity results from cytokines' binding on membrane receptors, producing a cascade of biochemical reactions inside target cells [9].

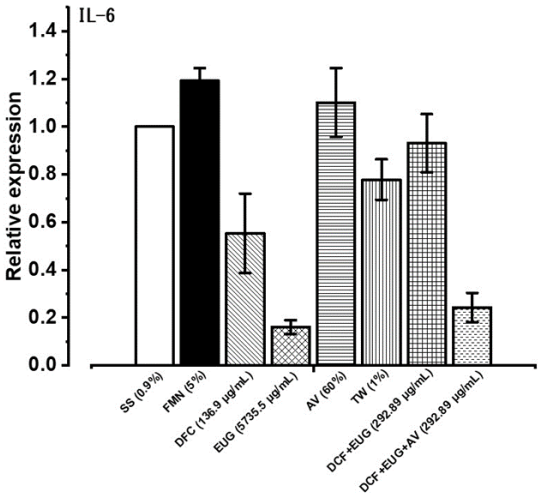

Figure 2.

Relative expression of IL-6 in muscle tissue after the localized administration of treatments

*p < 0.05 *vs.* SS, **p < 0.05 *vs.* FMN

Cytokines help repair injuries, eliminate pathogens, and maintain health. However, they can cause hypersensitivity reactions in tissues and act as pro-inflammatory, anti-inflammatory mediators, or both [29]. During an inflammatory process, granulocytes

are recruited into damaged tissue, producing inflammatory mediators, including pro-inflammatory cytokines such as TNFα, IL-1β, IL-6, maintaining an acute inflammatory response until the cause is eliminated [36]. TNFα (pro-inflammatory) produced by T-lymphocytes induces cytotoxicity or apoptosis and is almost undetectable under normal conditions, but production and secretion increase when an inflammatory process exists [28].

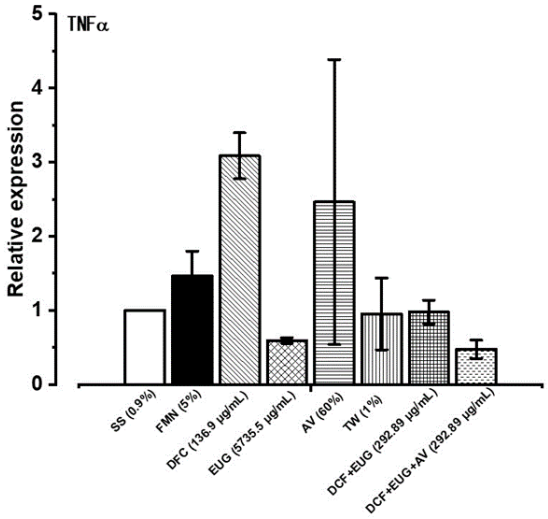

Figure 3.
Relative expression TNFα in muscle tissue after the localized administration of treatments
*p < 0.05 *vs.* SS, **p < 0.05 *vs.* FMN

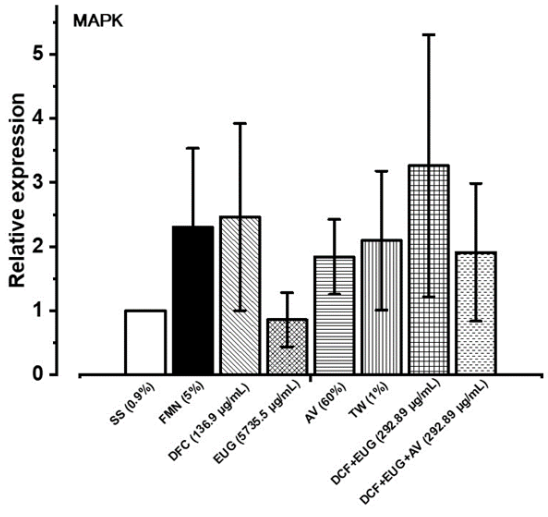

Figure 4.
Relative expression MAPK in muscle tissue after the localized administration of treatments
*p < 0.05 *vs.* SS, **p < 0.05 *vs.* FMN

On the other hand, IL-1β (pro-inflammatory) secreted by monocytes, dendritic cells, and neutrophils [5] can induce the expression of other pro-inflammatory factors that, together, maintain the nociceptive/ inflammatory process [30], such as interleukin 6. IL-6 (glycosylated protein) can be synthesized and secreted by many cell types, including monocytes, T-cells, fibroblasts, and endothelial cells [32]. It is generated in injured tissue and sent to the rest of the body as a warning signal. Besides, it stimulates NF-κB-associated signalling pathways; in turn, TNFα and IL-1β activate transcription factors to produce IL-6 [33]. Also, IL-1β, IL-6 and TNFα interact with TLR receptors: IL-1β receptor, IL-6 receptor, and TNFα receptor. These stimulate mitogen-activated protein kinase (MAPK) pathways producing osmotic stress, mitogens, heat shock proteins, and inflammatory cytokines, promotes cell proliferation, differentiation, survival and apoptosis [6].

