
BIOCHEMICAL EVALUATION OF ANTI-INFLAMMATORY ACTION OF A PHARMACEUTICAL COMPOSITION OF NIMESULIDE WITH CAFFEINE IN A FORMALIN PAIN MODEL IN RATS

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ABSTRACT

Background. Nonsteroidal anti-inflammatory drugs are widely used to treat pain and inflammation, while combination formulations may modify treatment effects through complementary mechanisms.

Aim. To evaluate the anti-inflammatory activity of a pharmaceutical composition containing nimesulide and caffeine based on serum ceruloplasmin levels and to assess its antioxidant activity based on serum conjugated triene levels in rats with formalin-induced paw edema.

Materials and Methods. The study included 36 male Wistar Albino Glaxo rats allocated to six groups: intact control, formalin-induced paw edema, nimesulide (15 mg/kg), caffeine (0.6 mg/kg), nimesulide with caffeine (15 mg/kg and 0.6 mg/kg), and diclofenac sodium (8 mg/kg). Paw edema was induced by 2% formalin. Serum ceruloplasmin was measured by enzyme-linked immunosorbent assay (Cusabio, China), and conjugated trienes spectrophotometrically. Statistical analysis used one-way analysis of variance with Tukey's post hoc test or the Kruskal–Wallis test with Dunn's post hoc test; $p < 0.05$ indicated significance. The study was performed within the research project "Chemical and Pharmaceutical Substantiation for the Development of Biologically Active Compositions with Anti-Inflammatory and Analgesic Activities" (2024–2026), state registration No.0124U002495.

Research Ethics. Procedures complied with the European Convention on experimental vertebrate animals and ARRIVE 2.0; the protocol was approved by the Bioethics Committee of Kharkiv National Medical University.

Results. Formalin increased serum ceruloplasmin and conjugated triene levels. The nimesulide-caffeine combination reduced ceruloplasmin to $[139.83 \pm 18.42]$ mg/L and conjugated trienes to $[0.124 \pm 0.012]$ arb. units/mL. These values were lower than with nimesulide or caffeine alone. Ceruloplasmin was also lower than with diclofenac sodium, while conjugated trienes did not differ significantly from the diclofenac sodium group or intact control.

Conclusions. Under the experimental conditions, the nimesulide-caffeine combination produced the largest reductions in the inflammatory and lipid-peroxidation biomarkers studied among the treatment groups. The findings support further investigation of this combination.

Keywords: *experimental medicine, biomarkers of inflammation, antioxidant activity, ceruloplasmin, conjugated trienes, lipid peroxidation.*

Abbreviations

ANOVA – ANalysis Of VAriance.

ARRIVE – Animal Research: Reporting of In Vivo Experiments.

COX-1 – CycloOXygenase-1.

COX-2 – CycloOXygenase-2.

CP – CeruloPlasmin.

CT – Conjugated Trienes.

ELISA – Enzyme-Linked ImmunoSorbent Assay.

FE – Formalin-induced paw Edema.

GIT – GastroIntestinal Tract.

HRP – HorseRadish Peroxidase.

IL-1 – InterLeukin-1.

IL-6 – InterLeukin-6.

LPO – Lipid PerOxidation.

M $\pm$ SEM – Mean $\pm$ Standard Error of the Mean.

NSAIDs – NonSteroidal Anti-Inflammatory Drugs.

TNF- α – Tumor Necrosis Factor-alpha.

WAG – Wistar Albino Glaxo.

Introduction

NSAIDs are widely used to reduce pain, inflammation, and fever. Combination pharmacother-

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rapy is used in several pain and inflammatory conditions and, depending on the components and clinical setting, may provide additive or synergistic effects compared with monotherapy [1–4].

Fixed-dose combinations can combine active ingredients with complementary mechanisms, potentially improving analgesic efficacy or allowing lower doses of individual components in selected settings [5; 6].

Reducing pill burden can also support treatment adherence, although adherence depends on multiple patient-, disease-, and treatment-related factors [7; 8].

Accordingly, the development of rational combinations requires evidence of both expected benefit and component-specific safety.

A pharmaceutical composition of nimesulide (N-(4-nitro-2-phenoxyphenyl)methanesulfonamide) with caffeine (1,3,7-trimethyl-3,7-dihydro-1H-purine-2,6-dione) has been developed by the staff of the Department of Medical and Bioorganic Chemistry at Kharkiv National Medical University.

Nimesulide is a sulfonanilide NSAID with relative selectivity for COX-2 and pharmacological actions that extend beyond cyclooxygenase inhibition [9; 10]. Its clinical use is limited by safety concerns, especially hepatotoxicity, for which systematic reviews and recent appraisals emphasize appropriate patient selection and duration of use [11; 12].

Caffeine is used as an analgesic adjuvant in some combination products; clinical evidence in acute pain supports efficacy advantages for caffeine-containing paracetamol or NSAID combinations in selected conditions [13]. The authors have previously studied caffeine-containing NSAID combinations in formalin-induced inflammation, including their effects on lipid peroxidation products [14]. More recently, the same nimesulide-caffeine composition and doses used in the present work were evaluated in a formalin-induced paw edema model and showed greater anti-exudative activity than nimesulide alone and diclofenac sodium [15].

CP was selected as an inflammatory biomarker. It is a copper-containing positive acute-phase protein with ferroxidase activity, and its serum concentration can increase during acute inflammation. Independent experiments in rats have demonstrated increased serum CP in carrageenan-induced foot edema and pleurisy [16].

Through its ferroxidase activity, CP oxidizes Fe^{2+} to Fe^{3+} , facilitating iron binding to transferrin

and contributing to iron homeostasis during inflammatory and oxidative processes.

LPO contributes to oxidative membrane damage. CT arise from the rearrangement of double bonds in polyunsaturated fatty acids during lipid peroxidation; therefore, their serum concentration was used here as an indicator of lipid peroxidation activity.

The combined assessment of CP and CT was used to characterize inflammatory and oxidative changes in the formalin-induced paw edema model and their modification by nimesulide, caffeine, the nimesulide-caffeine composition, and diclofenac sodium.

The study aimed to evaluate the anti-inflammatory activity of a pharmaceutical composition containing nimesulide and caffeine based on serum ceruloplasmin levels and its antioxidant activity based on serum conjugated triene levels in rats with formalin-induced paw edema.

Materials and Methods

Biochemical analyses were performed in serum from 36 male WAG rats. The study used the same six-group formalin-induced paw edema design as the authors' previous anti-exudative experiment with the nimesulide-caffeine composition [15]. Six animals were assigned to each group.

Animals in Group 1 served as the intact control and received a single intragastric administration of 3% starch mucilage (2 mL per 200 g of body weight).

In Groups 2–6, FE was induced by subplantar injection of a 2% formalin solution into the hind paw. Group 2 received 3% starch mucilage via intragastric administration. Groups 3–6 received single intragastric doses of suspensions prepared in 3% starch mucilage: Group 3, nimesulide (15 mg/kg); Group 4, caffeine (0.6 mg/kg); Group 5, nimesulide (15 mg/kg) with caffeine (0.6 mg/kg); and Group 6, the reference drug diclofenac sodium (sodium 2-[(2,6-dichlorophenyl)amino]phenylacetate) at 8 mg/kg.

The doses of nimesulide (15 mg/kg), caffeine (0.6 mg/kg), and diclofenac sodium (8 mg/kg), as well as the nimesulide-caffeine combination, were as in the authors' previous formalin-induced paw edema protocol [15].

FE was induced at $t=0$ h by subplantar injection of 2% formalin. The test substances or reference drug were administered intragastrically at $t=3$ h, 1 h before the reported 4-h peak of edema in the previous formalin-edema experiment [15]. Control animals received the corresponding volume of 3%

starch mucilage. Animals were euthanized at $t=4$ h. Blood was collected immediately, serum was separated by centrifugation, and used for biochemical analyses.

Animals were housed under standard vivarium conditions with free access to food and water. Experiments were performed during the first half of the day to minimize circadian variation.