Also, MAPKs can positively regulate the expression of genes involved in inflammation, such as those coding for TNFα, IL-1β, IL-6 and IL-8, cyclooxygenase 2 and collagenase 1 and 3 [16]. Under that context, in the present work, the administration of EUG (5735.54 µg/kg bw) did not produce changes in the relative expression (ER) of TNFα, IL-1β, IL-6 and MAPK (Figures 1, 2, 3 and 4) in the muscular tissue. On this subject, De Paula Porto *et al*., 2014 report that the administration of EUG at 0.62, 1.24, 2.48 µg/mL during six h in rat macrophages in the absence of cell stimulation does not generate changes in the expression of mRNA from TNFα [7].

Methyl eugenol form (100 mg/kg bw, p.o.) administered in rats for 30 days, does not produce changes in mRNA expression of IL-6 and TNFα [30]. In contrast, EUG at 13 µM in a culture of fibroblasts, obtained from human dental pulp, caused the expression of IL-1β 2.4 times more than the control group [27]. Chainy *et al*. reveal that anethole and its structural analogues (EUG and isoeugenol) are potent inhibitors of the effects produced by TNFα and that they can act inhibiting the phosphorylation of MAPKs involved in the signalling pathway, so if there is no phosphorylation of protein kinases, will be expected that there are no changes in their expression [4]. In turn, when it was evaluated the effect of localized administration of DFC (136.99 µg/kg bw) on the relative expression of IL-1β, IL-6 and TNFα, a significant increase in the relative expression of IL-1β was observed (Figure 1; ER 13.27), but not for the other mediators, using the dosing scheme. Comparatively, Yano *et al.* administered DFC (80 mg/kg bw, p.i.) in mice and reported that the mRNA expression corresponding to TNFα does not show significant changes before 24 hours [35]. In agreement, Barcelos *et al*. pointed out that the administration of DFC (10 mg/kg bw, p.o.) for seven days in rats does not cause changes in mRNA expression from TNFα and IL-6 in the lateral vascular muscle [1]. In contrast, an increase in the expression of IL-1β, between 3 and 12 hours, was observed.

In the case of DFC+EUG, there is no previous report that supports the safety of the administration of the combination. DFC+EUG combination effect on mRNA expression is similar to that obtained with the DFC

individual administration, where was observed a statistically significant increase in the IL-1β expression (ER 11.426) even though the dose of EUG and DFC in the combination is 20 times lower (Figure 1). Therefore, it is deduced that the combination components have a behaviour like the non-steroidal anti-inflammatory drugs (DFC ER 13.27). The effect does not occur when combined with AV (60%; ER 1.78) (Figure 1).
It is essential to highlight that isolated IL-1β is unable to stimulate the neurons of the dorsal root ganglion, but considering the presence of IL-6 and TNFα, it produces a rapid increase in the sensitivity of TRPV1 and the release of the peptide related to the calcitonin gene, which leads to thermal sensitization [2].
Furthermore, it is necessary to increase several pro-inflammatory mediators to observe a harmful effect on the tissue. Also, Yano *et al*. measured the hepato-toxic effect of DFC in rats and reports that cytotoxicity is reversible once the drug is withdrawn, suggesting that the combination also presents this behaviour at a localized level, but without causing changes in IL-1β levels at a systemic level.
When it was administered the mixture DFC+EUG+AV, IL-1β decreased, which indicates that *Aloe vera* may be a suitable excipient for the combination. Moreover, some studies report the effect of combining acetyl-salicylic acid with eugenol and, more specifically, a derivative of them, the aspirin eugenol ester. This combination was evaluated on other inflammatory mediators such as arachidonate 5-lipoxygenase, C-reactive protein, FII, cyclooxygenase 1 and 2, in healthy rats. From where they conclude, this type of combination presents a more significant effect than their precursors, significantly reducing the indicated mediators [25]. Therefore, the data support similar combination studies as an alternative to treat pain and inflammation with the advantage of using shallow doses of the active ingredients and a possible reduction of adverse effects.

Conclusions

The data suggested that DFC+EUG combination causes a slight increase in the expression of the pro-inflammatory cytokine IL-1β, but not for IL-6, TNFα and MAPKs in healthy rats. Furthermore, the DFC+EUG combination shows a mechanism of action like NSAIDs such as diclofenac.

Conflict of interest

The authors declare no conflict of interest.

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