Serum CP was measured using the "Human Ceruloplasmin ELISA Kit" (Cusabio, China) by a sandwich ELISA. Microplate wells pre-coated with CP-specific antibodies captured CP from samples; after washing, biotinylated CP-specific antibodies and then streptavidin–HRP conjugate were added.

After removal of the unbound conjugate, the substrate solution was added and converted to a colored product by HRP. The reaction was stopped with the stop solution.

Absorbance was measured at 450 nm. CP concentration (mg/L) was calculated as follows:

$$CP = \frac{E \cdot F}{t} \quad (1),$$

where:

E – optical density (absorbance) of the test sample at 450 nm;

F – calibration factor dependent on assay conditions, standard concentration, and instrumentation;

t – incubation time, 30 min.

Serum CT levels were determined spectrophotometrically using the method described in [17]. A 1:10 volume of cold isotonic NaCl solution was added to 1 mL of the sample.

The mixture was centrifuged for [3–5] min at 3,000 rpm. Four milliliters of cold heptane were added to 1 mL of the sample and mixed vigorously for [1–2] min. After phase separation, the upper heptane layer was collected. Absorbance was measured at 233 nm against a heptane blank. CT content (arb. units/mL) was calculated as follows:

$$CT = A_{233} \cdot 100 \quad (2),$$

where:

A_{233} – optical density of the sample at 233 nm;

100 – coefficient used to standardize the results.

Statistical analysis was performed using Statistica 6.0 (StatSoft Inc., USA). Data are presented as $M \pm SEM$. Normality was assessed with the

Shapiro–Wilk test. Normally distributed data were analyzed by one-way ANOVA followed by Tukey's post hoc test; non-normally distributed data were analyzed by the Kruskal–Wallis test followed by Dunn's post hoc test. Bonferroni correction was applied where appropriate. Differences were considered statistically significant at $p < 0.05$.

Research Ethics

The study was conducted in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes [18] and the ARRIVE 2.0 guidelines [19]. All experimental procedures complied with applicable Ukrainian legislation on the use of laboratory animals in biomedical research. The protocol was reviewed and approved by the Local Bioethics Committee of Kharkiv National Medical University. Measures were taken to minimize animal suffering and reduce the number of animals used.

Results

The effects of nimesulide, caffeine, their pharmaceutical composition, and diclofenac sodium on serum CP and CT were compared in rats with FE.

The intact control group had a serum CP concentration of $[145.83 \pm 17.47]$ mg/L. FE increased CP to $[474.00 \pm 26.84]$ mg/L, a 3.25-fold increase compared with the control group ($p < 0.05$).

Nimesulide reduced CP to $[311.00 \pm 18.00]$ mg/L, significantly below the FE group ($p < 0.05$), although the value remained significantly above that in the intact control. Caffeine reduced CP to $[334.50 \pm 25.45]$ mg/L, which was also significantly lower than in the FE group ($p < 0.05$). Diclofenac sodium produced a CP concentration of $[261.33 \pm 27.78]$ mg/L.

The lowest CP concentration was observed in Group 5 receiving nimesulide with caffeine: $[139.83 \pm 18.42]$ mg/L, which was 3.4-fold lower than in the FE group ($p < 0.05$). Group 5 was also significantly lower than Groups 3, 4, and 6 ($p < 0.05$), while no statistically significant difference was found between Group 5 and the intact control (Fig. 1).

The intact control group had a serum CT concentration of $[0.087 \pm 0.004]$ arb. units/mL, whereas FE increased it to $[0.442 \pm 0.004]$ arb. units/mL ($p < 0.05$).

Nimesulide reduced CT to $[0.165 \pm 0.085]$ arb. units/mL and caffeine to $[0.156 \pm 0.091]$ arb. units/mL; both values were significantly lower than in the FE group ($p < 0.05$).

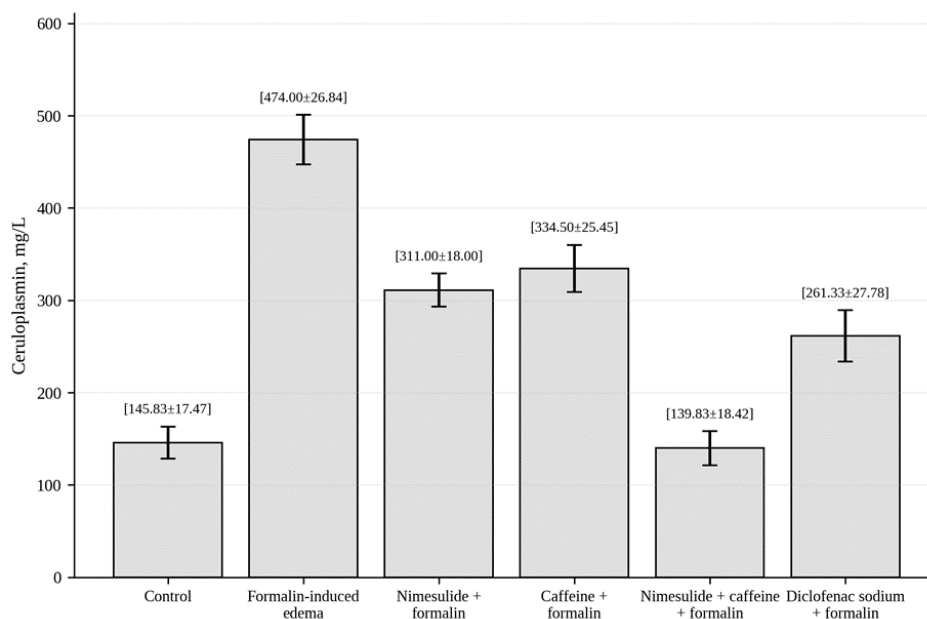


Fig. 1. Effect of nimesulide, caffeine, and their pharmaceutical combination on serum ceruloplasmin levels in rats with formalin-induced paw edema.

Notes: data are presented as $M \pm SEM$. Statistical comparisons are reported in the Results.

Group 5 receiving nimesulide with caffeine had a CT concentration of $[0.124 \pm 0.012]$ arb. units/mL, 3.6-fold below the FE group ($p < 0.05$) and significantly lower than Groups 3 and 4 ($p < 0.05$).

Diclofenac sodium produced $[0.157 \pm 0.015]$ arb. units/mL. No statistically significant differences in CT were detected between Group 5, the intact control group, and the diclofenac sodium group (Fig. 2).

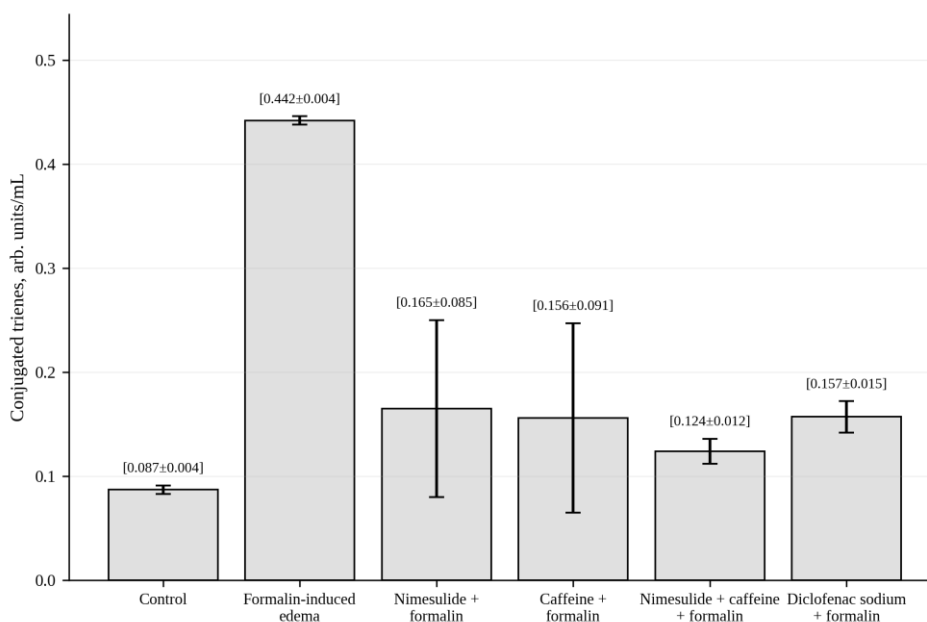


Fig. 2. Effect of nimesulide, caffeine, and their pharmaceutical combination on serum conjugated triene levels in rats with formalin-induced paw edema.

Notes: data are presented as $M \pm SEM$. Statistical comparisons are reported in the Results.

Discussion

Formalin-induced inflammation was accompanied by elevated serum CP and CT, indicating an acute-phase response together with increased lipid peroxidation activity. The 3.25-fold rise in CP is directionally consistent with independent rat inflammatory models in which serum CP increased during carrageenan-induced foot edema and pleurisy [16]. Direct quantitative comparison is limited by differences in inflammatory models and analytical methods.

Nimesulide significantly reduced both biomarkers. This is consistent with its anti-inflammatory pharmacology and experimental evidence that nimesulide can influence oxidative stress and antioxidant parameters under inflammatory conditions [9; 10; 20].

Caffeine alone also reduced both biomarkers relative to FE. The relative effect of caffeine and nimesulide was marker-dependent: the CP value was higher with caffeine [334.50 ± 25.45] mg/L than with nimesulide [311.00 ± 18.00] mg/L, whereas the mean CT value was slightly lower with caffeine [0.156 ± 0.091] arb. units/mL than with nimesulide [0.165 ± 0.085] arb. units/mL. The present data, therefore, do not support a general statement that caffeine was less effective than nimesulide for both outcomes.

The combination produced the lowest CP and CT values among the active-treatment groups. Compared with either component alone, this pattern is consistent with an enhanced combined effect; however, the study design and reported statistical analysis do not by themselves establish a specific pharmacodynamic interaction mechanism.

The CP value in Group 5 was [139.83 ± 18.42] mg/L, close to the intact control value of [145.83 ± 17.47] mg/L, and did not differ significantly from it, indicating normalization of this acute-phase marker under the reported analysis. The reduced CT value is consistent with lower lipid peroxidation activity.

Taken together, the biochemical findings complement the authors' earlier anti-exudative results for the same nimesulide-caffeine composition in the formalin edema model [15]. Further studies with mechanistic endpoints are needed before the interaction can be characterized more specifically.

Conclusions

1. Formalin-induced inflammation increased serum ceruloplasmin. The nimesulide-caffeine combination produced the greatest reduction among the treatment groups, bringing the value close to the intact control level.

2. Formalin-induced inflammation increased serum conjugated trienes. The nimesulide-caffeine combination produced the lowest value among the treated groups; under the reported analysis, it did not differ significantly from the intact control or diclofenac sodium groups.

3. Under these experimental conditions, combined nimesulide and caffeine affected both the inflammatory and lipid-peroxidation biomarkers more strongly than either component alone. The results support further experimental study of the combination.

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Declarations

Conflict of interest is absent.

All authors have agreed to the publication of the article under the terms of the Creative Commons BY-NC-SA 4.0 license and of the public agreement with Kharkiv National Medical University, and to the processing and publication of their personal data.

The authors of the manuscript declare that in the process of conducting research, preparing, and editing this manuscript, they did not use any generative artificial intelligence tools or services to perform any of the tasks listed in the Generative Artificial Intelligence Delegation Taxonomy (GAIDeT, 2025). All stages of the work (from the development of the research concept to the final editing) were performed without the involvement of generative artificial intelligence, exclusively by the authors.

Authors' Contributions

Contributions	A	B	C	D	E	F
Authors						
Syrova G.O.	+	+	+		+	+
Savelieva O.V.		+	+	+	+	+
Abramova L.V.				+		+
Kozub S.M.		+		+		+
Chalenko N.M.	+			+		+
Prysiaznyi O.V.			+	+		+

Notes: A – concept; B – design; C – data collection; D – statistical processing and data interpretation; E – writing or critical revision of the article; F – approval of the final version for publication and agreement to be accountable for all aspects of the work.

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